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GENE TRANSFER INTO HEMATOPOIETIC CELLS: PROGRESS, PROBLEMS AND PROSPECTS*

*M. Siraç Dilber MD, PhD***

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Gene therapy is the introduction of genetic material into somatic cells in order to correct a genetic defect or provide a new therapeutic function. Among the numerous potential cellular targets for gene therapy, hematopoietic stem cells (HSC) may be viewed as one of the best candidates for such genetic modification. More than 250 clinical gene transfer protocols have been reported from around the world, and almost one in three involves manipulation of hematopoietic cells. The introduction of a new gene into the DNA of HSC and expression of the gene product in their progeny are exciting approaches to treatment of congenital and acquired diseases. Although it has been shown that hematopoietic progenitor cells, or even LTC-IC, can be easily transduced with high efficiency by retroviral vectors in vitro, and that long-term expression and high transduction can be obtained in mice after transplantation of the gene manipulated cells, this has not been observed in primate models or in human clinical trials. The reason for this discrepancy is not properly known. The major problems of gene therapy for cancer have been transduction rate, selectivity, and effectiveness due to the heterogeneity of genetic alterations found in human tumors. Overcoming these limitations in gene therapy requires not only improvement in cell biology and immunology, but also development of better delivery systems. Nevertheless, gene therapy is still promising and offers encouragement for future applications in clinical practice. *Key words: hematopoietic stem cells, gene transfer, gene therapy, viral vectors, transduction.*

Developments in molecular biology have led to significant diagnostic methods and the characterization of disorders at the DNA level. In the last two decades, many molecular genetic techniques have been used to get more information about the DNA. The further characterization of the DNA helix, the discovery of restriction endonucleases, the development of vector systems, and the cloning of genes have been the milestones for gene therapy. Potential targets for gene therapy are hereditary diseases, especially single gene defects as well as acquired diseases such as cancer or certain infections.

Gene therapy is defined as the transfer of genetic material into the cells of an organism to treat disease. Molecular genetic techniques have been used to transfer genetic material into target cells for a diagnostic, preventive or therapeutic purpose. The material transferred may be one or a few genes in size. Gene

* From the Department of Hematology and Medicine, Huddinge Hospital, Karolinska Institute, Huddinge, Sweden.

** Internist and Hematologist, Department of Hematology and Medicine, Huddinge Hospital, Karolinska Institute.

therapy can be used for germ-line or somatic cells in humans, but for ethical reasons, only somatic gene therapy is currently being performed. The first genetic therapy to humans was actually attempted during the 1970s[1]. Shope rabbit papilloma virus which produces arginase was infected into children suffering from arginase deficiency. The clinical trial was based on animal experiments showing that the direct injection of a wild type virus could deliver the gene encoding arginase and decrease plasma arginine levels. Unfortunately, the results were not successful. During the 1980s, bone marrow cells of two children with thalassemia were transfected with the herpes simplex virus thymidine kinase (HSVtk) gene as the dominant selective marker by a calcium phosphate precipitation method[2]. The persistence of the transfected gene was observed for up to nine months, but then it became undetectable, probably because of the techniques used. Most current gene-therapeutic approaches are based on viral vectors used for transferring a foreign gene to the target cells. In 1989, a retroviral vector carrying a marker gene, the neomycin phosphotransferase II (neoR), was used to transduce autologous lymphocytes, and the cells were then infused into the patients in association with immunotherapy for malignant melanoma[3]. In 1990, the first gene therapy experiment with the possibility of producing clinical benefits was initiated[4,5]. Since then, great progress has been made on gene transfer strategies, but new developments are still required for substantial improvements in current gene transfer technologies[6-10].

Delivery Systems

Gene therapy requires not only the introduction of foreign genes into cells, but also their stable and appropriately regulated expression in the new environment. Normally, this can be obtained by replacement of the defective gene with a normal gene via homologous recombinatilon. This procedure alone is likely to ensure that the foreign gene will be regulated similar to the endogenous gene. Although this technique has been used successfully for mouse embryonal stem cells, it has not yet been applied in cells that are used for gene therapy. The currently available gene therapy approach is to insert additional functional genes into target cells. Many techniques have been developed for the introduction of foreign DNA into mammalian cells. These include chemical, physical, and fusion methods, receptor-mediated endocytosis, and recombinant viral vectors.

Nonviral Gene Delivery System

Gene transfer via these methods is called "transfection". The efficiency of gene transfer by these techniques is quite variable, depending on the cell type and cell cycle phase. Stable integration of a gene into DNA can be obtained, but it is very low, ranging from one transformation event in 10^2-10^5 cells. The integration

of a foreign gene occurs in random sites of the genome of target cells. The expression of a transgene depends on the site of integration. Variable expressions can be obtained from the same transgene due to the site of integration, and the corresponding gene products may also show differential stability in the target cells[11-13]. These methods, including $CaPO_4$ precipitation, microinjection of DNA and particle bombardment, electroporation. cationic lipids and liposomes, receptor-mediated gene delivery, and direct injection of naked DNA, can be used in some non-hematological diseases, but they are not ideal techniques for hematopoietic cells due to their low integration into DNA and transient expression.

Viral Gene Delivery Systems

Gene transfer via viral vectors is called "transduction". As a result of severe limitations of gene transfer efficiency and obvious difficulties for in vivo application by chemical or physical techniques, viral vectors have been widely used in gene therapy. Thus far, clinical protocols have used viral vectors for cellular gene transfer more frequently than nonviral methods. Commonly used viral vectors are retroviruses, adenoviruses, and adeno-associated viruses, but herpes viruses and vaccinia viruses are currently being developed for use in humans as well[14, 15].

