YALE JOURNAL OF BIOLOGY AND MEDICINE 75 (2002), pp. 215-219.

MEDICAL REVIEW

Ribozyme Therapy: RNA Enzymes to the Rescue

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INTRODUCTION

According to the central dogma of biology, ribonucleic acids (RNA)[a] were thought to play the role of messengers (mRNA) that are mere intermediates between DNA and proteins. Recent advances in understanding the capabilities of RNA have proven this wrong.

The catalytic reaction required for making proteins is carried out by RNA [1]. RNA evolved *in vitro* can bind small molecules with high specificity [2]. Cellular mRNAs can control gene expression directly in response to metabolites [3, 4]. The aforementioned studies and many others have proven that RNA exhibits all the properties of proteins that are key players in practically every cellular process. This realization has provided impetus to therapeutic approaches that use RNA to correct disease states [5].

The object of this review is to introduce one such exciting possibility for therapeutic intervention: the use of RNA enzymes (ribozymes). The progress in this field will be summarized, and some of the obstacles that need to be overcome will be evaluated.

EARLY DAYS OF CATALYTIC RNA

Ribozymes are RNAs that catalyze a chemical reaction, e.g., cleavage of a covalent bond (reviewed in [6] and [7]). Cech and coworkers first described RNA self-splicing in their studies of the 413-nucleotide group I intron from *Tetrahymena thermophila* [8]. Several years later, Zaug and colleagues described a variant of the *T. thermophila* ribozyme that could act *in trans* (i.e., as a sequence specific endoribonuclease acting upon other RNA substrates; [9]). The work of Forster and Symons [10, 11] on plant virusoid RNAs suggested smaller RNA domains were capable of ribozyme activity. Uhlenbeck was the first to demonstrate a small active ribozyme, the hammerhead ribozyme, in which the catalytic and substrate strands were separated [12]. Such ribozymes bind substrate RNAs through base-pairing interactions, cleave the bound target RNA, release the cleavage products, and are recycled so that they can repeat this process multiple times. Haseloff and Gerlach enumerated general design rules for simple hammerhead ribozymes capable of acting *in trans* [13]. Armed with these simple

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[a] *Abbreviations*: HCV, hepatitis C; HIV, human immunodeficiency virus; mRNA, messenger ribonucleic acids; RNA, ribonucleic acids; VEGF, vascular endothelial growth factor.

rules, ribozyme engineers were ready to intervene with gene function *in vivo*. One of the earliest studies on the *in vivo* efficacy of hammerhead ribozymes showed a 60 percent reduction in specific gene expression in monkey cells [14]. In order to make ribozyme therapy viable, further study was required.

IN VITRO AND CELLULAR STUDIES

To have a ribozyme that is effective *in vivo*, extensive *in vitro* and cell culture studies are needed. The understanding gleaned from these studies is then used to obtain physiologically acting ribozymes with good pharmaco-kinetic properties. Below are some of the important considerations for such *in vitro* and cell culture studies.

Ribozyme kinetics

Ribozymes appear to obey simple Michaelis-Menton type kinetics [12, 15]. Under saturating conditions, cleavage rates are determined by the ribozyme K_{cat}/K_m. The most efficient wild type ribozymes display K_{cat}/K_m values of 10^8 M^{-1} min^{-1} (about the order of the apparent hybridization rate constant for two oligonucleotides [6]). It has been suggested that within the intracellular environment, the hybridization step may be rate limiting for trans-acting ribozymes [16]. Kinetic and mutagenesis experiments have indicated that for both natural and engineered ribozymes, at substrate saturation, product release may be rate limiting [16, 17]. This release rate-limitation is due to the length of the sequence of RNA to which the ribozyme hybridizes. Therefore, studies have been carried out to optimize the length of the hybridizing arms of a ribozyme [e.g., 17, 18].

Analysis of ribozyme activity

Demonstration of ribozyme activity *in vitro* is relatively straight-forward: incubation of the ribozyme and its substrate followed by polyacrylamide gel electrophoresis analysis, allows detection of the cleavage products. Studying ribozyme activity in cells is more complex. First, it requires a means of delivering the ribozymes into the cell. This is usually accomplished in one of three ways: delivery of pre-formed ribozymes by microinjection or by cationic liposomes; delivery by transfection with plasmid constructs designed to express the ribozyme RNA; or delivery by infection with a retroviral vector bearing the ribozyme. Second, cleavage products produced by the ribozyme usually cannot be detected within cells, possibly due to the activity of intracellular ribonucleases that quickly degrade the cleaved substrate RNA [14, 19]. Therefore, indirect evidence is usually presented from which ribozyme activity is inferred.

RIBOZYMES IN CLINICAL TRIALS

Several phase I and II clinical trials have been initiated using *trans*-cleaving ribozymes on patients with infectious diseases or cancer. In these studies, the ribozymes have been delivered to the patients either by gene therapy methods or by direct injection of a synthetic ribozyme.

