

RNA Interference: It's application in Veterinary Medicine

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Abstract

RNAi is a relatively new scientific discovery which enables genes to be “switched” on and off. Researchers hope that this discovery will lead to advancements in the treatment of viral infections, genetic disorders and other diseases caused by genetic malfunctions. This paper describes the research into the use of RNAi in Macular Degeneration, against Respiratory Syncytial Virus and in Cancer in humans. In the future the techniques that are being developed in human medicine will hopefully become available to Veterinary Surgeons as methods for treating both large and small animals. Examples of suitable diseases for this method of treatment are Bovine Viral Diarrhoea, Genetic defects in small animals and Cancer. However, before this is possible there are numerous obstacles that have to be overcome to make the widespread use of RNAi an achievable goal.

Introduction

RNA interference was first observed by plant specialists in the USA and the Netherlands in the 1990's, after an attempt to produce petunias of a more intense purple colour. The scientists introduced copies of the gene which encoded for the colour, in this case Chalcone Synthase, but instead of producing darker flowers, the increase in this gene resulted in less pigmented or even fully white flowers (Fig 1.). At the time, this phenomenon was unexplained but after further research it was found to be caused by post-transcriptional inhibition of gene expression via an increased rate of mRNA degradation. This was called co-suppression of gene expression but at this stage the molecular mechanism which caused it was unknown.

After these initial findings many scientists across the world began to search for examples of this occurrence in other organisms. The most notable of these findings was discovered by Craig C. Mello and Andrew Fire. They carried out experiments on the worm *C.elegans*, and found that the double-stranded RNA (dsRNA), in the antisense RNA preparations that they were using to silence genes in their investigations into embryonic cell differentiation, were responsible for the inducing of the interference [1]. Because of the importance of their discovery to the medical world, they were awarded the Nobel prize in Physiology or Medicine in 2006.



Fig 1.

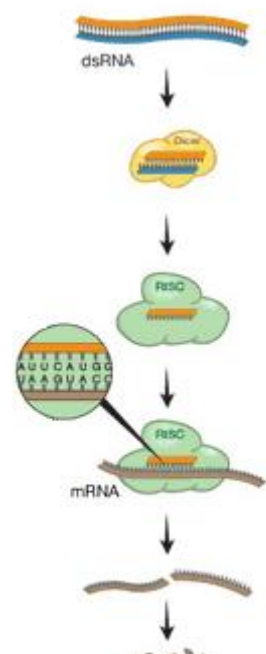


Fig 2.

RNA interference

works in the following way (Fig 2.) : Upon entering the cell the dsRNA is cleaved into small fragments by an enzyme called Dicer. Each of these small fragments or short interfering RNAs (siRNAs) contain 21-23 nucleotides with two-nucleotide 3' single-stranded overhangs. These siRNAs bind to proteins and form the RNA-induced silencing complex (RISC). One of the proteins in this complex (Slicer) separates the double stranded RNA so that just one strand is left bound in the RISC. This complex then binds to the messenger RNA (mRNA) in a sequence specific manner - the nitrogenous bases pair up. It then cleaves the mRNA and the fragments are recognised by the cell as abhorrent and are destroyed. This prevents the translation and transcription of

the genes and the protein cannot be formed.

Although RNAi is not being used in the medical world at the moment, many trials are being carried out into possible uses. There are two main areas of research at the moment; targeting the genes of viruses attacking the body and targeting genes within the human body itself. This second category is subdivided into many other areas including stimulating immune responses and switching specific genes on or off.

Some specific areas that have been researched recently are macular degeneration (loss of vision in the centre of the visual field because of damage to the retina - sometimes caused by gene mutations), respiratory syncytial virus (a negative-sense, single-stranded RNA virus that causes respiratory tract infections especially in young children - at present there is no vaccine and only supportive care) and cancer (a group of cells showing uncontrolled growth, invasion and sometimes metastasis).

The research that is being carried out into Macular degeneration has come up with some unusual finds. Macular degeneration is caused by blood vessels overgrowing in areas of the eyes which are needed for vision, blocking out light and therefore impairing vision. This process is known as choroidal neovascularisation (CNV). To begin with, researchers targeted receptors that promote blood vessel growth (a VEGF receptor) in the eyes, in the hope that this would prevent CNV. In order to compare the results of this treatment, a number of controls were used; RNAi that targeted proteins not found in the eye and RNAi that was directed to proteins found in insects and bacteria. Instead of not working, as the researchers expected, these experiments still resulted in improvements in sight. This was very unexpected but eventually it was worked out that as part of a cell's innate immune system there are many surface receptors that attach to the double-stranded nucleic acids used by viruses. The researchers tested a couple of these receptors using mutant mice, and found that protection from CNV required the presence of a protein called Toll-like Receptor 3 (TLR3). Using other mutations, the authors concluded that binding of double-stranded RNAs causes TLR3 to activate immune signalling molecules like Interferon- γ and Interleukin-12, both of which can block blood vessel growth. During this research, the scientists also discovered that some people carry variations in the TLR3 gene that make them more or less receptive to RNAi treatment. This means that RNAi will not be suitable for all patients and that genetic profiling should be carried out before treatment is decided on. [2]