Retroviral Vectors: Oncoviral vectors: Oncoviruses consist of a large class of enveloped viruses that contain single-stranded RNA as the viral genome[16]. They replicate via a chromosomally integrated DNA intermediate (provirus). During the normal viral life cycle, the viruses can bind to target cells via specific cell surface receptors the amphotropic receptor on primate and rodent cells[17] or the ecotropic receptor on rodent cells[18] and enter into the target cells via endocytosis[19]. Viral RNA is reverse transcribed to yield double-stranded DNA and is then transported into the nucleus where it is integrated in the host genome by a second virus-specific enzyme, the integrase. The double-stranded proviral DNA integrates stably and heritably into random sites of the host genome as a single copy colinear with the original viral genome. The viral genome is about 10 kb in size.

Most commonly used retroviral vectors are obtained from the Moloney murine leukemia virus (MoMLV). The MoMLV is a single-strand RNA virus consisting of three genomic parts. The long terminal repeats (LTRs) contain the viral promoter and enhancer regions which are responsible for the initiation or termination of transcription. LTRs also contain some sequences which are required for integration of retroviruses into the host genome. Psi (or encapsidation) is responsible for the packaging of the RNA viral genome into viral particles. The third region consists of the viral genes gag, pol, and env which encode viral structural proteins, enzymatic proteins (reverse transcriptase etc.) and the envelope protein, respectively.

Retroviral-mediated gene transfer is dependent on two important components: the viral vector (Fig. 1) and the packaging cell (Fig. 2). A packaging cell line (usually a murine fibroblast) is a cell that contains a retroviral genome from which the signals that are responsible for encapsidation (psi) of the viral genome into virus particles have been deleted[20, 21]. The viral genome in the packaging cells encodes all the components necessary for virus replication, but cannot create an infectious particle because of the lack of encapsidation. Packaging cells receiving a recombinant retroviral vector form a producer cell line. All important components are deleted in a retroviral vector except psi and replication signals. Up to seven kb genes of interest can be inserted into a retroviral vector by cloning, allowing incorporation of most cDNAs but very few full-length genomic genes. Vectors can contain a single therapeutic gene or several genes, including selectable markers such as the neoR. Several genes can be simultaneously expressed by the retroviral LTR or one gene may be expressed by the viral LTR and the second by an internal heterologous promotor. Using picarnovirus 5' non-translated sequences between the transgenes allows internal ribosomal attachment, resulting in translation of those genes from a single template. These vectors are called "polycistronic"[22-25]. Much effort has been made on the modification of the retroviral envelope to incorporate specific ligands such as erythropoietin, or the c-Kit receptor. Such genetically modified retroviral vectors should be able to target cells with specific ligand receptors, improving the specifity of vector attachment[26, 27]. The transduction of the gene into the target cells can be achieved either by cocultivation of the vector-producing cell line and the target cells, or by incubation of the target cells in the cell-free vector containing supernatant.

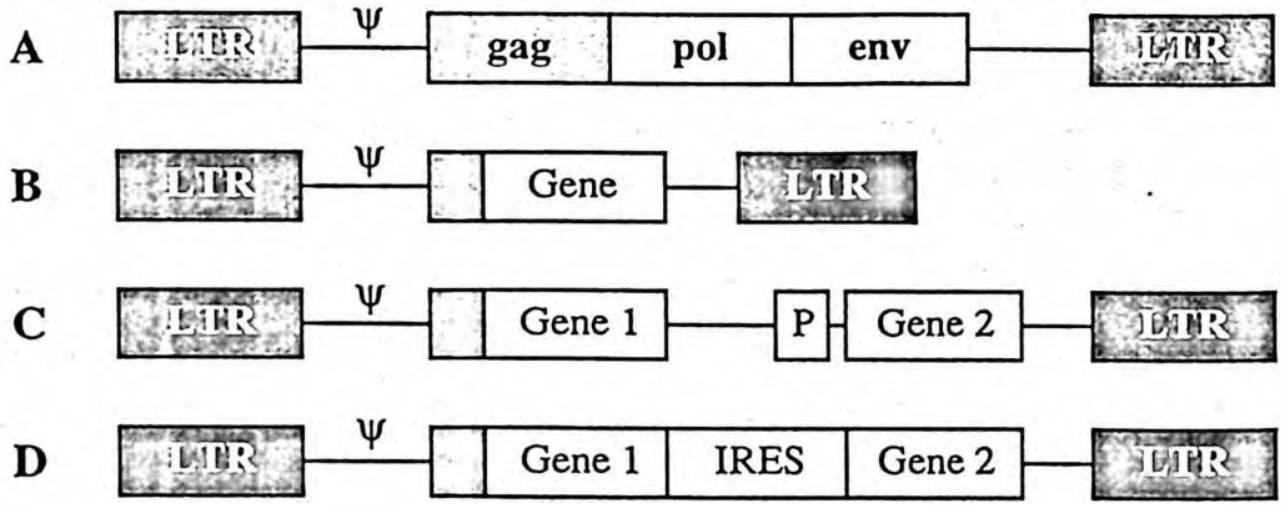

Fig. 1: Structure of retrovirus and retroviral vectors A) A retrovirus, with long terminal repeats (LTRs) at both 5' and 3' ends, the packaging signal ψ, and the viral replicate and the viral replicative and envelope protein genes (gag, pol, and env). B, C, and D) Replacement of the structural genes by gene or genes of interest. B) Single-gene vector. C) Two-genes vector with internal promoter. D) Bicistronic vector with internal ribosome entry site.

