

RNA Interference (RNAi) for the Future

By

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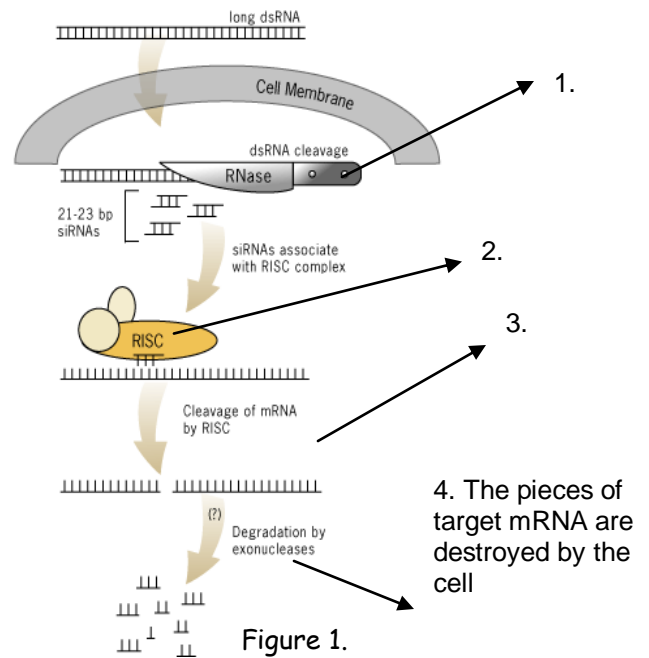
Abstract

RNAi is the method of gene silencing using siRNA (small interfering ribonucleic acid) to stop and/or manipulate gene expression to create desired characteristics, for example the silencing of a gene causing cystic fibrosis or Hepatitis in animals. One of the problems associated with RNAi is establishing a theoretical method as to how to manipulate a cell to turn off any genes eg which cause Hepatitis as well as how to potentially ensure siRNAs reach the correct cells and are effective. Using RNAi for silencing a variety of genes can create ethical issues brought up by this development in gene control. RNAi could be used to cure Hepatitis B, eg. by 'turning off' the genes that enable the cancerous and HBV infected cells to divide. There are potential ethical issues that need to be combated before RNAi can be extensively used. Another problem could be how to administer siRNA to the correct cells requiring them eg using the same virus for different cases of RNAi, which has been genetically altered to render it useful and safe.

Introduction

RNAi ultimately was discovered by 'Fire' and 'Mello' but were aided by earlier research three years prior conducted by 'Guo' and 'Kemphues' on *Caenorhabditis elegans* while trying to ascertain the function of the par-1 gene. 'Guo' and 'Kemphues' attempted to use antisense RNA to stop the expression of the par-1 gene in order to establish its function but found that the sense strand had the same effect of disrupting the gene expression. After this discovery, 'Fire' and 'Mello' injected dsRNA (double stranded ribonucleic acid), both sense and antisense strands into *C. elegans*, producing total silencing of the homologous gene in the worm; a more efficient method of silencing using a few molecules of dsRNA per cell. This was then passed onto the offspring. Further research demonstrated that bathing worms in dsRNA also produced silencing results as demonstrated in the article by 'Applied Biosystems' 'RNA and Gene Silencing et al'. RNAi, the mechanism, is the effect of using Dicer to dissect dsRNA into siRNA after transcription and cause cessation of certain genes. The process is 1. dsRNA enters a cell and is recognized by the enzyme Dicer, a part of the family dsRNA specific endonucleases. Here it cleaves it into shorter dsRNAs, named siRNAs (small interfering ribonucleic acids), as shown by 2 nucleotide long 3 prime overhangs. 2. The siRNAs form a ribonucleoprotein complex, RISC. RISC is a 'SLICER' made of argonaute protein. 3. The RISC complex formed controls the unwinding of the siRNA when this is affixed to RISC, binds to target mRNA in a certain sequence. This binding creates a cleavage plane that is complementary to the siRNA sequence as it severs the target mRNA in the middle of the siRNA sequence. 4. The target mRNA, now in smaller pieces, is recognized by the cell as abnormal and unusual and therefore is destroyed, thus impeding translation and silencing the gene expression, as described on the 'Applied Biosystems' website 'RNA interference an Overview et al'. This is also described by the video of 'RNA Interference' by 'Nature Reviews et al'. This process is illustrated in figure 1. (found on the 'Applied Biosystems' website 'RNA interference an Overview' et al). These processes are effective in plants. However humans, as well as other higher mammals, are incapable

of utilizing this defense as the introduction of long dsRNAs, for example those in viruses, creates an interferon based inflammatory response which causes all protein production to cease despite the presence of RISC and dicer. Effectively, the inflammatory interferon based response blocks the RNAi pathway; therefore, short RNA strands (siRNA) are needed to create the interference response, but not the inflammatory response. The main problem associated with this is delivering the siRNAs to cells in need of them rather than triggering responses elsewhere or not reaching the specific site and therefore becoming ineffective.



