REVIEW ARTICLE

Accepted 24 April 1998
Received 29 March 1997
Indian J. Pharm. Sci., 1998, 60(5) pp. 259-264

Gene Therapy

S.S. MAHAJAN* AND H.V. PATANKAR,
The Bombay College of Pharmacy,
Kalina, Santacruz (East), Mumbai – 400 098

This article describes the prospects of gene tharapy of blood disorders, cancer, HIV infection and cystic fibrosis. The article outlines the approaches currently explored for gene therapy of these diseases and experimental details are not mentioned except for their conclusion. The Sickle cell anemia and beta thalassemia have been cured in mice and rabbits by gene therapy. The monkey virus is the gene transfer vector and kidney cells are the target cells. The clinical trials for the gene therapy of hemophilia B have been carried out in human beings with some success. In the gene therapy of cancer, cytokine genes, specific drug activating genes, and alteration of immune response to the cancer are the major approaches, currently explored. The gene therapy for HIV infections appears to be cotherapy, to be used in lower magnitudes of infections with other therapies. The causative mutation for the cystic fibrosis has been identified and the research is directed towards construction of an artifical chromosome which will act as a vector for transfer of therapeutic gene.

IT is now possible to engineer retroviruses so as to delete their natural genes and transform them into vectors to transfer the desired gene into a cell. These viruses are engineered such that they retain their infectivity but lose their ability to replicate. This is achieved by deleting some of the genes necessary for replication and substituting them by the desired gene.[1,2]

GENE THERAPY OF BLOOD DISORDERS

The most widely occurring blood disorders include:

1. Thalassemia
2. Sickle cell anemia
3. Hemophilia

Gene Therapy of Thalassemia and sickle cell anemia

Both of these diseases are caused due to errors in the formation of globin chains of the hemoglobin i.e. gene coding for globin. The alpha and beta chains of globin are coded by two separate genes situated on the sixteenth and the eleventh chromosome respectively. These extend upto sixty kilobases. Thalassemia is caused due to mutation of the gene coding α or β- or both the globin chains. Accordingly thalassemia is classified into various categories. Sickle cell anemia is caused due to a point mutation at the sixth position of the beta chain. The change of a single amino acid residue causes formation of a knob like projection at one end of the beta chain whereas at the other end an articulating cavity is formed. The two beta chains join together in this manner leading to the formation of a continuous cluster of globin chains. The erythrocytes formed with this hemoglobin are cleared from the blood stream during their passage through spleen[3]. Thus, there is severe anemic condition of the patient. Presently, except blood transfusion, there is no other therapy available for Sickle cell anemia. The cure rates of this therapy are very low. Thalassemia can be treated with certain drugs, however, the therapy is very expensive, as patients are required to take the medicines throughout the life[4].

*For correspondence

Mulligan[5] successfully transformed a monkey virus (a retrovirus) to introduce the gene coding for globin into the viral RNA. This engineered virus was then used to transfect the monkey kidney cells. As the virus was genetically engineered to delete the code for replication, upon transfection of the monkey kidney cells it could not replicate, however, viral DNA was integrated into the cell genome and the transfected kidney cells produced globin. In the same experiment, it was observed that monkey virus caused cleavage of the globin sequence and transfected the host cells with fragments of the desired gene. It was later found that viral vectors can effectively transfer DNA sequences upto a length of 7kb only. The globin produced by such transfected kidney cells was in insufficient quantities, if this is to act as a cure for thalassemia or Sickle cell anemia, also curing of disease would require the infection of the bone marrow stem cells. In this experiment the frequency of infection of the bone marrow stem cells was found to be extremely low which was related to the inability of the viral vector to carry such a long DNA sequence. It was observed that transfected bone marrow stem cells from the above virus mature to both erythrocytes as well as to leukocytes and only the erythrocytes produced globin contrary to the expectation that any cell derived from such transfected cell would produce globin. Hence, it was concluded that a promoter sequence is required to induce the transcription of any gene. It was also showed that for the cells to produce globin in biologically significant quantities an enhancer DNA sequence is essential. It was proposed that enhancer sequence unwinds the highly coiled portions of the chromosomes containing the gene for globin and the promoter sequence catalyses the action of the enzyme to induce transcription and translation of the gene to synthesize the protein globin. Using the genetically engineered monkey virus containing the gene for globin, beta thalassemia has been cured in mice and rabbits.