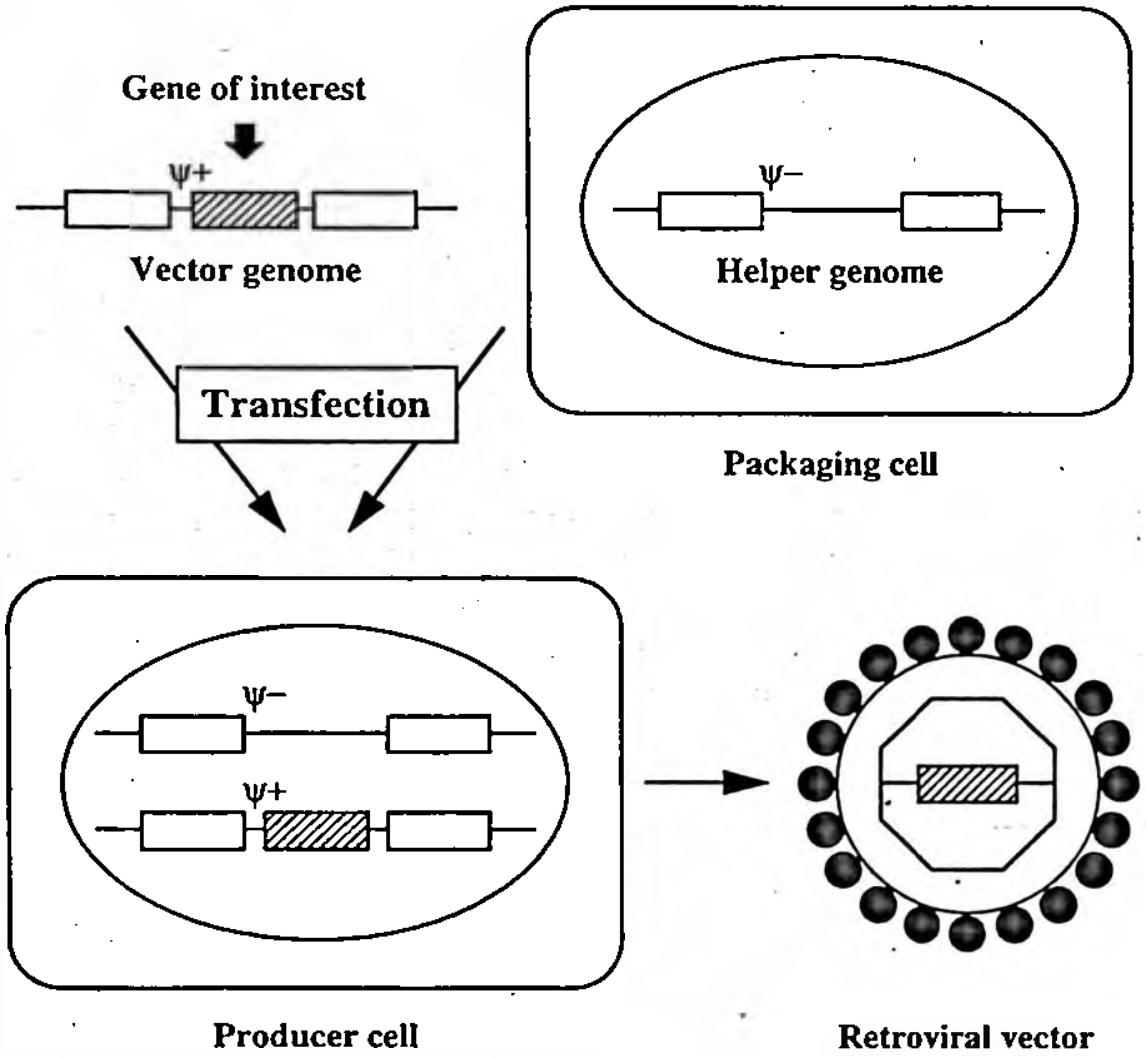

Fig. 2: Retroviral vector production using packaging cell lines. Packaging cell lines contain a retroviral genome which lacks the encapsidation sequence (ψ-) and therefore cannot incorporate the full-length viral genome into viral particles. Vector DNA introduced into the packaging cell line creates a producer cell line. Since the vector DNA contains the encapsidation sequence (ψ+), the vector genome is packaged into retroviral vector particles.

Retroviral vectors have some advantages over other viral vectors. They have been extensively investigated, therefore their features are well known. They are generally not harmful to target cells. The most important benefit is that retroviral vectors have the capacity to integrate into the genome of target cells. Another important feature of retroviral vectors is that the target cells need to be in the mitotic phase to allow gene transfer[28]. Depending on the target cells, this can be an advantage or a disadvantage. Amphotropic virus is rapidly inactivated in vivo in primates via a complement-mediated process.

Although retroviral-mediated gene transfer is an essential gene transfer method, it involves some potential risks[29-31]. Insertional mutagenesis, recombination with endogenous retroviral sequences, transfer of exogenous genetic material, and

production of replication-component murine retroviruses are major theoretical risks of this method. Insertional mutagenesis occurs when the integration of a retrovirus or retroviral vector causes disruption or abnormal regulation of a gene. This side effect has so far never been reported from using replication-incompetent retroviral vectors. The human genome contains many endogenous retroviral sequences. Because of mutations, they are replication-defective. It is theoretically possible that recombination can occur between these sequences and a retroviral vector, although the probability of this side effect is very low due to little homology. Another possible side effect is the contamination of the supernatant with replication-competent murine retrovirus (RCR). Packaging cell lines are designed to be free of replication-competent retrovirus but the generation of replication-competent virus via recombination in the producer cell line has been observed. The RCR could cause insertional mutagenesis via repetitive infections. Immunocompetent primates are able to clear and deactivate the RCR before it causes any damage. However, rhesus monkeys transplanted after total body irradiation with bone marrow cells transduced with a RCR-forming producer cell line did not develop antibodies against the RCR and developed T cell lymphoma nine months after transplantation[32]. Retroviral vectors in a clinical setting require extensive testing for the presence of RCR. So far, side effects related to RCR have not been reported in humans.

