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Review Article

siRNA and microRNA for the treatment of cancer

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CONTENTS

Abstract

RNA interference (RNAi) is a recently discovered RNA-silencing process that occurs in plants and animals. In this process, small segments of RNA, either delivered to the cell or derived naturally from the cell genome, are involved in the repression and/or regulation of hundreds of complementary target messenger RNAs (mRNAs). The discovery of RNAi has led to a revolution in our understanding of genomic and cellular regulation. In addition, RNAi molecules and methods are being employed to identify unique cancer biomarkers as tools in target validation and as a new class of anticancer therapeutics, among many other biomedical applications. Since cancer is a disease of genetic mutation and deregulation, RNAi approaches hold tremendous potential to enhance our understanding of this disease and to offer a variety of new and more effective tools in the treatment of cancer. This article will review the general process of RNAi and the possible applications of this recent discovery for the treatment of cancer.

Introduction

The 2006 Nobel Prize in Physiology and Medicine was awarded to Craig Mello and Andrew Fire for their description of the process of RNA interference (RNAi) just eight years after their original landmark 1998 *Nature* publication, which described their work in *Caenorhabditis elegans* (1). In that article, the investigators demonstrated that noncoding regions of double-stranded RNA (dsRNA), presumed to be biologically inert, produced highly selective gene silencing and that this process is a natural and important regulatory mechanism. RNAi occurs when a short dsRNA segment triggers a cascade resulting in the destruction of a specific messenger RNA (mRNA) that is complementary to all or part of the dsRNA sequence. Another form of RNA silencing involves the microRNA pathway in both plants and animals. MicroRNAs are also small, noncoding segments of dsRNA that originate in the plant or animal genomes and function in the natural repression of hundreds of target mRNAs that are complementary to the microRNA regulators. Earlier work demonstrated that microRNA operates as a mechanism for viral defense in plants and flies (2, 3). At the present time, over a thousand microRNAs have been identified in plants and animals and several hundred are known to exist and to be involved in the regulation of genetic function in humans (4-6). Many of the microRNAs are evolutionarily conserved, which suggests that they are involved in the regulation of important physiological mechanisms (6).

High-throughput RNAi-based screens for whole genomes have been developed to identify loss-of-function phenotypes for individual genes in plant and animal cells (7-9). Furthermore, it has been shown that short interfering RNA (siRNA) molecules can be synthesized for use in the selective inhibition of specific genes for periods of days or weeks. Thus, RNAi has the potential to be used in the treatment of many types of human diseases related to the enhanced expression of genes and related proteins (10, 11), and trials to test the response, sensitivity and toxicity of siRNA in humans are being conducted (6). Cancer is known to be related to aberrant genetic regulation, and siRNA and microRNA thus hold the potential to more clearly define the genetic mechanisms involved in cancer initiation, validate new therapeutic targets for cancer treatment and silence the overexpression of genes known to be responsible for cancer initiation and/or progression. This review article will focus on the potential applications of RNAi in the treatment of cancer.

The molecular mechanism of RNAi

The RNAi silencing of specific gene expression begins with the cleavage of large dsRNA into relatively small (approximately 19-22 base pairs) pieces of siRNA by the Dicer enzyme (see Fig. 1). These small pieces of

siRNA combine with RNA polymerase and form a protein assembly called the RNA-induced silencing complex (RISC), which leads to unwinding of the bound siRNA. The antisense strand of siRNA retained in the siRNA-RISC complex then binds to mRNA complementary to the siRNA, and the RISC complex produces single-site cleavage of the target mRNA (12). The cleaved mRNA fragment is thus destabilized and eventually completely degraded by cellular ribonucleases. The activated RISC complex is then available for another cycle of mRNA degradation. The activated siRNA-RISC complex is very stable and silencing has been reported to persist for weeks in some tissues (13-15). Since this process is highly selective, it provides a tool to create functional gene knockouts which can be used to identify gene function and for therapeutic target validation.