GENE THERAPY OF HEMOPHILIA

Hemophilia A

It is caused due to the deficiency of factor VIII in the blood. For factor VIII hepatic cells are the main site of formation. Some other tissues such as spleen, kidney and lymphocytes also synthesize factor VIII. Factor VIII is secreted as a glycoprotein into the blood stream and it circulates as a complex. The complex has a half life of ten hours, whereas free factor VIII is extremely unstable. The lower half life of factor VIII requires frequent expression of its gene. Fibroblasts are transfected with the cDNA of factor VIII. The cDNA of factor VIII was engineered to exclude the non essential B domain of factor VIII. The levels of factor VIII produced were lower which was attributed to the fact that the A_2 and A_3 domains of cDNA of factor VIII do not allow accumulation of the mRNA transcribed from it. The studies taken up in the mice are not reported to be encouraging[6].

Hemophilia B

It is caused due to the deficieny of factor IX. The gene for factor IX is expressed in cells as diverse as fibroblasts, endothelial cells, myoblasts, keratocytes and hepatocytes which yield from 0.01-4.6 $\mu g/10^6$ cells/24 hour of factor IX. The enzyme, γ-glutamyl carboxylase, transforms inactive factor IX to active factor IX[7]. The molony murine sarcoma virus (a retrovirus) was genetically engineered to contain the cDNA for factor IX and the enzyme. Fibroblasts when transfected with this virus did not produce active factor IX due to failure of the fibrobalsts to express the gene of γ-glutamyl carboxylase. When the gene for γ-glutamyl carboxylase was replaced with the gene of polypetidase, active factor IX was produced and the expression of the gene persisted for longer periods[6].

In another study, Kay and coworkers[8] transfected hepatocytes with the retroviral vector engineered to contain the cDNA for factor IX and an appropriate enzyme. A suspension of the hepatocytes, transfected by the viral vector, was directly injected into the portal vasculature. The blood was found to contain detectable levels of factor IX and clotting time was improved only marginally. A clinical trial was carried out in Chinese patients. It was found that younger patients responded more favourably compared to the older volunteers in the trial. It is probably because of the faster growth of the fibroblasts and hepatocytes in the younger patients. When an adenoviral vector, genetically engineered to contain cDNA for defective factor IX was injected into hepatic vasculature, large quantities of active factor IX were produced. The levels of factor IX declined to base level in a period of 1-2 months. This may be caused by destruction of the transfected hepatocytes by the T-lymphocytes[6]. No clincal trials in human beings using this technique have been reported.

GENE THERAPY OF CANCER

The oncogene and the tumor suppressor gene are the constituents of normal human genome. The oncogenes encode a series of molecular cogs that control the growth of cells by transmitting the signals from the cell surface to the nucleus. A mutation in them leads to either their increased activity or increased biosynthesis, both leading to cancer. The tumor suppressor genes code for proteins that act as cell's braking system. A decrease in their activity leads to cancer. Usually 4-6 genetic changes are required to produce cancer.

For the gene therapy of cancer, the usual procedure of *ex vivo* gene transfer or introduction of transfected cells into the host body is adapted. The cells most commonly chosen for this purpose are blood cells, fibroblasts, hepatocytes, and myoblasts, as these cells are readily accessible. However, the limited life span of these cells requires the gene transfer procedure to be carried out repeatedly[9].

The following strategies are being explored for the gene therapy of cancer.

Genetic tagging of the cancer cells

The biopsy sample cells of the cancer patient are transfected with a genetic marker. These cells are reinjected into the patient's body. This is followed by the usual chemotherapy. The presence of these transfected cells in the further biopsy sample is a useful sensitive indication about residual disease. These indications are used in designing an optimum chemotherapy[9]. In acute childhood myoblastic leukemia, the bone marrow cells were labelled with neoR gene and bone marrow transplantation was performed. The recurrent tumor cells were anlayzed for the presence of neoR gene. The presence of the marker gene (neoR) indicated the failure of chemotherapy to suppress the growth of tumor cells[10].