The main aim of the research into respiratory syncytial virus (RSV) is to design a drug which can be used as a prophylactic or as a therapeutic antiviral agent and to develop a method which allows sustained protective immunity. The research has discovered that RSV can be successfully targeted by an intranasal delivery of in vitro-active siRNA which is directed at the P gene of the RSV. This has been shown to significantly inhibit the replication of the virus. This method of treatment is so effective because the RSV replicates in the respiratory epithelium of humans. This has allowed the treatment of this disease to progress further than in many other research areas as one of the main problems with RNAi is the difficulties in introducing the dsRNA into the cells. However, although the new improvements in the treatment of RSV are very promising, the researchers have still not developed a method which

allows an improvement to the memory responses of the body towards this virus. There is also research being carried out into the development of this treatment for other viruses, for example, human immunodeficiency virus (HIV) and hepatitis. [3]

The research into RNAi treatments for cancer is based in two different areas. When a cancer begins to develop, there are two main genetic abnormalities. The first is the activation of cancer promoting oncogenes. These cause hyperactive growth and division, protection against programmed cell death, the ability to establish themselves in different tissue environments and the loss of respect for normal tissue boundaries. The second is the inactivation of tumour suppressor genes. This results in changes to the normal functions of cells, for example, they lose control over the cell cycle, they no longer replicate DNA accurately, they change their orientation and adhesion within tissues and they no longer interact with the immune system.

Because of these two types of genetic abnormalities, the research into RNAi treatments can be directed at both sets of genes - either 'switching off' the activated oncogenes or restoring the normal functions of the inactivated tumour suppressor genes. There has also been investigations into using RNAi alongside chemotherapy as a way of silencing genes that make some cancer cells resistant to this treatment. [4]

Although all the research at the moment is targeting the treatment of human diseases, there are many possibilities for using this treatment in veterinary medicine as well.

Discussion

In animals, as with humans, there are a large number of different areas where research could be focused. These include, treatment and prevention of viral diseases which at the moment are difficult to cure, treatment and prevention of genetic disorders and treatment of cancer. These areas themselves could also be targeted at different markets- animals used in farming, or animals kept as pets. Both of these areas have different considerations which need to be taken into account when developing the treatment, for example, treatments for farm animals need to be economic and easy to administer.

There are many viral diseases which could be treated using RNAi. One example is Bovine Viral Diarrhoea (BVD). This disease is very common in the UK and at the moment is untreatable. It is caused by a small RNA virus of the Togavirus family which can survive storage at -4°C for at least 16 months. This, combined with the fact that there are at least 13 strains of the virus, means that it is very difficult to control. Paton et al. (1999) report that 95% of 1071 dairy herds in England and Wales were positive for BVD virus antibodies in bulk milk, with 65% of the herds likely to have suffered an outbreak in the recent past and currently having persistently infected animals in the herd. The effects of the disease can cause large financial losses for the farmers, and this is one of the reasons why RNAi treatment for this disease may be so successful.

The disease has many short term and long term effects, other than just an outbreak of diarrhoea. These include infertility, embryonic death, foetal mummification, abortion, congenitally damaged calves and persistently infected (PI) calves that are likely to die

of mucosal disease before two years of age. There has also been evidence found which suggests that the BVD virus may act as an immunosuppressive agent. This would increase the likelihood of contracting common infections such as respiratory disease in calves, salmonellosis, interdigital dermatitis and mastitis. [5] [6]

Research into uses of RNAi to deal with BVD could target the virus in a similar way to RNAi uses for RSV (a viral disease of humans also caused by an RNA virus). However there are problems with this as the BVD virus does not reproduce in the respiratory epithelium like the RSV does so introducing the siRNA into the infected cattle may be more difficult. Also, the longevity of the siRNA once they are inside the cells needs to be improved, to allow prolonged prevention of the disease. Treatment of the disease, once it has been contracted, would also be an important area of research, as this is one of the main problems with BVD; it is preventable by isolation of diseased cows, but there is still no treatment which is able to cure the infected animals.

Many viral diseases of small animals are preventable via yearly vaccination, for example, Feline immunodeficiency virus and Feline leukaemia in cats. However there are still no treatments available for many of these diseases and this is another area into which research could be directed.