The siRNA and microRNA approaches appear to have therapeutic advantages when compared to other types of gene-based therapy. For example, antisense DNA gene silencing is stoichiometric, which means that one molecule of antisense is required for the inactivation of each molecule of target DNA, therefore requiring large and potentially toxic doses of antisense DNA. In contrast, cellular levels of target mRNA are much lower, and the activated siRNA-RISC complex is capable of destroying many copies of target mRNA. In addition, siRNA-induced inhibition is generally much longer acting than antisense DNA-induced inhibition. Furthermore, RNAi is generally reversible, so that RNAi effects before and after silencing can be compared in the same sample.

siRNA and microRNA in the treatment of cancer

RNAi has the potential to be used in a variety of diseases associated the elevated expression of specific genes, and numerous biotechnology companies are involved in the development of novel RNAi therapeutics (16). It appears that an early clinical application may be the siRNA targeting of vascular endothelial growth factor (VEGF) for the treatment of age-related macular degeneration (AMD), with the use of intraocular injection to simplify nucleotide delivery (17). The treatment of various viral and inflammatory diseases may represent other potential clinical applications for siRNA. For example, siRNA has been shown to reduce the replication of HIV, influenza, polio and hepatitis C viruses (HCV) (18-23). Since viral infections and tissue inflammation are associated with the initiation of numerous cancers, RNAi approaches may be used as a form of chemoprevention. Furthermore, since cancer is known to be a disease of genetic mutation and deregulation, the application of RNAi technology for the treatment of cancer has also received a great deal of attention (24, 25).