Lentiviral vectors: Lentiviruses also belong to the retrovirus family, but they can infect both dividing and non-dividing cells[33]. Therefore, much focus has been placed on the establishment of these vectors. The best-known lentivirus is the human immunodeficiency virus (HIV), which has been disabled as a vector for gene therapy. Up to 7.5 kb genes of interest can be inserted into the lentiviral vectors. HIV has the three gag, pol, and env genes, but it also carries genes for six accessory proteins called tat, rev, vpr, vpu, nef and vif. In HIV, the nuclear transport of preintegration complex is guided by nuclear localization signals present in the viral matrix.

Lentiviral vectors have been made with the transgene enclosed between the LTRs and a packaging sequence. Five genes (env, vif, vpr, vpu, and nef) can be deleted without affecting production of the vector or efficiency of infection[34]. The env gene of HIV is in dispensable for generating fully complement HIV-derived vector particles, as these can be pseudotyped with the surface glycoproteins of other viruses such as MoMLV or vesicular stomatitis virus (VSV). In spite of these advantages, a most legitimate concern about the clinical use of HIV-based vectors is linked to the possibility that the parental pathogenic virus might be reconstituted. Although such occurrence becomes unlikely, it nevertheless cannot be formally excluded. By contrast, a much high or degree of biosafety would be achieved if sequences coding critical virulence factors could be eliminated completely.

Adenoviral vectors: Adenoviral vectors are larger (35 kb) and more complex than retroviral vectors[35-37]. They are non-enveloped linear double-stranded DNA viruses that are taken up by the target cells via receptor-mediated endocytosis. After entering the target cells, they migrate to the nucleus and remain extrachromosomal. Adenovirus is a minor pathogen in humans and is not considered responsible for tumor formation. Adenoviruses they are easy to grow in large amounts and can infect nondividing cells. Many different cell types and tissues can be easily transduced by adenoviral vectors. Usually, the E1 coding region of the virus with the gene of interest is replaced. The human 293 cells are used as a source of the E1 proteins to produce vector particles. One major disadvantage of adenoviral vectors is that viral vectors remain extrachromosomal. Therefore, the expression of the foreign gene is particularly transient in dividing cells. The host immune response to the virus is also an important problem of the adenoviral vectors.

Adenoviral/Retroviral Chimeric Vectors

A new vector, combining the high titers and broad range infective capacity of the adenoviruses with the low immunogenicity and potentially stable, heritable expression of the retroviruses, has been developed[38, 39]. Adenoviral vectors have been used to deliver retroviral packaging plasmids directly to target cells, thereby converting them into retroviral packaging cells in situ, the retroviral particles subsequently being released at high local concentrations to infect surrounding cells. This chimeric system may allow delivery of high titers of retroviral particles coding a gene of interest into cell layers where the elusive stem cells may be found.

Adeno-Associated Virus: Adeno-associated virus (AAV) is a small, linear single-stranded DNA virus (4.68 kb)[36, 40, 41]. Although AAV is a simple virus, the expression and function of the AAV genes and gene products are less well characterized. Particularly, the AAV-2 has been investigated much more among AAVs. Heparan sulfate proteoglycan is considered a primary receptor for the AAV-2[42]. The virus requires coinfection with a helper virus such as adenovirus or some other virus in order to replicate, but AAVs can integrate into the host genome and remain as a provirus without a helper virus. The integration of wild-type AAV usually occurs in a specific site of chromosome 19. AAVs can infect almost all cultured cells that have been tested. Although more than 80 percent of humans are seropositive for antibodies against the AAV, infection has not been associated with any disease.

AAV-based vectors are simple vectors having two coding sequences: rep, which produces four proteins that mediate replication and integration, and cap, which produces the three capsid proteins. They contain the viral terminal repeat (TR) sequences flanking the transcription unit of interest. In most commonly used recombinant AAVs, the rep gene has been deleted because of a negative effect

of its protein to the transduction rate. In the absence of rep, AAV vectors may not always integrate into the host genome, but even if they do, the integration occurs at random. Also, AAVs tend to integrate in multiple copies in the genome (average 2-3 copies), but they have a high frequency of rearrangement. It is not known whether primary cells must undergo a round of cell division for integration to occur. A gene of about 4.5 kb in size can be inserted into the AAV vectors. Long-term gene expression can be obtained in non-dividing cells by these vectors. Production of recombinant AAV requires coinfection of a permissive cell line such as Detroit-6 with a plasmid containing TR sequences flanking the gene of interest as well as plasmids containing the cap and rep genes. In addition, transfected cells require superinfection with adenovirus to provide additional helper functions. Even though the vast majority of the helper adenovirus can be removed from AAV vectors by isopyknic centrifugation and heat treatment, denatured adenoviral protein can still be a potential source of contamination. Also, cell lysis is necessary to release recombinant AAV vectors. Therefore, vectors are quite difficult to grow in large amounts. High titer viral stocks can be obtained, although the percentage of infectious particles could be quite low. In a recent study, a mini adenoviral genome incapable of expressing adenoviral structural proteins but still able to supply the essential helper function required for AAV was generated[43]. These constructs have increased AAV production 40-fold over the conventional packaging strategy and appear to have resolved the high-titer AAV production problem without any contamination of adenovirus.