Ribozymes delivered using gene therapy methods

The gene-therapy-based trials have focused upon developing ribozyme-based treatments for individuals infected with human immunodeficiency virus (HIV). Three separate groups have used retroviral vectors to introduce expression cassettes for anti-HIV ribozymes into CD4+ lymphocytes or CD34+ haematopoietic precursors *ex vivo* that have been taken from the infected patient or from an identical twin [20-22]. The transduced cells are then infused into the patient, and the engraftment and survival of the ribozyme containing cells are monitored. Initial results from these studies suggest that transfer of ribozyme-encoding genes to HIV-infected individuals is well tolerated, and transduced cells can persist in the patient [21].

Moreover, preliminary reports suggest that anti-HIV ribozyme-containing cells may possess a transient survival advantage in the patient compared with cells transduced with a control vector [21]. Larger clinical trials must now be performed to evaluate the efficacy of anti-HIV ribozymes. Critical factors that will influence the success of such trials will be the development of gene-transfer systems that can efficiently transduce pluripotent haematopoietic stem cells and the generation of improved ribozyme expression cassettes that can increase the survival advantage of transduced cells.

Synthetic ribozymes delivered using direct injection

Three different nuclease-resistant synthetic ribozymes are being evaluated in clinical trials [23]. Each of these trials uses a hammerhead ribozyme derivative that contains chemical modifications that greatly enhance the ribozyme's stability in biological fluids [24]. Moreover, methods have been developed that enable large-scale synthesis of this new class of therapeutic agents under good manufacturing practice protocols [25]. All three of these synthetic ribozymes target RNAs whose expression is associated with the induction or progression of cancer, and all three have shown promising results in pre-clinical cell and animal experiments [26, 27]. In 1998, the first of these ribozymes entered a phase I trial targeting *flt-1* mRNA, which encodes the high affinity receptor for the angiogenic protein vascular endothelial growth factor (VEGF). Results from this and two subsequent phase I trials show that daily intravenous or subcutaneous delivery of this compound is well tolerated and that plasma levels could be maintained for prolonged periods after subcutaneous delivery [23]. Currently its therapeutic efficacy is being evaluated in phase II clinical trials for breast and colorectal cancer. The other two synthetic ribozymes to enter clinical trials target mRNA of human epidermal growth factor receptor type 2, which is overexpressed in many breast cancers, and hepatitis C virus (HCV) RNA which is associated with liver cirrhosis and hepatocellular carcinoma. Results from these initial efficacy studies will provide the first significant into the long-term utility of *trans*-cleaving ribozymes as therapeutic agents. The clinical factors that will most likely determine the success of these synthetic ribozyme efficacy trials are the ability to deliver ribozymes efficiently into the appropriate cell *in vitro*, and the level and duration of target-gene inhibition that is required to alter disease pathology or slow disease progression.

OBSTACLES FACING *IN VIVO* APPLICATION OF RIBOZYMES

Ribozymes must hybridize to RNA targets that possess their own secondary structure, a feature of the target known to affect the efficacy of ribozymes [28]. Computer folding programs for RNA may help determine regions of RNA likely to be involved in RNA structure or help predict the activity of ribozymes [29, 30]. However, these algorithms have not eliminated emperical approaches since the algorithms achieve an 80 percent accuracy at best [31, 32]. Targeting of the ribozyme by anchor sequences able to hybridize to the RNA at sites distant from the actual cleavage site may provide an alternate strategy for circumventing secondary structure at the ribozyme cleavage site [33].

The efficacy of a ribozyme depends strongly on the persistence of the affects caused by it *in vivo*. None of the modalities proposed to date can eliminate the disease/target. For ribozymes to benefit individuals with chronic disorders such as cancer and HCV (or HIV) infection, long-term, high-level inhibition of the target transcripts will probably be required. This may be dif-

ficult to achieve in practice, especially when targeting highly expressed viral RNAs.

Another critical determinant of the efficacy of a ribozyme in a physiological setting is the ability to deliver the ribozyme to the sub-cellular compartment that has the target RNA. If the target is outside the vascular system, the agent will have to extravasate. Non-gene nucleic acid drugs have molecular weights in the 3-10 kDa range, so extravasation is not a particular problem for the agent itself. However, as these drugs do not permeate into the cytoplasm of cells but are found primarily in the endosome compartment [34, 35], they will most likely require some covalent modification or delivery system to mediate their efficient entry into the cytoplasm of the target cells [36].

NEXT GENERATION RIBOZYMES: FINE CONTROL OF ACTIVITY

RNA catalysis can be modulated allosterically just as finely as protein catalysis [37]. These allosteric ribozymes are activated or inactivated by small metabolites. The recent findings on the use of such modulation by cellular mRNAs to control gene expression [3, 4], raises the possibility of allosterically activated therapeutic ribozymes. This would increase the level of control that one has over the therapy. For example, if an allosteric ribozyme were delivered using gene therapy approaches, then it can be specifically activated only when needed by administering a small molecule (the "metabolite"). This approach thus provides both temporal and dosage control. Furthermore, RNA is capable of binding its metabolites cooperatively [38]. Incorporating a series of domains in the design of an allosteric ribozyme allows for it to be activated only when two or more metabolites were present at the same time. This provides a mechanism to increase specificity of ribozyme action.

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