EXPLORATION OF DELIVERY VECTORS OF SHORT INTERFERENCE RNA TO CANCER CELLS

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Abstract
RNA interference is a relatively recent discovery. After looking at the science and research behind it, I began thinking about how it could be possible to apply this to the world of veterinary science. Cancer has always been a big issue for the medical world so I thought about how RNAi could be delivered to cancer cells to control them. My first thought was of monoclonal antibodies as these can be made to be specific to cancer cells. As I did more research, I thought of perhaps more simple transport vectors such as bacteria and viruses. The bacteria then lead me towards plasmids and the viruses to retroviruses. These all have the possibility of being used in the future each with their own individual benefits and problems. Many factors need to be taken into account, including ethical issues, costs and efficiency, before a suitable delivery vector can be used in medicine.

INTRODUCTION

RNA interference (RNAi) is an exciting recent discovery in the field of genetics. It is a phenomenon where double-stranded RNA (dsRNA) initiates post-transcriptional gene silencing (PTGS). This is the switching off of a gene after it has been transcribed onto messenger RNA (mRNA) by the destruction of the mRNA, preventing translation. It was first witnessed by Napoli et al (1990) in plants when they introduced a pigment producing gene into petunias: They expected a deeper purple flower but instead observed a variegated or a completely white flower.

The mystery of what had happened was not revealed until Fire and Mello (1998) injected sense and antisense RNA together into *Caenorhabditis Elegans* (round worms). The sense strand RNA would have had the same nucleotide sequence as the mRNA produced in the cell and the antisense is the complimentary sequence to this. These recombined as dsRNA and produced an effect in the worms as if the worms completely lacked the functioning gene for the resultant muscle protein. After more simple experiments into this area, Fire and Mello came to the conclusion that the dsRNA was preventing the expression of the gene for the muscle protein and that what they saw was in fact a form of gene silencing.

There was then further analysis by Elbashir et al (2001) in fruit flies (*Drosophila melanogaster)* who indicated that the mechanism of RNAi was a two-step process. First the long dsRNA are cleaved by an enzyme known as DICER into smaller 21 to 23 nucleotide fragments. DICER is an endonuclease enzyme, a type of enzyme that cuts the phosphodiester bond (a bond between two sugar groups and a phosphate group) within a polynucleotide chain, and is specific to dsRNA. These smaller fragments are called short interfering RNAs (siRNAs) and are not completely double stranded but in fact have an overhang of two nucleotides at each end. These overhangs allow for a protein complex called the RNA-induced Silencing Complex (RISC) to recognise the siRNA and incorporate them into the complex. From here the activated RISC, an adenosine triphosphate dependent process, unwinds the siRNA and using this sequence as a guide targets the messenger RNA (mRNA) of the cell. RISC then binds to the mRNA where an endoribonuclease (a nuclease enzyme specific to ribonuclease which catalyses the degradation of RNA into smaller components) cleaves the smaller strands of mRNA before they are degraded by exoribonucleases. (An exonuclease is an enzyme which cleaves the phosphodiester bond at the end of a polynucleotide, and is specific to ribonuclease.) This process results in the loss of expression of the gene and has therefore, lead to post-transcriptional gene silencing.

In worms and flies, where most of the original research has been carried out, only a few molecules of the dsRNA are required per cell to silence thousands of mRNA molecules. This suggests that dsRNA either acts catalytically, or is amplified, or both since the small amount of dsRNA is silencing a broad region.

In humans, an inflammatory based response is produced if long dsRNA enters the cell. However, the small 21 to 23 nucleotide RNA strands do not cause this response and instead only

activate the RISC. This is important because the inflammatory response is a complex interferon process that shuts down all protein production in the cell and therefore the RNAi process does not work.

The discovery of RNAi has many medical applications. If a gene can be silenced throughout the whole body then many diseases can be cured. This relates to veterinary medicine also as many diseases caused through faulty genes that occur in animals may now have a potential cure. Cancer in both humans and animals may be treatable via the entering of siRNA directly into the cells. There are many issues that need to be addressed before this can be put into practice- one of them being how to deliver the siRNA complexes to all cells requiring them which is what I will be addressing in this paper. Current research has been done into the delivery of siRNA into the body with many different vectors being explored. SiRNA has already been injected directly into melanoma cells to treat them and now it is the systemic delivery that needs to be addressed. However, the siRNA is not just limited to treating cancer, other diseases such as foot and mouth or HIV may now have more chance of being successfully treated as research into this area can now move forward.