Herpes Simplex Virus Vectors: Herpes simplex virus (HSV) vectors may provide a unique strategy for the persistence of foreign DNA in cells of the central nervous system because of their tropism to these cells[44]. HSV vectors are non-integrating and therefore their use in dividing cells will probably not provide long-term expression. Although they have a complex regulation system, a replication deficient viral stock has been achieved, but it seems that even replication-incompetent virus is toxic to the target cells. Studies are being performed to make these vectors safer. Recently, a high rate of gene transfer into hematopoietic cells has been achieved with a HSV type-2 vector[45].

Gene Transfer into Hematopoietic Progenitors

The Biology of Hematopoietic Progenitor and Stem Cells

The turnover of cells in the hematopoietic system in human beings is estimated to be close to 10^{15} cells per day, including 200 billion erythrocytes and 70 billion neutrophilic leukocytes. All these cells are produced by a small population of bone marrow cells called hematopoietic stem cells (HSC)[46]. A lymphohematopoietic stem cell is defined as a cell with great self-renewal and proliferative capacity as well as the capacity to differentiate into the progenitors

of all the blood cell lineages, including erythrocytes; neutrophilic, eosinophilic, and basophilic granulocytes; mast cells; monocytes and macrophages; platelets; T lymphocytes; B lymphocytes; and natural killer (NK) cells[46-48].

Very recently, it has been shown in a murine model that myogenic progenitors are recruited from the bone marrow and access a site of muscle regeneration via peripheral circulation[49]. Genetically marked bone marrow, obtained from a transgenic line in which the lacZ gene is under the control of the muscle-specific promotor, has been transplanted into SCID-beige mice. When muscle regeneration is induced in the transplanted animals, lacZ-positive nuclei are found incorporated into the regenerated fibers. These data indicate that bone marrow-derived cells can migrate into areas of muscle degeneration, undergo myogenic differentiation, and participate in regeneration of the damaged fibers[49].

Self-renewal refers to the capacity to produce daughter cell(s) with identical characteristics. Another common definition of HSC is that the cells are capable of long-term reconstitution of the hematopoietic system of recipient animals. HSC represent up to 0.05 percent of the cells in the bone marrow. Many functional or indirect techniques have been developed to get more information about HSC, including spleen colony assay, clonal and suspension culture assays, and reconstitution of lethally irradiated or genetically deficient mice. These primitive cells can be obtained from bone marrow, spleen, or bood with the support of some cytokines. One important type of cell is the long-term culture initiating cell (LTC-IC). These cells require the presence of an adherent marrow stromal cell layer. The cells are grown on irradiated stromal layers and assayed with semi-solid clonal assays five weeks after culturing. Xenogeneic reconstitution systems have proven to be useful in vivo models for human hematopoiesis. Several groups have shown that the long-term engraftment of human hematopoietic primitive cells, which are called the long-term repopulating cells (LTRCs), can be obtained in different animal models such as immunodeficient mice[50, 51] or pre-immune fetal sheep[52]. The majority of HSC are in G_0 phase of the cell cycle in the steady state; just a small population of HSC go to mitosis randomly. It has been claimed that the G_0 state corresponds to time during which sten cell repairs DNA damage, thus allowing maintenance of the genetic integrity of the stem cell populations[46]. The product of HSC is progenitor cells. A progenitor cell is the differentiated product of a stem cell that has restricted its lineage potential and that has little, if any, renewal capability. Progenitor cells normally become restricted to a single blood cell lineage such as colony forming unit-granulocyte-macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E), or colony-forming unit-megakaryocyte (CFU-MK). These progenitor cells are responsible for short-term engraftment in vivo. The expression of cell-surface antigens by HSC is being investigated and allows us to obtain more information about HSC. The demonstration that progenitor

cells in human bone marrow cells express the CD34 antigen has enabled many investigators to explore the hematopoietic stem-cell compartment in humans[53]. CD34 is a highly glycosylated membrane glycoprotein of unknown function[54, 55]. Expression of CD34 is only found in hematopoietic precursors and vascular endothelial cells. The CD34+ cell population is heterogeneous. CD34 expression is highest in the earliest progenitors, and progressively reduced with maturation. Recently, CD34+ progenitors have been extensively used in a clinical setting[56]. The average of CD34+ cells in the bone marrow is 0.5-2 percent. High enrichments of human hematopoietic progenitor cells can also be obtained from CD34+ cell populations. These cells are CD34+CD38-HLADR-. They are also Thy+, c-Kit+, and have low retention of the mitochondrial dye, rhodamine 123, but are negative for lineage markers. Survival and proliferation of the HSC is regulated by cytokines which prevent cells from apoptotic death[57]. A number of direct-acting and indirect-acting cytokines have been identified and their genes cloned. IL-1, IL-3, IL-6, IL-11, stem cell factor (SCF), leukemia inhibitory factor (LIF), and GM-CSF are early-acting cytokines that regulate the proliferation of the HSC. It has been claimed that IL-3 and GM-CSF maintain survival in the G_0 of the HSC[46].