Vectoring cytokines to tumor

The cytokines have profound side effects and their parenteral administration is not possible[9]. Insertion of the cytokine gene into the cells, that can potentially home in tumor, would release the appropriate proteins. The tumor infiltrating lymphocytes were transfected with the gene for tumor necrosis factor (TNF) in melanoma patients. The lymphocytes would home into the tumor and release cytokines.[11,12] However, the controversy whether expression of cytokine gene is sufficient for their therapeutic use and whether lymphocytes have sufficient ability to target the tumor cells questions the success of the therapy.

Insertion of drug activating gene

Major problem of cancer chemotherapy is the lack of selectivity of drugs in their action on normal and tumor cells. If the tumor cells are selectively transfected with the gene of a drug activating enzyme then the action of the prodrug would be slective[13]. Certain genes and their promoters are known to be expressed preferentially in the tumor cells, e.g. *α-fetoprotein, tyrosinase and c-erb* B_2.[14]* A retroviral vector engineered to contain the gene for specific promoter and drug activating enzyme form, a unique vector which would infect all the cells but would be preferentially expressed in the tumor cells[9].

Another approach of this kind was employed for the oncogene c-erb B_2 and enzyme cytosine deaminase. This approach has been termed as "virally directed enzyme prodrug therapy". In the brain tumor, the retroviral vector, as engineered above, was introduced the neurons locked in the G_0 phase of cell growth cycle were not infected and the proliferating tumor cells are infected by the viral vector. Simultaneous administration of 5-flurocytosine causes it to be converted to 5-flurouracil (5-FU) in the tumor cells only, whereas, 5-flurocytosine remains unaltered in the normal cells. The cytotoxic 5-FU affects the tumor cells[3].

*The names of the receptors, oncogenes and proteins are according to the literature.

Suppression of oncogene expression[9]

Down regulation of abnormal oncogene expression can revert the malignant phenotype in various *in vitro* tumor lines. The insertion of genes coding for the complementary (antisense) mRNA to that produced by the oncogene would specifically inhibit the formation of the abnormal protein product. The mutant form of *c-ras* oncogene is the obvious target for this approach because it has been proved that upto 75% of the pancreatic cancer cases are caused due to the mutation of 12th amino acid of this protein and inhibiting the effect of this change will lead to the restoration of normal growth control.

Alteration of the immune system by the gene therapy for the treatment of cancer

Recognition of a tumor specific peptide presented on major histocompatability (MHC) class I proteins by the CD_8+T-cells, and signals generated from that recognition are insufficient to activate T-cell precursor to discharge its lytic machinery. At least one set of additional signals as represented by the lymphokines is necessary for the complete activation of T-cells. The lymphokines are produced by the helper CD_4+T-cells. To produce lymphokines, CD_4+helper T-cells must recognize separate set of antigen peptides presented on MHC class II protein molecules by dedicated antigen presenting cells as dendriatic cells or macrophages, which must endocytose these antigens from the exterior and place them into the lysosomal compartment. The macrophages have costimulatory molecules such as B_7 that occupy receptors on T-helper cells (as CD_{28}) concomitant with the recognition of the antigen by the T-cell receptor[15].

With this much understanding about the generation of immune response to the tumor, following approaches are being explored to modify the immune response to a tumor.

Transfection of tumor cells with gene for MHC proteins

Transfection of tumor cells with this gene resulted in the reduced tumorogenic capacity and metastasis. However, a good correlation between tumorogenicity and expression of MHC gene has not been found[15].

Introduction of B_7 gene

B_7 is a surface ligand binding to CD_{28} and CTL A_4 receptors on the T-cells. Crosslinking of B_7 with CD_{28} has been shown to increase the levels of lymphokines by CD_4+T-cells after engagement of T-cell receptor. This effect is caused due to increased transcription and stability of mRNA.[16,17] It is suggested by some workers that CD_{28} receptor on the CD_8+T-cells must be occupied by B_7 for their lytic activity.

Introduction of the cytokine gene

Increased cytokine levels in the tumor result in altered local immunological environment to the tumor, resulting in either increased presentation of antigen to immune system or enhanced activation of tumor specific lymphocytes. It has been shown that at high levels of cytokines the injected tumors are rejected and also inflammation is produced. This elucidates the role of lymphocytes in antitumor action.[16,18] The tumor cells transfected with the cytokine gene show reduced tumorogenicity and increased immunogenicity. However, the number of potential cytokine genes makes them difficult to be constituted into a vaccine product.

