Molecular medicine—from diagnosis to gene therapy

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The development of molecular medicine will be illustrated by several approaches to the diagnosis and possible gene therapy of some haematological and malignant disorders. Firstly, the history of the prenatal diagnosis of the hereditary blood disease thalassaemia will be traced from blood test to DNA test. New developments that may allow a foetal diagnosis to be made from a blood sample taken from the mother will then be presented. Finally, various approaches for gene therapy will be reviewed and, as examples, experimental approaches in treating hepatoma will be outlined.

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Introduction

Professor Sir David Todd has influenced my career tremendously and in a long-lasting way. What I would like to do today is to present two examples of advancements in molecular biology that have influenced medicine and in which I have played a small part.

Prenatal diagnosis

The first of the topics I would like to cover is prenatal diagnosis, which has evolved over a number of years. Initially, one would perform globin chain analysis of foetal blood; then, molecular diagnoses at the DNA level were introduced; and now, one can detect mutations directly by a variety of different methods. Recently, attempts have been made to isolate foetal cells from the maternal circulation; if the methodology proves efficient, it will allow the diagnostic state of the foetus to be made by using blood that has been drawn from the mother.

Nucleic acid hybridisation

Foetal blood sampling is routinely performed using a foetoscope. If the blood from a normal foetus is analysed at around 18 weeks of gestation, the ratio of the amount of β-globin chain (adult haemoglobin) to γ-globin chain (foetal haemoglobin) present is 1:10. In patients with β-thalassaemia who are homozygous, the β-globin chain is absent from the haemoglobin. In a foetus that has homozygous α-thalassaemia due to hydrops foetalis, diagnosis at the DNA level reveals a most distinctive feature (as shown in research I performed with Sir David). The α-thalassaemic trait in hydrops foetalis was detected by the presence of haemoglobin H; by using DNA hybridisation, however, we found that the expression of the α-globin gene was lacking in a foetus that was homozygous for the α-thalassaemic trait. When probes for the α- and β-globin genes were used, there was progressively less and less hybridisation to the α-globin cDNA probe with the progression of gestation, while hybridisation to the β-globin gene was normal throughout. This discovery with Sir David was really the first time that DNA had been used to diagnose a human disease. This technology evolved very quickly thereafter.

Restriction fragment length polymorphism

The detection of a restriction fragment length polymorphism (RFLP) involves the digestion of DNA with a restriction enzyme, followed by electrophoresis and visualisation of the separated nucleic acid bands. A polymorphic site that is present in some people would yield a fragment of DNA of a certain length; but in other people, for example with a certain disease, there may be a mutation at this site which may remove or create a restriction site. Electrophoresis of DNA

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digests, followed by Southern blotting and hybridisation to specific probes would reveal the RFLP as longer or shorter DNA fragments. The results can be used for linkage analysis to detect the occurrence of the disease. This is now a very common tool for identifying disease-related genes.

Sometimes detection of an RFLP identifies a gene alteration that is the cause of the disease. Sickle cell anaemia is caused by a mutation in the *Mst*II restriction site which abolishes the site, thus creating a longer piece of DNA in an *Mst*II restriction digest. In this way one can distinguish between the heterozygote, the normal homozygote, and the homozygote for the sickle cell anaemic trait. This technique is now used for the diagnosis of many diseases.

The dot blot

One can use a dot blot to distinguish between the wild-type and mutated globin sequences. If one uses wild-type DNA, it would hybridise to the wild-type probe and be visualised subsequently. Only the DNA from a patient with sickle cell anaemia will be visualised following hybridisation to the probe detecting the mutation. In this way, one can scan for mutations. The new technology that is currently being developed is the biosensor microchip. In one chip, it should be possible to place about 10 000 dots in a dot blot, and it may be possible to scan for very complex mutations such as the multiple breast cancer mutations.

Extraction of foetal cells

Another area of recent progress is the development of new approaches for prenatal diagnosis. This is currently done by amniocentesis or chorionic villus sampling. There are two new research developments in this area. One is pre-implantation diagnosis which can be done during in vitro fertilisation. A single cell is removed from the fertilised ovum for a DNA test. Although pre-implantation diagnosis has a high success rate, it is a very expensive procedure, because in vitro fertilisation itself is quite a difficult and expensive procedure. The alternative method is to isolate foetal cells from the maternal circulation. I would like to present some of the work which we are doing in this field.

The protocol involves drawing approximately 20 mL of maternal blood, using a centrifugation gradient, and separating and enriching nucleated red blood cells by affinity chromatography, using an anti–transferrin receptor antibody. The method relies on the fact that nucleated red blood cells are very rich in transferrin receptors which are needed to import iron for haemoglobin production. The extracted cells are displayed on a slide and are stained with anti–embryonic or anti–foetal haemoglobin antibodies. From about 20 mL of maternal blood, one can identify 10 to 20 nucleated red blood cells that belong to the foetus, and which can be subjected to polymerase chain reaction analysis to determine foetal genotype.