DISCUSSION

The applications of siRNAs have been, and are still continuing to be, explored. One area included in this is the use of siRNA in cancer therapies. Possible applications for siRNA are in cancer research, screening and detection. Perhaps the most exciting is the use of siRNA for the destruction of cancer cells where if used in a drug it might possibly become a cure for cancer. However as with all possibilities there are problems that first need to be overcome and research needs to be carried out in more depth before anything can be used.

One of the issues that has constantly arisen is that of how to deliver the siRNA to the cancer cells with many different theories evolving. Research is currently being undertaken into this and at present, it is practical to deliver siRNA into well localised tumours without metastases, (a complicated process where the tumour spreads to other parts of the body,) through simple injection. Chemically synthesised siRNA has been injected straight into skin melanomas which then successfully activated the RISCs of the cells. However, the challenge facing researchers is the wide spread systemic delivery of RISCs in a targeted fashion. Targeted systemic delivery is putting something into the body and it getting to the required place through systemic circulation such as the blood. There are many different vectors that could possibly be used, each with their own benefits and problems. SiRNA can be chemically synthesized as short RNA duplexes or they can be expressed as short hairpin RNA (shRNA- which is a sequence of RNA that makes a small hairpin turn), from plasmid or viral vectors. This shRNA is then cleaved into siRNA once in the cell allowing it to also bond into the RISC. The shRNA therefore gives the possibility of using certain plasmid and viral vectors that transcribe the RNA polymerase III into shRNA. A polymerase is an enzyme that catalyses the formation of polynucleotides.

Plasmids are a natural occurrence in certain cells such as bacteria cells. They are a separate form of DNA capable of replicating independently from the bacteria’s chromosomal DNA. They are small circular double-strands of DNA that can easily be chopped by enzymes such as an endonuclease and have different parts of DNA fused to the plasmid. When the plasmid is chopped they have what are called sticky ends so the DNA can fuse better to the plasmid. These sticky ends are overhangs like those in the siRNA and therefore make it easier for siRNA to be fused to the plasmid. These features allow for plasmids to be used as vectors as the required DNA can be fused into the plasmid where it can then replicate and achieve the desired effect. This is a relatively simple process therefore allowing for plasmid vectors to be used for the delivery of shRNA to the cancer cells requiring it. Once within a cell shRNA joins to RISC, and these vectors direct the transcription of shRNA from a polymerase III

RNA gene promoter. A gene promoter is a DNA molecule to which RNA polymerase then binds, initiating the transcription of mRNA. This method of systemic delivery has many advantages; unfortunately it also has many disadvantages which include low and variable transfection efficiency. Transfection is the infection of a cell with purified viral nucleic acid which then results in the replication of the virus within the cell. Another disadvantage is the difficulty with combining the host DNA to the plasmid to allow it to become stable.

A more efficient siRNA delivery system is then needed. Viral vectors such as adenoviruses and retroviruses could provide this and be used for the delivery of siRNA into cancer cells. These could possibly target primary cells with high efficiency carrying, like the plasmid vectors, the shRNA for in vivo RNAi mediated gene therapy against cancer cells. As shown by Devroe and Silver's (2002) results retroviruses have so far proved to be extremely useful delivery vehicles in mammalian cells. A retrovirus is an RNA virus which uses an enzyme called reverse transcriptase to transcribe DNA from its RNA so it can then be incorporated into the host cell's DNA by another enzyme and therefore be replicated within the cell. Though it is possible for some retroviruses to be a potential cause of cancer they are a large family of viruses with viruses such as lentivirus promising to help provide a cure for cancer along with shRNA. Though some retroviral infections are rapid, uniform and stable therefore making them viable vectors for the delivery of siRNAs, the lentivirus is a slow virus capable of carrying a significant amount of genetic information into the DNA also making it a viable vector for the delivery of siRNA. It is also a simple system which consequently could be widely used by most laboratories providing a significant advantage over plasmid vectors which possibly takes months and thus more money to produce. Another advantage is that they enter a wide range of hosts including human cells and will only be integrated into replicating cells- which is a useful characteristic in targeting neoplastic cells; cells which cannot regulate cell division (so all tumour cells are neoplastic).