Under normal circumstances, the majority of HSC are present within the bone marrow, but it has been shown that a detectable number of HSC circulate in the peripheral blood in normal individuals[58-61]. The reason for peripheral blood progenitor cells (PBPC) being in circulation is unclear. Steady state peripheral blood contains approximately 0.1 to 0.2 percent CD34+ cells, about one-tenth of the level in normal bone marrow. Therefore, it is difficult to collect enough PBPC from normal individuals for therapeutic purposes, but PBPC levels can be markedly increased by mobilizing strategies based on cytokine injections and/or on the hematopoietic rebound effect that occurs following chemotherapy. The advantages of PBPC over bone marrow autologous transplantations are that 1) PBPC can be harvested without the prerequisite of a healthy pelvic bone marrow and without a general anesthesia, 2) rapid hematopoietic reconstitution is seen in those patients transplanted with PBPC collected after mobilization, and 3) tumor contamination of the harvest is less in PBPC than in bone marrow. For these reasons, PBPC transplantation is commonly used world-wide. Moreover, allogeneic PBPC mobilized by cytokines has become a new source for stem cell expansion and transplantation[62]. Ex vivo stem cell expansion has been developed by using cytokines. Incubation of selected CD34+ cells in combination with high-dose cytokines has been the most commonly studied technique of ex vivo hematopoietic culture[63-65].

Another source of HSC is cord blood. The presence of hematopoietic progenitor cells in umbilical cord blood was first reported in 1974[66]. The main sources of cells for transplantation and engraftment of the hematopoietic system are adult

bone marrow and PBPC. Recently. cells from the umbilical cord and placental blood of children at birth have been used for transplantation purposes. Approximately one percent of the nucleated cells present in unfractionated cord blood express the CD34 antigen.

Gene Transfer Studies

Since methods were developed allowing the transfer of foreign genes into eukaryotic cells, the HSC have been an obvious and desirable target for gene therapy[67-71]. There are some advantages of HSC for transferring a new gene: 1) the HSC can be easily removed from the patient, 2) they can be manipulated ex vivo, 3) they can be cryopreserved for later use, 4) they can be returned to the patient, 5) transduced cells can be expanded without losing the new gene, and 6) successful gene transfer into a few pluripotent HSC may be sufficient to repopulate the recipient with enough modified cells to treat some diseases. Therefore, these cells have been extensively used in gene transfer experiments.

Animal Models: The successful gene transfer into hematopoietic progenitor cells by recombinant retroviral vectors was first reported in 1983[72]. Murine bone marrow cells were transduced with a retroviral vector carrying the dominant selectable marker gene neoR. As a result, 0.3 percent of G418 (analog of neomycin) resistant CFU-GM was obtained. After this result, several other groups also showed that more primitive progenitors cells could be transduced by means of retroviral vectors, although the transduction rate was quite low[73-75]. After the discovery and cloning of many of the hematopoietic growth factors, they have been used to stimulate and maintain the HC, thus these cells are made susceptible to retroviral transduction in culture. Particularly, the combination of IL-3 and IL-6 with or without SCF was found to be one of the best cocktails[76, 77]. One other way to stimulate HSC was to inject 5-fluorouracil (5-FU) into donor mice before the harvest of bone marrow in an attempt to induce mitosis of primitive cells[78]. This method was later modified to optimize for a better yield of cycling HSC[79, 80]. A high transduction rate of a foreign gene into long-term repopulating cells has been obtained in recipient mice with engraftment of multiple hematopoietic lineages by using these methods. The expression of the recombinant gene in the murine HSC was observed for up to 12 months in some animal experiments indicating the possibility of gene transfer into pluripotent stem cells[81-84]. In spite of successful gene transfer into murine HSC, long-term engraftment of the cells containing a foreign gene in large animals was not too effective. Both in rhesus monkeys and in dogs, the long-term engraftment of cells carrying the recombinant gene was obtained, but the transduction rate was quite low[85-89].

Gene Transfer into Human Hematopoietic Cells: Committed human progenitor cells as well as LTC-IC have been transduced by retroviral vectors in the

presence of cytokines, or in the presence of marrow stromal cells with or without cytokines[90-93]. It has also been shown that specific extracellular matrix component, such as fibronectin, increase retroviral-mediated gene transfer into HSC[94]. Apart from in vitro experiments, SCID, NOD-SCID, or SCID-beige mice have been used for evaluation of normal HSC as well as HSC carrying a new gene[50, 95, 96]. The human HSC are capable of generating human hematopoiesis in these xenograftic mouse models. As previously discussed, since PBPC have some advantages over bone marrow, they are very attractive target cells for retroviral-mediated gene transfer[97-100]. PBPC have been chosen as target cells for many clinical protocols dealing with gene therapy[101]. Cord blood cells are also other important target cells for retroviral-mediated gene transfer[102].

Applications of Gene Transfer into Hematopoietic Cells

Gene Marking

Gene marking can be used to determine whether harvested progenitor cells in marrow or blood are contaminated with tumorigenic cells. The technology can also provide some information about the contribution of marrow or blood HSC to long-term engraftment. Autologous bone marrow or peripheral blood stem cell transplantation has been extensively used for malignant diseases such as leukemia and lymphoma, but relapse remains the major cause of treatment failure. For many malignancies, it is not known whether tumor relapse following autologous stem cell transplantation results from residual malignant cells in the patient, from the tumor cells in the harvest or from both sources. Because the source of relapse is unknown, it was difficult to evaluate whether marrow purging, which has some side effects to the normal HSC, was necessary. Studies to address these issues have been performed by gene marking experiments.