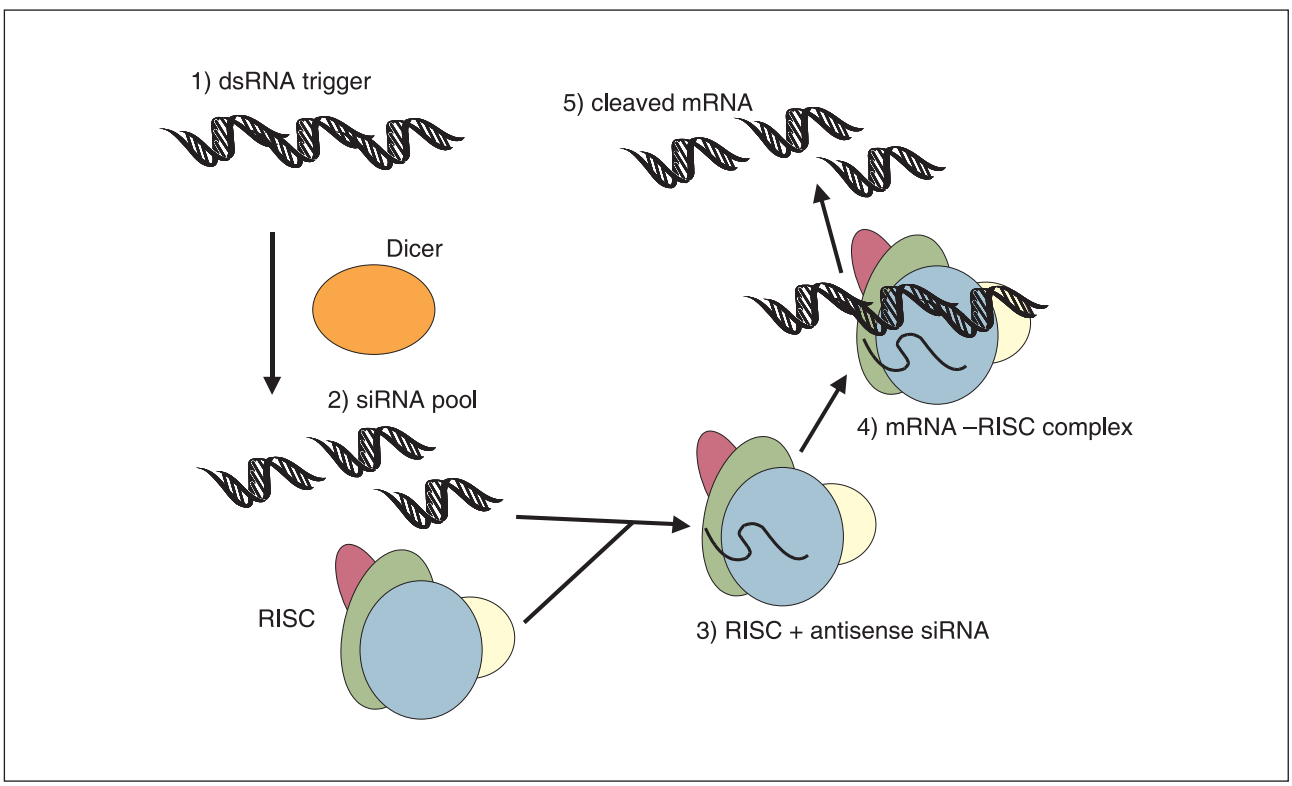

Fig. 1. The RNAi molecular mechanism. 1) dsRNA in the cell cytoplasm initiates the RNAi process. 2) Dicer is an enzyme that breaks the dsRNA into a pool of siRNA approximately 19-22 base pairs in length. 3) The RNA-induced silencing complex (RISC) interacts with siRNA to cause its unwinding. 4) The antisense siRNA-RISC complex directs interaction with complementary mRNA. 5) The RISC complex contains endonuclease activity which produces degradation and silencing of the selected mRNA.

The use of synthetic siRNA is an approach that is ideally suited to the treatment of cancers which are known to be associated with the overexpression of specific genes (26). For example, tumors do not create a vasculature system, so that as the tumor mass grows larger than a few millimeters in diameter, it must acquire blood vessels from surrounding normal tissue. VEGF is known to be involved in the neovascularization of solid tumors and to be essential for tumor growth and progression (27). Accordingly, many groups have employed VEGF siRNA to inhibit cancer angiogenesis (28) and reduce the growth and viability of many types of cancer, including gastric (29) and breast cancer (30-33), melanoma (34), retinoblastoma (35) and leukemia (36). It should be possible to target any gene that is overexpressed and/or associated with the progression of cancer, such as epidermal growth factor (EGF) (37), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF) (38), fibroblast growth factors (FGF), transforming growth factor (TGF), tumor necrosis factor (TNF) (39), signal transduction intermediates and cell cycling regulators (11, 40). Silencing of promoters for genes associated with cancer using RNA-directed DNA methylation is another viable approach. Kawasaki and co-workers have employed this method to inhibit erbB2, which is often overexpressed in aggressive breast, ovarian and other cancers (41).

Another group of potential therapeutic targets for siRNA in the treatment of cancer are the oncogenes, proto-oncogenes and mutant tumor suppressor genes. Transforming oncogenes such as Ras and overexpressed inhibitors of apoptosis such as c-Myc and Bcl-2, which are known to be associated with many human malignancies, appear to be suitable targets (24, 37, 42-44). Concerning tumor suppressor genes, *p53* is a tumor suppressor that is known to be mutated and dysfunctional in the majority of human cancers (16). The mutated p53 protein represses the tumor suppressor action of nonmutated p53, and siRNA has been shown to block the expression of mutant *p53* and to reactivate *p53* tumor suppressor activity (45). Furthermore, RNAi-based genome screening has been employed to identify novel tumor suppressor genes (46, 47).

An apparently ideal target for siRNA therapy is the BCR-ABL fusion protein, also known as the Philadelphia chromosome, which is created by the rearrangement and fusion of the *BCR* gene on chromosome 22 and the *ABL* gene on chromosome 9, resulting in a hybrid composite oncogene (48, 49). Several BCR-ABL-associated tyrosine kinases enhance downstream phosphorylation and are associated with the development of chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL) (50). It has been reported that siRNA silencing of the BCR-ABL fusion protein alone or in combination with chemotherapy could be used to induce apoptosis and reduce cancer cell proliferation in CML (10, 51-53).

Therapeutic approaches based on siRNA that antagonize endogenous microRNA should be effective because microRNAs are known to be involved in the regulation of cell growth and differentiation, processes that are in turn known to be directly associated with cancer initiation and progression (26, 54-56). In addition, some of the microRNAs may function as oncogenes or oncogene activators (55-58). There is strong evidence for a relationship between the expression of certain microRNAs and the development of various cancers (26, 43), including colorectal (59), lung (60), breast (61), thyroid (62) and pancreatic cancers (63), glioblastoma (64), lymphocytic leukemia (65, 66) and B-cell lymphoma (67). Some of these studies have used expression profiling to identify aberrant expression of microRNA in cancer specimens compared to normal tissue. There appears to be a significant overlap in the aberrant expression of microRNA among different types of cancer (63).