As the retrovirus is an RNA virus it would be possible to incorporate siRNA into its genetic information so, instead of using the reverse transcriptase to make it into DNA, the siRNA will start the interference process of the cancer cells activating RISC and destroying the matching mRNA. This would then turn off the otherwise uncontrollable genes that keep the cancer cells replicating. Even if the retrovirus was not specific to cancer cells and when entered into the body penetrated into other cells, the siRNA of the virus would activate RISC but, as the siRNA would be synthesised to match the mRNA of the genes within a cancer cell they should have no affect on other cells as they would not be translating that particular mRNA. Also, as the siRNA would be fused to the RNA of the virus it may cause the virus to be less harmful to the host and affect the cells in a different way. It must be remembered that the gene in cancer cells that causes them to continue replicating may also be present in normal genes but is switched on and off as it is needed. Cells once differentiated rarely replicate. However, stem cells continue to do so. As cancer cells started as normal cells which could not stop replicating, it may be that the mRNA that translates from the genes for the replication is the same in all replicating cells.

Therefore, the siRNA may have an effect on these cells that are also replicating in the body and possibly stop these duplicating as well. This could have disastrous effects on the body. For example in a young animal it may stunt growth if bone cells are turned off and it could possibly kill the animal if the siRNA turns off all replicating cells. It is not just limited to young animals, examples of cells that replicate all the time are skin cells and epithelial cells in the alimentary canal. These cells are constantly destroyed and constantly need replacing. There is, therefore, the need for a more specific delivery vector which could not interfere with other cells in the body and will only affect the desired cancer cells and hence limit the risks involved.

Monoclonal antibodies could be this possible vector as they have been designed to be more specific. This is the vector that has been researched the least but could have great potential in this area as it is relatively simple and specific. Monoclonal antibodies have been created so that they are specific to certain cells. They have currently been developed to target some cancer cells. The antibody binds

specifically to the cancer cells antigens. An example of one of these is trastuzumab, a monoclonal antibody specific to breast cancer cells. Therefore if siRNA or even the RISC could be bonded to these monoclonal antibodies, then when injected in the blood or lymphatic system they would go directly to the cancer cells. The siRNA could then enter the cells turning off the genes necessary for DNA replication stopping the cells dividing, and therefore stopping the aspect of a tumour that makes it so dangerous. The siRNA or the RISC could be bonded to the monoclonal antibodies at the end of their two heavy polypeptide chains simply and easily. Monoclonal antibodies can therefore be considered as a possibility for a delivery vector of siRNA into the cancer cells

To improve systemic delivery it is necessary to make the siRNA behave like a normal drug. This could be in the form of monoclonal antibodies as these could be entered in the body via injection the same way as normal antibodies providing that the addition of the siRNA does not change this aspect of the antibody. Viruses and plasmids may also help make the siRNA behave like a drug, something which is not common practice at the moment. There may be other vectors not mentioned in this paper that could do this better. For example in current research chol-siRNA administered to rats by injection showed improved drug qualities when compared to unconjugated siRNAs.

CONCLUSION

As with all areas of scientific research there are many ethical issues that have to be addressed especially when dealing with genetics. These ethical issues and the risks involved with RNAi must all be outweighed by the benefits the research into RNAi can have in the veterinary and medical world before being widely used. All of the original research was carried out using fruit flies and roundworms and now, as the research is becoming more advanced more and more mice are being used.

Mice are used in the manufacture of monoclonal antibodies. These mice are caused great distress and pain especially in the production of monoclonal antibodies. Surely this is uncalled for pain? How can veterinary surgeons claim to be trying to help animals when to save one you hurt another? Why is it the veterinary surgeons choice to decide which animals are free from pain and which are subjected to it?

Once research has gone past this stage of research on mice, new drugs will then need to be tested on other animals. Initially the results may appear to provide the desired outcome ie a cure for cancer. It is important to remember though that genetic mutations and disruptions can take many years to show their full effects and it may not be until future generations that catastrophic effects become apparent. What we believe to be a wonderful revelation in the world of science could actually prove to be something we know too little about to interfere with, for fear of its long term effects on many animals.

If taking in to account these ethical issues, RNAi still has a place in cancer research then a suitable delivery vector needs to be found and thoroughly tested. Plasmids and viruses are simple and relatively easy to use. However when used as delivery vectors for other genetic information they have not been found to be that effective with many disadvantages and problems involved. On the other hand monoclonal antibodies may be the answer as they are more specific. Nevertheless there has not been enough research into this area to establish whether or not they are viable vectors. At present it is just another logical idea that might possibly amount to something great.

When choosing the right delivery vector for a cancer treatment, many factors other than the biological ones will have an input. These include the cost of using the treatment, transportation of the drug, and which laboratories have the funding and equipment to mass produce it. Therefore even if an ideal vector can be found, it still might not provide the cure to cancer. There are many other problems that need to be addressed before siRNA can be used in everyday medicine.

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