The first gene marking study was to transduce tumor infiltrating lymphocytes to determine whether they homed selectively to tumors[3]. In 1991, nucleated bone marrow cells of patients with acute myelocytic leukemia (AML) and neuroblastoma were transduced with a retroviral vector carrying the neoR gene[103, 104]. In the AML study, four out of 12 patients relapsed, and in two, the marker gene was present in the tumor cells. In the neuroblastoma study, five patients relapsed, and gene-marked tumor cells were found in four patients. Similar results have been obtained in patients receiving autologous bone marrow transplantation for chronic myelocytic leukemia (CML)[105]. These results clearly show that harvested bone marrow in remission could ontain residual tumor cells that can contribute to the relapse of diseases. Moreover, in the studies of AML and neuroblastoma, the recombinant gene was found in about 2-15 percent of hematopoietic progenitor cells after transplantation. The marker gene continued to be detected and expressed for up to five years in the mature progeny of marrow precursor

cells, including peripheral blood T and B cells and neutrophils, suggesting successful stem cell gene transfer[106]. A similar attempt was made for patients with multiple myeloma and breast cancer[107]. In this study, the levels of gene transfer into HSC were quite low and the marker gene (neoR) was not detected in the tumor cells. After showing that myeloma cells can be tranduced by a retroviral vector carrying a marker gene[108], some clinical protocols for gene marking of tumor cells in patients with multiple myelomas are in progress[109-112].

Correction of a Single Gene Defect

One of the major targets of gene therapy is the replacement of defective or missing genes in congenital diseases affecting the hematopoietic cells.

Adenosine Deaminase Deficiency: The first application of modern gene therapy was initiated in September 1990[4,5]. Two children with adenosine deaminase deficiency (ADA) were treated by autologous lymphocytes which had been transduced with a retroviral vector carrying the ADA gene following stimulation with IL-2 and anti-CD3 monoclonal antibodies. After ex vivo expansion, the cells were repeatedly infused intravenously. Over two years following the lasat infusion of transduced cells, T lymphocyte clones were found to contain the foreign gene. Both children showed improved immune function, increased T cells and ADA levels. In spite of these encouraging results, lymphocyte gene therapy has some problems. Particularly, the lifespan of most circulating T cells is probably short. Therefore, repeated collections and infusions of T cells will probably be necessary. The attempt has been made to transduce CD34+ progenitor cells. One of these patients was treated by transduction of G-CSF mobilized peripheral blood CD34+ cells, using another retroviral vector carrying the ADA gene with an internal promotor. In this way, the vector could be distinguished from the first vector by polymerase chain reaction (PCR), but peripheral blood leukocytes containing the second vector were not detected by PCR after therapy[113]. A number of patients with ADA deficiency have also been treated by other groups. In one study, T lymphocytes and bone marrow CD34+ cells of two children were transduced with two different vectors carrying an ADA gene[114]. The patients currently have about one percent transduced CFU-GM in their marrow and about one percent of their circulating T lymphocytes express the ADA. In a second study, the bone marrow of three patients was transduced with a retroviral vector carrying the ADA gene[115]. They cocultured the patients' CD34+ cells on the fibroblasts producing the ADA vector. Vector-containing cells from the bone marrow of one patient were detected six months after treatment. In a third study, three newborn children were treated with CD34+ cord blood cells transduced with a retroviral vector carrying the ADA cDNA[116]. Four years later, they continued to produce leukocytes containing the ADA cDNA, with aproximately 100-fold

higher frequencies of gene-containing T lymphocytes (1-10%) than other lineage (0.01-0.1%). In the summer of 1997, PEG-ADA replacement was discontinued in one patient; multiple laboratory and clinical parameters of immune function declined during the two-month period and the enzyme treatment was restarted[117].

Gaucher's Disease: Gaucher's disease is a lipid storage disorder and results from deficiency of the lysosomal enzyme glucocerebrosidase (GC). Accumulation of large amounts of glycolipids in reticuloendothelial cells causes hepatosplenomegaly, pancytopenia, lytic bony lesions, and neurologic symptoms with a wide range of severity. The disease is the most common disease among the lysosomal storage disorders. There are three variants of Gaucher's disease. In the type I variant, generalized clinical manifestations may be treated largely by the genetic correction of HSC. The GC gene has been succcessfully transduced into murine and human hematopoietic progenitor cells[25, 81, 82, 93, 118].

A number o patients with Gaucher's disease have been treated by different institutions. In one study, CD34 separated bone marrow cells or G-CSF mobilized PBPC from three adult patients with moderately-severe Type I disease were transduced with a retroviral vector expressing the human GC cDNA. Gene-corrected cells were engrafted and persisted for at least three months post-infusion, even without myeloablation[119]. However, the level of corrected cells was too low to result in any clinical benefit. In the second study, a G-CSF mobilized and enriched population of CD34 cells from PBPC was also transduced with a similar vector[120]. The results to date reveal the presence of the GC transgene and an increase in GC enzyme activity in peripheral blood leukocytes in the patients studied. In one case, the levels increased to above 50 percent of normal and this effect has persisted for 12 months following the last transplantation[120].

Fanconi's Anemia: Fanconi's anemia is a rare genetic disorder characterized by progressive bone marrow failure and a predisposition to leukemia[121]. The Fanconi's anemia group A (FAA) and group C (FAC) genes have been cloned and encode DNA repair proteins[121]. Transduction of hematopoietic progenitor cells from Fanconi patients with retroviral vectors expressing FAC and FAA cDNA corrected the hypersensitivity of DNA cross-linking agents, such as mitomycin, to hematopoietic cells and improved their viability in vitro[122, 123].

Three patients with Fanconi's anemia group C have been treated with gene therapy[124]. Each patient received three or four cycles of gene transfer, each consisting of one or two infusions of autologous PBPC that had been transduced ex vivo with a retroviral vector carrying the normal FAC cDNA. Although there was an in vivo selective advantage resulting from the FAC gene transfer, long-term hematopoietic reconstitution with gene-corrected clones was not observed.