One development in animals using RNAi was the reduction of eye angiogenesis, which is due to new blood vessels being formed from pre-existing vessels that can lead to blindness in the form of age-related maculopathy and diabetic retinopathy as described in 'Principles of Inhibition of Angiogenesis in the Eye' et al. RNAi also has been developed to treat SARS virus in primates by delivering the siRNA of the virus, which produced a decrease in fever and damage to the alveoli described in the article 'Performing RNAi experiments in animals' author unknown. One previous development of RNAi leading to particular beneficial uses, is the silencing the production of leptin secreted by the brain, in vitro. In order to ascertain the physiological effects of this type of leptin rather than the pituitary gland and C6 glioblastoma cell leptin, siRNA 7 was used to reduce leptin mRNA by 50% causing a rate of cell death twice the normal rate of C6 cells; as described in the article 'RNAi-mediated Silencing of Leptin Gene Expression et al'. Leptin is detected in the hypothalamus using receptors as a hormone that inhibits appetite by negating the effects of neuropeptid Y. It causes organisms to loose weight if injected with leptin when excessively obese as shown by the experiments carried out on obese mice with the genetic mutation forcing them to gain weight. Further information can be found on the Wikipedia website regarding leptin production. This could be a vital development for veterinary Medicine because obese animals, suffering from degenerative diseases associated with obesity, could be treat using RNAi to turn off the mutated gene, thus allowing them to loose weight more easily using fast, natural and effective methods. This could be used if they have the degenerative diseases or as a preventative measure as they begin to develop these problems and start to suffer. Other findings have shown the obese people have high concentrations of leptin in their systems and similar to type 2 diabetics, are not sensitive enough to its effects. Therefore RNAi could be used to turn off the

leptin producing gene temporarily to allow the body to become sensitized to little/no leptin, before resetting the gene to produce leptin again that the body can be sensitive to. In humans, RNAi research has led to trials on wet age - related macular disorder in the eyes, respiratory syncytial virus and for acute kidney injury according to the paper written by Johannes Fruehauf et al. Age related macular disorder is caused by deposits of drusen- protein and lipids, outside the cell. It can cause blindness of central vision and has genetic and environmental implications. If proved successful in humans, there may be more advancements in some of these disorders for animals.

Discussion

This could be useful for veterinary medicine as RNAi could be used to turn off the genes that have mutated causing the mice, or any animal with the mutation to become obese. A simple genetic test could be carried out if the animal is suspected to carry the mutated gene. Then a novel use could be to inject dsRNA sliced into siRNA into the fat storing cell surrounding a certain area, for example the major organs, or for results closer to the surface, directly under the skin. This then could associate with RISC and cut the target mRNA in a sequence specific manner before being recognised as unusual by the cell, thus destroying any target mRNA. This would effectively stop translation and replication of any mutated genes leading to obesity. The change in the genetics of the fat storage cells would spread throughout the body naturally as the fat storage cells replicate by mitosis producing genetically identical cells. This alteration in the genes would then pass onto any offspring as a characteristic of RNAi, which would also help to eradicate obesity gene mutations. Another novel use of using RNAi could be using siRNA to destroy the target mRNA of the genes producing leptin for a certain amount of time, then to re-introduce the gene that produces leptin, so that leptin production increases to the level it was before the interference. This would be effective on patients that, much like type two diabetics, had become desensitized to the effects of leptin.

The current research regarding wet age-macular disorder could be useful with regards to rhesus macaques as they share a structure similar to humans with a macular, which is susceptible to drusen the macular pathology, including a foveal pit. Their eyes have a large amount of features that allow spatial perception for example a large concentration of cone photoreceptors, layers of retinal cells that are displaced (similar to some vessels), as well as an area void of capillaries. This supports why there has been a discovery of a large number of colonies of macaques with drusen and pigmentary changes similar to intermediate stage human age-related macular disorder (one of the colonies studied reside at the Oregon National Primate Research Centre). This was backed up by showing that the drusen, of increasing size build up over time in the results of the same article 'Rhesus Monkeys and Humans Share Common Susceptibility Genes et al'. The effects of