Gene Therapy of HIV infections

Following approaches are being explored for gene therapy of HIV infection:

i) Immunization with HIV protein

In vivo expression of the viral proteins as a genetic vaccine is one approach to attacking the virus. HIV mutates very fast so only highly conserved portions of HIV-RNA fragments are used as immunogens. Delivering the viral gene together with a gene coding for imunostimulatory molecules as B_7, BB is being tried[19].

ii) Interferon mediated inhibition

Interferon system is a powerful natural mechanism to combat viral infections. Interferon cDNA has been cloned and inserted into vectors for constitutive or inducible expression in target cells. HIV replication has been inhibited in culture media in this manner. Probably interferons induce proteins such as RBP-9-27 which interfere with viral protein expression.[20,21]

iii) Autolytic destruction of infected cell

Diptheria toxin A gene has been used as an inducible suicide gene. A single molecule of toxin is potent enough to kill the cell. The diptheria toxin A gene under control of HIV gene promoter is inserted into the cells. The HIV infected cells express the gene and are destructed not affecting the non infected.[19] Similarly gene for herpes simplex thymidine kinase under the control of HIV gene promoter is introduced in the cells. On administration of gancyclovir or acyclovir only the infected cells are destructed as they express the enzyme thymidine kinase under the control of HIV gene promoter.[22]

iv) Inhibiting entry of virus

A gene coding for only the part of receptor CD_4 truncated beyond its membrane anchoring segment (soluble CD_4, sCD_4) has been cloned. Viral protein GP120 binds to CD_4 for entering the cell. The lymphocytes are transfected will sCD_4 to produce soluble CD_4 protein in their cytoplasm. HIV can infect such transfected cell but as the virus replicates, sCD_4 binds to it, thus, viruses incapable of infecting the cells are produced. Cell cultures transfected with sCD_4 showed a ten fold decrease in the activity of HIV[19].

v) Antisense

Antisense RNA is a sequence of RNA that is the base pair complement of a mRNA. The binding of the antisense to mRNA blocks its translation. Antisense to genes of HIV such as structural genes, regulatory genes including primer binding site (PBS) and long terminal repeats (LTR - a repeatitive nucleotide chain at 5' - and 3' - terminal of the HIV-RNA) sequence have been cloned. Antisense to the HIV-LTR delivered through the adenovirus vector produced 90% reduction in the HIV replication. A single antisense can be effective against a large number of variants in an individual. Any deleterious effect due to expression of antisense has not been reported.[19]

However, it appears that none of the above mentioned methods is outstandingly more effective for the gene therapy of HIV infections. The escape of HIV after certain time is common due to its higher mutagencity. These can be considered as cotherapies useful at lower magnitudes of infections.

Gene Therapy of cystic fibrosis[23,24] (CF)

The disease is characterized by increased viscosity of pancreatic and respiratory secretions and higher salt content of sweat. It is an inherited condition caused due to a recessive allele and for its expression individual should be homologus for this gene. It shows no sex-linked inheritance and out of every 2000 to 2500 births one suffers from cystic fibrosis and normally dies at late childhood.

In the normal respiratory tract, the liquid that lines the airway is a colloid composed of a watery sol phase which bathes pericapillary region allowing villial movement and a gel glycoprotein rich phase. Patient shows abnormal mucosal secretions especially in lungs and gastrointestinal tracts. There is chest deformation, bronchioectstasis with continuous lung infection due to poor drainage of secretions and infected mucus. Infection by the microorganisms, secreting alginic acid salts, worsens the condition. A repeated oral and intravenous therapy of antipseudomonal and staphylococal antibiotics is essential with physiotherapy.

Due to diminished function, the pancreatic cells are replaced by fibrous scar tissue, forming a cyst. Due to decreased bicarbonate secretion, function of digestive juices is hampered.

Trans epithelial electric potential is high in patients showing 3 fold increase in passive conductance of cations and anions. The sol phase of colloid in air-way epithelial tissue is controlled by the electrolyte transport.