The reason for using nucleated red blood cells is that they have a very short life span. In the past, this type of test used lymphocytes since foetal lymphocytes leak into the maternal circulation. The problem, however, is that foetal lymphocytes can persist in the mother's circulation for many years. Isolating lymphocytes therefore would not work since one could be making a diagnosis of a previous pregnancy; thus nucleated red blood cells are now widely preferred.

Although the procedure is very reliable, it is unfortunately also very tedious by way of the purification step and the visual search for the foetal nucleated red blood cells. Out of the many millions of cells which belong to the mother, there will still be a few thousand to scan visually after purification. We are now working to make the procedure more streamlined and easier to perform, so that it can become a routine procedure.

Gene therapy

I will present the different gene therapy approaches that are being tried and then I will use the treatment of hepatoma as an example to show the current status of gene therapy.

Gene therapy is broadly defined as the use of genetic material to treat human diseases. It does not necessarily have to be for a genetic disease; for example, gene therapy to treat rheumatoid arthritis is currently being tried. There is a potential to treat many diseases with gene therapy. Diseases for which gene therapy is currently being tested include cancer, genetic, metabolic, cardiovascular, infectious, and autoimmune diseases.

Vectors

A number of methods for gene delivery have been attempted. The most popular is to use vectors or carriers that have been derived from viruses, such as retroviruses, adenoviruses, adeno-associated virus, and herpes virus. The latter, for example, has been the most promising in the treatment of central nervous system diseases. There are also some non-viral vectors, such as naked DNA, DNA in liposomes, or DNA attached to a ligand that targets a specific cell type.

Retrovirus

The genome of a retrovirus is made up of the 'gag-pol-env' genes. One can remove these genes and put a gene of therapeutic value in their place. The retrovirus vector system usually works ex vivo; one removes the target tissue, inserts the gene into the cells using the vector, and returns the cells to the patient. This method results in permanent gene transduction, because the gene is integrated into the host chromosome of a cell, so it will persist during cell division, and the gene will be carried into the progeny. The retrovirus vector has the advantage of having a broad host range—it can infect many different types of cell. This in turn, however, is a disadvantage since the retrovirus lacks tissue specificity; in other words, it is indiscriminate. The method thus cannot be extended to an in vivo situation. Another problem is low titre—it is difficult to produce a large number of transduced cells. This is because a retrovirus can transduce only dividing cells; transducing normal liver cells, for example, would not work since normal liver cells do not divide.

Adenovirus

Using the adenovirus as a vector for gene transfer also has its advantages and disadvantages. As with the retrovirus, broad host range is both an advantage and a disadvantage. Adenovirus, however, has the added advantage of being able to transduce non-dividing cells. Unfortunately, it remains extra-chromosomal and sooner or later, the cell will lose the DNA that entered by virtue of the adenovirus vector. A big advantage, however, is the very high titre; one can actually obtain about 10^{12} to 10^{13} particles per mL.

There are two main disadvantages of using adenovirus as a DNA vector: cytopathogenicity and immunogenicity. Cytopathogenicity was demonstrated during the early treatment of cystic fibrosis with adenoviral vectors. It had been thought that adenovirus was the best type of vector because it enters the lungs and respiratory tract. It turned out that the necessary adenovirus dose caused inflammation, and clinical trials had to be stopped very rapidly. The immunogenicity of adenovirus means that theoretically, it can only be given once; the second time round, the person would have already developed antibodies against the virus.

Adeno-associated virus

Adeno-associated virus is a small DNA virus of about 4700 base pairs. As the name implies, infection with the virus accompanies an adenoviral infection because it needs the adenovirus to help it multiply. The virus itself, however, is totally harmless as far as we know. Probably all of us have been infected by adeno-associated virus through past adenoviral infections, but it has never been associated with any human diseases. Thus one advantage is that it is non-pathogenic; it can also transduce non-dividing cells and can be made in a high titre.

The disadvantage is that it is a small virus, so only about 4500 base pairs of DNA can be accommodated. For example, cystic fibrosis could ideally be treated by the introduction of an adeno-associated virus vector into the respiratory tract. Unfortunately, the gene for cystic fibrosis is too large for this virus to accommodate. In addition, it integrates rarely and thus transduction is transient. With respect to treating a respiratory condition, however, this is not disadvantageous since it enables one to repeatedly administer treatment without it being immunogenic and cytopathic.

Non-biological vectors

I want, very briefly, to mention the use of non-biological vectors. There is no size limit in these methods, but entry of DNA into the cell is very inefficient, especially in vivo. The DNA remains extra-chromosomal and thus is only transiently expressed, but liposomes may increase the efficiency of cell transduction.

Application of gene therapy

I will now describe some of the targets in which gene therapy has been tried. The first one is the haemopoietic cell; the first example was in the treatment of adenosine deaminase deficiency whereby several patients were treated using gene therapy, but long-term follow-up has not yet been published.

There is a potential to treat human immunodeficiency virus (HIV) infection by gene therapy. By introducing an antiviral gene into an infected T-lymphocyte, the HIV might be killed; alternatively, expressing an antiviral gene in a normal T-lymphocyte might protect it from future HIV infection.