wet-age related macular disorder include slow degeneration of central vision, which is the same as initial stages of the dry macular disorder, but it occurs before blood vessels develop abnormally under the macular, which burst in the wet type as described in the article reviewed by Sunir J. Garg et al. The developments in RNAi as a cure for this degenerative disease could be vital for these animals as currently, there's no cure or specific treatment, other than natural treatments such as extra antioxidants, eating more omega 3 and leafy green vegetables. The cure could be injecting siRNA that leads to destruction of any target mRNA of the gene causing AMD or any target mRNA of mutated genes causing AMD. The other choice of treatment involves injecting medicines into the eye, for example ranibizumab, to stop the excessive bleeding. This in itself can be dangerous for any animal as they would have to undergo surgery to have this treatment, which as the name suggests occurs in older animals who are less likely to recover and 'wake' from any anesthetic. This coupled with the fact that any animals would have to be caught could lead to high stress levels, particularly in wild or semi wild animals, which may also increase the chances of death. These factors may be ethical issues in themselves as it may be too traumatic for the animal at an old age to be forced into this situation. However, it could provide any animal with a better quality of life and could provide research for the human market. This is also a contentious issue as in many people's opinion it is barbaric and cruel to test or carry out research on animals for a human's benefit when it causes pain, distress or puts the animal in an unusual and unfair position, for example a high demand for Rhesus Monkeys could increase black market sales of the breed and increase poaching.

Acute Kidney injury, formerly known as acute renal injury, is a swift loss of the functions of the kidney according to the Wikipedia website. This implies that the kidney failure it is referring to is when the nephrons are lost due to toxins, infections or cancer as well as old age, as mentioned on the 'Abbey Vets' newsletter. As detailed in the newsletter, 2/3 of nephrons are usually lost before pronounced symptoms occur, which cannot be replaced but can be treated using injections of vitamins and minerals to replace any that's not re - absorbed in the blood. Steroids and antibiotics along side a specially formulated diet eg 'Renal Failure' food by 'Royal Canin' can also used in treatment. Research that could turn off cancerous genes that metastasize would be effective treatment for early kidney failure. SiRNA could be used to destroy any target mRNA of the cancerous cells, thus destroying all traces eventually. Another way that kidney failure could be tackled could be to turn off the gene that is controlling the lack of replacement of the nephrons so that ones that are lost are continually replaced. This could be achieved by using siRNA to destroy any target mRNA of the gene that stops the replacement. This could lead to ethical issues again as animals that normally suffer from kidney failure are old, for example geriatric cats, so it could be perceived as unfair to the animal to be put through this kind of treatment in which the invasiveness is currently unknown.

Hepatitis B, the inflammation of the liver has many causes, for example a virus carried in the liver called Hepatitis B or HBV as detailed on the 'British liver Trust' website. Becoming infected with HBV, for example by the exchange of bodily fluids, leads to constant intrahepatic inflammation as a result of the constant infection, so the host's liver attempts to regenerate by attacking the cells infected using apoptosis. Apoptosis is a genetically controlled cellular death program different to necrosis, which is caused by cellular injury as detailed on Wikipedia. As a result of being genetically controlled and regulated, it is used for the regular destruction of liver tissue cells. The intrahepatic inflammation causes the liver to undertake a large amount of cellular destruction, then regeneration. This in itself could cause liver tumors by one of two ways, either that the mitotic division creates cells with slightly altered DNA due to the increased mitotic division during a chronic disease, or that the removal of infected cells is not occurring fast enough. The liver cell destruction usually occurs via the Fas/FasL pathway as cell death receptors, which could be as a result of evolution and the increasing need to destroy hepatic cells infected with viruses. Once infected, the T lymphocytes destroy the cells expressing the HBV antigens on their surface membranes using the Fas apoptosis pathway, which can be initiated by interleukins or inflammatory cytokines, as demonstrated in the article 'Apoptosis: a mechanism of acute and chronic liver injury' et al. The article demonstrates that studies have shown there are some tumors that are showing a loss of Fas regulation but an increase in FasL which allows the tumor to kill immune cells creating an area for tumors to proliferate. In order to stop this, cancerous cells can be prevented from metastasizing by switching off the genes FasL which allows the tumor to kill T lymphocytes and other killer cells. This could be completed by using siRNA which will associate with RISC and destroy the target mRNA of the genes of a certain amount of FasL cells. This should stop FasL being replicated and reproduced at the level which generates too much FasL. Then siRNA could be used to switch off the cancerous cell genes and the genes of the cells with HBV to stop their mitotic division and reproduction. This could be done by introducing siRNA to the cells containing the virus and the cancerous cells after transcription. The siRNA should couple with RISC and destroy the target mRNA, thus stopping their division and any metastasis allowing the protein Fas, to work at a level which would be fast enough for the cellular death pathways to work efficiently as well as not producing extra cancerous cells. This would allow the liver to regenerate after being infected with Hepatitis B.