Genetic disorders are less common in animals than they are in humans, but there are still opportunities for research to be carried out into these areas. Many genetic disorders in animals are caused by mutations to several genes, which would make it harder to treat, as different siRNA would need to be used to target each gene. With further advancements into the genetic basis of diseases in animals, RNAi may become more useful as a method of treating and dealing with these disorders.

In animals, genetic disorders are mainly breed specific. This is due to a reduction in the gene pool of pure bred animals, meaning that inbreeding is more common and genetic mutations are more likely. Also, especially in farm animals, the use of artificial insemination (AI) has meant that some genetic disorders have become more common, as the sperm of one bull can be used to fertilise the eggs of thousands of cows. This means that if the bull carried the gene for a genetic disorder (even if it was only a carrier rather than being directly affected) the gene could be passed on to all its offspring. An example of this is Citrullinaemia, a genetic disorder of Holstein and Friesian cows in Australia. The mutation responsible for this came from a bull which had been used for AI to increase the butter fat content of milk. However, by breeding so many cows with this bull, the genetic disorder became quite common. It has now been controlled due to screening of sperm before using for AI, but at the time it was untreatable. [7]

A lot of research has been carried out into the treatment of cancer using RNAi in recent years and this is an area in which the research in humans could be directly translated for use in animals. Cancer is common in domestic animals and farm livestock (although incidence of cancer in the latter is reduced due to most farm animals being slaughtered while they are relatively young). In fact the incidence of tumours in dogs is higher than in any other species including humans, showing that this is an important area of research. [8]

At the moment the options for cancer treatment in animals are as follows:

Surgery - this can result in either partial or complete removal of the tumour.
- the goal of complete removal is to remove all of the tumour cells present in a given location, thereby curing the patient or at the least relieving symptoms for an extended period of time.
- partial removal or debulking of tumours is carried out when the tumour is too large or in a location where they cannot be removed completely. The aim of this is to remove as much of the cancer as possible, while minimizing damage to surrounding normal tissues and vital structures. Because cancer cells have been left behind this procedure is usually combined with either chemotherapy or radiotherapy. [9]

Chemotherapy - this treatment is likely to be recommended for cancers that have already spread to other areas of the body (metastatic disease), for tumours that occur at more than one site (multicentric disease), or for tumours that cannot be removed surgically (nonresectable disease). In some cases, chemotherapy can be used to try to shrink large tumours prior to surgery .

- the drugs involved in this treatment attack cells in the process of growth and division. However, because the chemicals cannot distinguish between malignant cancer cells and normal cells, there are many side effects.

- these side effects include decreased appetite, vomiting and diarrhoea, hair loss, and a drop in the white blood cell count resulting in increased susceptibility to infection.

- chemotherapy will not cure the cancer, but will improve the length and the quality of the pet's life

- chemotherapy is also very expensive and this means that it is not a feasible option for all pet owners [10]

Radiotherapy - this is used to treat cancers which cannot be removed by surgery or treated by chemotherapy alone. It is also used after debulking surgery as a way to destroy any cells left after removal of the tumour, or before surgery in an attempt to shrink tumours down to more manageable sizes. It can also be used as a permanent method of controlling the tumour, without actually destroying it (palliative treatment).

- there are some risks of using radiation, including side effects caused by the destroying of healthy cells ("radiation dermatitis" and hair loss are the most common), and risks associated with anaesthesia (as radiotherapy requires the patient to remain still throughout the process) [11]

Although these methods of treatment are reasonably successful at controlling the development of cancer tumours, there are still reasons to try and develop RNAi treatments. RNAi, because it only targets specific genes, would cause fewer side effects than many of the treatments being used at the moment. It could be used as a treatment on its own, as a method of preventing the growth of tumours, or combined with other procedures, for example, either before or after surgery as an alternative to chemo- or radiotherapy. It could also be used in conjunction with chemotherapy, in a similar way as it is being developed in humans; to silence genes that make some cancer cells resistant to this treatment.

Conclusion

At the moment, the understanding of RNAi and its uses are not advanced enough to allow the types of treatments outlined above to be carried out. In anti-viral treatments the main problems are transporting the dsRNA / siRNA to the area that is attacked by the virus without being destroyed, and increasing the longevity so that the effects on the virus are more prolonged. For treatments within the body, the main problems are again, transporting the dsRNA / siRNA to the area, and enabling them to enter the cells without being destroyed.

At the moment the methods for introducing the dsRNA/siRNA are topical applications or nasal sprays (which allow them to reach the eye, the respiratory tract and the skin) and there is research being carried out into the use of intravenous injections using liposome particles (which would allow the dsRNA /siRNA to reach the liver and the jejunum). However, there are still problems with introducing them to other areas of the body.

The rate of development of RNAi treatments in humans will also affect the rate at which developments in the veterinary profession can happen. Understandably, research will be concentrated on human uses, but as these become more established and use becomes more widespread, the techniques may be developed for use in animals.

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