Considerations for the use of RNAi in the treatment of cancer

Although siRNA technology holds the potential for the development of highly specific and effective treatments for cancer and other diseases associated with altered gene expression, several obstacles must be overcome to make this treatment a reality.

Drug delivery

Nucleotide delivery to a specific tissue at the required concentration has been an important obstacle for all forms of gene therapy (25). Although siRNAs are readily taken up by invertebrate cells, uptake into mammalian cells often compromises siRNA bioactivity (11). Liposomal encapsulation and other lipid-based transfection systems can be employed to facilitate uptake into many cells *in vitro*, although some cells are refractory to lipid transfection. Furthermore, transfection is often less effective *in vivo* than it is *in vitro*. However, direct application of siRNA-lipid complexes may be possible with cancers of surface tissues that are easily accessible, such as the skin, oral mucosa, nasopharynx, lungs, vagina and cervix. For the treatment of internal cancers, it has been possible to couple the siRNA to cholesterol to facilitate uptake via cell-surface LDL receptors in the liver and intestine (13, 68). In addition, conjugation of the siRNA to an antibody or natural ligand for a cell-surface receptor known to be overexpressed by the cancer cells is another viable mechanism to deliver the nucleotide specifically to the desired cancer tissue (69). The use of viral vectors has been successfully employed with other types of gene therapy (70-74). Retroviral, adenoviral and lentiviral delivery vectors have been used with siRNA to silence the expression of specific genes and related proteins both *in vitro* and *in vivo* (75-77). Thus, it appears that a variety of systems are available for the effective delivery of siRNA for the treatment of most cancers.

Target specificity

It is known that nontarget microRNA and other mRNAs containing sequences that are similar to the

siRNA may also be silenced and lead to undesirable side effects (78). The fact that most microRNAs are only partially complementary to mRNA targets further contributes to this problem (16). Methods such as the use of mRNA microarray screening can be used to reduce or avoid off-target effects (78). Also, siRNA could trigger an immunostimulatory response via an interferon pathway. Natural immune systems designed to recognize foreign RNAs associated with viral infections can activate the immune system (79, 80). In most cases, activation of the interferon pathway requires dsRNA of at least 30 nucleotides, so that siRNA of 20-22 nucleotides should not create a high degree of immunotoxicity (81).

RNA stability

Unmodified siRNAs have a very short half-life on the order of minutes in the circulation (25). In order to be used as therapeutic agents, the siRNAs may have to be modified to increase stability and/or target specificity in the body, as previously discussed (68, 69). Other approaches to enhance stability might consist of changes to the nucleotide backbone, modification of sugars or alteration of the overhangs on each end of the dsRNA nucleotide (17). Also, the gene-silencing efficacy of the siRNA can be improved by reducing the guanine-cytosine (GC) content and reducing internal repeats, which often results in superior thermodynamic characteristics (82).

Drug resistance

Rapidly multiplying cancer cells may become resistant to siRNA by the selection of cancer cells with mutations to the mRNA target. Since target microRNA is from a non-coding region, the coding mRNA, protein expression and thus cancer cell proliferation may not be significantly altered by these mutations. The use of several siRNAs that target different mRNAs or different regions of the mRNA may be used to avoid this problem and to enhance the efficacy of siRNA therapy (78). Another type of drug resistance involves the overexpression of multidrug resistance (MDR) genes which are known to be responsible for drug resistance to many cancer chemotherapeutic agents (83). However, it has been demonstrated that siRNA treatment can be used to effectively knock out MDR expression and restore chemotherapeutic efficacy (84, 85).

Conclusions

Our understanding of siRNA, microRNA and RNAi methodology has developed very rapidly over the past decade. The RNAi methods provide an important array of tools that are being used to gain insight into the genetic regulation of normal and malignant tissue. Furthermore, these methods are being used in the development of new, highly specific approaches to the treatment and/or prevention of cancer and to the identification of new cancer biomarkers. It appears that the combination of RNAi approaches with existing chemotherapy may enhance the therapeutic efficacy of both treatments. As with any new therapeutic approach, there are obstacles related to delivery, selectivity and resistance which must be overcome for the commercial development of this new class of anti-cancer therapeutics. In summary, RNAi holds tremendous potential for the early diagnosis and highly selective treatment of cancer.

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