One major problem with using siRNA as a method of gene silencing is how to transport the siRNA to the correct cells requiring them and into the cytoplasm of every cell intended. This is also complicated by the fact that the nature of the siRNA molecules mean that they undergo a form of disintegration in the blood. This is due to being broken down easily by enzymes when in contact with RNases in serum, thus potentially ruling out injection of siRNAs into the blood stream far from their target cells. There is also another problem posed even if the siRNA

manage to reach their target cells, which is that siRNAs are polar and therefore, cannot enter a cell through the cell surface membrane which are lipophilic and hydrophilic as mentioned in 'TransKingdom RNA interference et al'. One effective method of delivery could be the use of viruses as they have the innate 'machinery and programming' to invade cells and reproduce. The viruses with the specific genes to invade and replicate can be used if, for example the gene that damages the human host cell is removed by genetic modification to be replaced by the desired siRNA and still contains the gene that encodes the invasion of cells. The virus could also be genetically altered to have antigens complementary to the antibody receptor of the target cell so that the target cell are virus are attracted by chemotaxis once inside the body This could be achieved for example in a form of vaccination allowing the virus to invade the target cell. This would reduce the problems of reproducing enough to target every cell, ensuring that all the target cells were reached and the virus enters each cell. Another way of administering siRNAs, for example to treat leptin by using siRNAs in adipose tissue, could be achieved by using key-hole surgery and a tiny endoscope with small needle attached. Then using the endoscope, you could find an area of high density of tissue and inject the siRNAs, at a nanoparticle size, into various cells in the tissue. This would ensure that the siRNAs would reach their target cells and eventually over time would divide by mitosis to comprise the whole tissue. There are two other forms of delivery of siRNA, liposomal delivery and local delivery. The first comprises of using methods used for other drugs, where as other uses methods such as stabilized nucleic acid lipid particles. Although these have only been tested in vitro, in the future these methods could be used in living multicellular animals. The second comprises of inhalation of the siRNAs or injection of siRNA, but as siRNAs are unstable, it's not the optimum method, as described in the article 'TransKingdom RNA interference et al. There are certain ethical issues associated with RNAi, for example the ethical issues mentioned earlier as well as others. An example of this would be how to know when to accept that euthanasia is the next step in some cases. This could be due to a geriatric cat having problems but the owner not wishing to accept that the best option is euthanasia and opting for potentially invasive surgeries because of the love for their pet and attachment to the pet, which could be worse for the animal. Another ethical issue is that with time and new advancements, RNAi may become cheaper and more affordable or accepted by insurance companies as a benefit and so are fully included in policies, causing it to be viewed as a viable option rather than safer options, for example changing the diet and exercise of pet. These surgeries may also be more invasive than initially thought and until the surgeries have become the norm and less invasive with experience, may be seen as cruel as they are forced into painful surgeries. It may also lead to more deaths on operating tables as more animals experience different surgeries. The surgeries may also be high risk as they are involving new and innovative technology that has not been tried and tested for as long as other

surgeries eg standard neutering, which could lead to other unforeseen problems. This could be classed as a form of cruelty to the animal if they felt new pain or were in more pain than before the surgery. The cost of the research could be another ethical issue, as in order to complete just one section of the process i.e. generating enough nanoparticle lipid siRNAs could cost an estimated \$100,000 per year according to the 'Transkingdom et al article, which certain members of the public may find excessive and should be used on other specific areas, for example donated to charities such as 'WSPA' or human based charities such as water aid.

Conclusion

Overall, RNAi could be the future to curing many genetic problems for example obesity problems and cancers, both in humans and animals but there will need to be a colossal amount of extra research into the subject before it is fully operational and then monitoring as to how effective it is, how painful it is and dealing with any problems that arise. Each problem that does arise can be theoretically solved using new methods which will also require further research. The ethical issues associated with RNAi will also have to be monitored eg. by a board of vets to ensure that whatever work is carried out is in the best interests of the animal. Some problems faced with using viruses as a vector could include: the cost to work at such a tiny level using viruses, mapping the genome of the required viruses so that the correct genes were silenced and/or removed in the virus to make it slightly safer than using a 'live' virus and/or using a virus which could damage the host cells if the genes are incorrectly silenced or removed which could initiate a specific immune response as opposed to the desired gene silencing response and public safety. Injecting viruses into animals, at least not in the widely accepted form of a vaccine, could cause public concern. This could be combated using nanotechnology and computers to map the genome, as well as using one species of virus to cut down on the workload. A way to keep the costs relatively low would be to use volunteers and secondhand, but still correctly functioning equipment. As part of the research, the animal could be closely monitored for any signs of the virus and being stocked up on treatments if they were to become ill. If the animal did become ill, it would be another ethical issue as more harm is being done than what is benefitting the animal. The owners of the animals could be educated as to how RNAi works and the risks and benefits to keep public concern to a minimum, as a way to combat the problem of public concern. A problem associated with the key-hole surgery could be that it may take too much time to occur depending on how ill the animal is, which could be combated using more siRNAs and specific close monitoring.

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