Other diseases in which gene therapy has been tried are sickle cell anaemia and thalassaemia; gene targeting and gene expression in both these diseases, however, are very difficult to achieve. If one is treating an enzyme deficiency, very little expression of that particular enzyme protein is needed. If one wants to treat a haemoglobin disorder, however, a very high expression of that protein is needed, and that is something that is not easy to achieve at the present time. Similarly, there is a long way to go in treating muscular dystrophy using gene therapy since it is difficult to transduce enough of the muscle.

Delivery of hormones and blood factors

An interesting area is the production and delivery of factors such as hormones; examples are factor IX and erythropoietin in muscle cells. If an erythropoietin gene or factor IX gene is put into an adeno-associated viral vector, that vector will spread very well in the muscle, and effect a prolonged expression of the relevant protein. When this is done in mice for example, the secretion of erythropoietin by the muscle can continue for about 1 year. Factor IX is now being tested in the factor IX–deficient dog. This method may thus be a way for producing long-term expression of hormones or growth factors in general.

Gene therapy in cancer

Tumoricidal and tumour suppressor genes

There is much effort placed in the attempt to use gene therapy to treat cancer. There are two main approaches. One is to directly kill the tumour cells; in other words, introducing a gene such as tumour necrosis factor, ricin (a plant toxin), or diphtheria toxin into the cancer cell to kill it. For this to be successful though, it appears that every tumour cell must receive the gene—this is the major drawback. The other approach is to utilise tumour suppressor genes such as the p53 and retinoblastoma susceptibility genes. Again, for this to work, however, every cancer cell must receive and express the gene.

Metabolic killing

Another approach is to use genes that need not transduce every cell. One way of doing this is to metabolically kill tumour cells by first introducing the herpes simplex virus thymidine kinase (TK) gene in a viral vector, and then by administering ganciclovir. Another way is to use immunotherapy. In this method, one uses cytokines, such as interleukins 2 and 4, which help to present tumour antigens to sensitise the lymphocytes; human leucocyte antigen incompatibility can also be used. The sensitised lymphocyte will then trigger the killing of tumour cells. These two approaches—metabolic killing and immunotherapy—do not require the transduction of every tumour cell.

The herpes simplex virus TK method relies on the use of ganciclovir, a nucleotide analogue. In transduced cells, the TK enzyme very efficiently converts ganciclovir into the monophosphate and then triphosphate forms; untransduced mammalian cells cannot, or at least do so very inefficiently. The triphosphate form gets incorporated into the DNA during cell division and subsequently interferes with DNA replication, thus killing the cell. This is the most common metabolic method used, although there are a few other examples. Why is it not necessary to transduce every cancer cell? It turns out that ganciclovir triphosphate can be transmitted from one cell to its neighbours, probably through the gap junctions which connect them. Depending on the type of tumour, about 20% of cells need to be transduced with the TK gene, because when you give ganciclovir to the patient, all the tumour cells will be affected. Other tumours may require a higher percentage of transduction, it apparently depends on how efficient the gap junctions have formed. Cancer can thus be treated by tumour suppressor gene therapy, immunotherapy, and metabolic killing.

Tissue-specific gene therapy

One method to achieve tissue-specific gene therapy is ex vivo transduction. In this procedure, bone marrow is extracted, subjected to gene transduction, and replaced in the patient. Another approach is to use tissue-specific gene expression, where the gene may enter different cells, but a specific gene promoter is utilised so that the gene is expressed only in the appropriate cells. The third way involves in vivo transduction and is the best approach because the gene enters the desired cell only. Efficient in vivo gene therapy may be possible in the future.

I would like to discuss tissue-specific gene expression using hepatoma as an example. The theory is that in many hepatomas, cells reactivate α-fetoprotein (AFP) expression. One can take advantage of this to achieve tissue-specific expression of the herpes simplex virus TK gene, if it is first inserted in a construct adjacent to an albumin promoter and an AFP enhancer. The effect of adding these regulatory elements can be illustrated by transducing liver cells with this construct and comparing with cells that have been transduced with the construct lacking the promoter, or lacking the TK gene (using a neomycin resistance gene as a selectable marker). Culturing the transduced cells in the presence of ganciclovir kills only the cells that have been transduced with the virus containing the TK gene with the AFP enhancer and albumin promoter. This means that the two regulatory elements impart tissue specificity.

Nude mice experiments have been performed to test whether the methodology described above works in vivo. A tumour was transplanted into each nude mouse which was then infected with transducing virus that contained the construct mentioned above. The mice were then given ganciclovir. Tumours continued to

grow in the mice that received a saline control, whereas tumours in ganciclovir-treated mice started to shrink. In the future, we plan to treat hepatomas by introducing the TK gene with a cytokine gene into patients, by using hepatic artery catheterization and chemical embolisation to deliver the viral vector.

Conclusion

In conclusion, I hope that this overview will give an idea of how molecular biology has helped in the diagnosis of diseases and how it will perhaps introduce new modes of treatment by way of gene therapy.