The gene has 24 axons, spanning about 250,000 base pairs and 1480 amino acids. The proposed 3-D model of protein coded by this gene proposes its role in transport of macromolecules. The segment of protein called 'R-domain' acts as molecular flap which interferes with chloride transport across membranes. The cause for around 68% of CF cases is supposed to be a three base pair deletion at position 508, resulting in loss of a key phenylalanine residue in R-domain. At present, around 200 mutations have been reported. Also a considerable number of cases have reported to be containing normal gene indicating the need for screening DNA chain beyond protein coding region.

The present therapy of CF consists of nasal aerosol of amiloride, a diuretic Na^+ channel blocker and enzyme deoxyribonuclease which reduces mucus viscosity due to its mucolytic activity. The therapy is far from satisfactory.

Gene therapy is proposed to be promosing alternative to existing therapy. The retrovjrus and adenovirus, incapable of replicating, have been used as vehicle to carry normal gene. The giant size of the gene does not allow efficient gene transfer with other problems as instability of gene expression and loss of gene due to replacement. An entirely different approach of building a mini-chromosome has been taken at Royal Brompton Hospital, London. The mini-chromosome will be able to function as an independent

chromsome so that it can replicate during cell division and separate just as normal chromsome. It will take quite some time for this approach to reach clinical trial phase.

REFERENCES

1. Brooks, G., **Pharm. J.,** 1994, 252, 256.
2. Cooper, D.L., Narayanan, R., and Bernito, J.R. In: Tabor, J. M. Ed., **Genetic Engineering Technology in Industrial Pharmacy - Principles and Application,** 1st Ed., Marcel Dekker Inc., USA, 1989, 299.
3. Mohanty, D. In; **Molecular Genetics and Gene Therapy**: The New Frontier; **Proceedings of the first Annual Ranbaxy Foundation Symposium.** Scientific Communications, USA, 1994, 45.
4. Dover, G.J. and Valle, D., **New Eng. J. Med.,** 1994, 331, 609.
5. Mulligan R.C., Howard, B.H. and Berg, P., **Nature,** 1989, 227, 108.
6. Brownhee, G.G. **Brit. Med. Bull.,** 1995, 331, 91.
7. Palmer, T.D. Thompson, A.R. and Miller, A.D., **Blood,** 1989, 73, 438.
8. Kay, M.A., Rothenberg, S. and Landon, C.N., **Science,** 1993, 262, 117.
9. Sikora, K., **Brit. Med. J.,** 1994, 308, 1217.
10. Brenner, M.K. Rill, D.R., Moen, R.C. Krance, A.R. Mirow, J. and Anderson, F., **Lancet,** 1993, 341, 85.
11. Rosenberg, S.A. **J. Am. Med. Assoc.,** 1992, 268, 246.
12. Rosenberg, S.A. and Aebersold P., **New Eng. J. Med.,** 1990, 323, 570.
13. Huber, B.E., Richards, C.A. and Krenitsky, T.A. **Proc. Natl. Acad. Sci.,** USA, 1991, 88, 8039.
14. Culver, K.W., Ram, Z., Walbridge, S., Ishi, H., Oldfield, E. and Blease, M., **Science,** 1992, 256, 1550.
15. Pardoll, D.M., **Immunol. Today,** 1993, 14, 310.
16. Lindsten, T., June, C., Ledbetter, J., Stell, G. and Thompson, C., **Science,** 1989, 244, 339.
17. Fraser, J., Irving, B., Crabtree, G. and Weiss, A. **Science,** 1991, 251, 313.
18. Townsend S. and Allison, J., **Science,** 1993, 259, 368.
19. Lever, A.M.L., **Brit. Med. Bull.,** 1994, 331, 150.
20. Mace, K., Sief, and Anjard, C., **J. Immunol.,** 1991, 147, 3553.
21. Bednarik, D.P., Mosca, J.D. Raj, N.B.K. and Pitha, P.M. **Proc. Natl. Acad. Sci.,** USA, 1989, 86, 4958.
22. Caruso, M., Klatz, M. and Mann, D., **Proc. Natl. Acad. Sci.,** USA, 1992, 89, 182.
23. Mehta, M.R., Alper, O. and Smith, A.W., **Pharm. J.** 1993, 251, 435.
24. Redei, G.P. In **Genetics** 1st Ed., Mcmillan Publishing Co., USA, 1982, 456.