Chronic Granulomatous Disease: Chronic granulomatous disease (CGD) is characterized by granuloma formation and recurrent life threatening infections. Neutrophils, eosinophils, monocytes and macrophages are unable to inactivate bacteria, fungi, and parasites. The main defect is in the enzyme NADPH oxidase which consists of at least four subunits. The most common autosomal form results from a failure to produce the $p47^{phox}$. Transfer of the $p47^{phox}$ gene into PBPC derived from $p47^{phox}$-deficient patients has shown that gene expression and metabolic correction can be obtained[99].

A phase I clinical trial of gene therapy for $p47^{phox}$-deficient CGD was conducted by treating five adult patients with a single cycle of therapy with ex vivo transduced PBPC[125]. The ex vivo correction of oxidase activity in neutrophils differentiated in culture from the transduced PBPC as measured in chemiluminescence assay ranged from 23 to 65 percent, but very small number of oxidase-positive neutrophils appeared in the peripheral blood of all five patients and could be detected for three to six months.

Myeloablation was not used in these clinical protocols dealing with Gaucher's disease, Fanconi's anemia, and chronic granulomatous disease. After transduction, the cells were infused into the recipients in the form of as blood transfusion. This could be one important reason for low engraftment of gene-corrected cells into bone marrow. Although several studies have currently reported that a high level of engraftment of bone marrow in the mouse may be obtained without myeloablatilon, a very high number of marrow cells were used in these experiments[126, 127].

Hemoglobinopathies: Particularly, β-thalassemia is the one of the main target diseases for gene therapy[128-131]. The genetic correction of thalassemia major requires insertion of a normal β-globin gene. The new β-globin gene must produce an amount of β-globin equal to the amount of α-globin produced within the cell. Otherwise, the patient will remain anemic. This is very difficult with currently available vectors. The β-globin genes are coordinately regulated at the level of gene transcription. It has been shown that both gene proximal promoters and enhancers as well as a far upstream 15kb dominant regulatory region, termed the locus control region (LCR), are required for high level expression. LCR appears to be responsible for regulating the β-globin production to match the α-globin quantity in the cell. Addition of LCR to the constructs containing genomic fragments of the β- or γ- genes resulted in high level expression in tissue culture cells[132]. However, the expression level of these constructs in primary cells was quite low. Recently, constructs containing LCR elements and a human β-globin gene altered to reduce recombination were reported to induce high levels of expression in MEL cells and relatively high expression in mice[133], but these results require confirmation in non-human primate models. Further work on the role of the cis- and trans-acting elements in globin

gene regulation, vector development, and improved stem cell transduction is required before gene therapy becomes a treatment modality for hemoglobinopathies. An alternative approach, gene conversion utilizing an RNA-DNA oligonucleotide, was used for the correction of the mutation in lymphoblastoid cell lines from a patient with sickle cell disease[134]. Further studies are required to assess the feasibility of this approach in primary cells.

Hemophilia: Coagulation factor deficiencies such as hemophilia A and B do not require cell-specific gene expression. The production of coagulation factors can take place in hematopoietic cells[135]. Another important factor is that only 10 percent of normal factor concentration will probably be enough for effective therapy[136, 137]. Hematopoietic cells, bone marrow stroma cells, skin fibroblasts, hepatocytes, myoblasts, and vascular endothelial cells have been used as target cells in gene therapy studies. Some encouraging results have been obtained from mice and dog experiments[138, 139]. A phase A clinical trial of gene therapy for hemophilia B was conducted by treating four patients with autologous fibroblasts which had been transduced ex vivo with a retroviral vector carrying hFIX cDNA[140]. The genetically modified cells were amplified and implanted into the patients subcutaneously[140]. It was reported that the level of hFIX protein was elevated (5% of normal level), blood clotting activity increased, and hemorrhage tendencies were alleviated. Treatment-related side effects have not been observed so far (4 years).

Modifying the Drug Sensitivity of Progenitor Cells

Bone marrow suppression is one of the most common toxicities of chemotherapy regimens. A number of drug resistance genes have been identified that might be useful in gene therapy strategies to prevent chemotherapy toxicity. The ability to render HSC resistant to one or more chemotherapeutic drugs can be implicated to prevent some side effects of these drugs on marrow cells, or can be used as a dominant selectable marker, allowing the in vivo selection of transduced over-naive cells. Murine hematopoietic progenitor cells have been successfully transduced with a retroviral vector carrying the multiple drug resistance (MDR1) gene in vitro[141]. Mice with MDR1-gene modified bone marrow have been shown to tolerate much higher doses of chemotherapy[142-144]

Clinical trials using this approach are currently in progress and more than 40 patients have been treated so far[101, 112]

Gene Therapy for Cancer

There are several potential applications for the use of gene therapy in the treatment of cancer, including solid and hematological malignancies[106, 145, 146]

Suicide Gene Therapy

Another potential principle for use of gene technology in the treatment of cancer is the insertion of a suicide gene into tumor cells. A suicide gene is a gene encoding a protein that is toxic to the genetically modified cell. Some suicide genes may express products that are directly toxic for the cell, such as diphtheria toxin or pseudomonas exotoxin, both of which inhibit protein synthesis. Other suicide genes are encoding enzymes that selectively convert nontoxic prodrugs into highly toxic metabolites.

One of the most commonly used and investigated suicide gene is the HSVtk[147]. The transfer of the HSVtk gene into tumor cells using retroviral vectors or adenovirus vectors followed by the administration of GCV or acyclovir provides a potential strategy for the treatment of some malignancies[148-151]. GCV is converted by the HSVtk enzyme into a monophosphate form and subsequently to GCV-di-and triphosphate by endogenous mammalian kinases. GCV-triphosphate competes with normal nucleotides for DNA replicatilon in mammalian cells and can cause cell growth inhibiton and cell death[152]. Therefore, cells that express HSVtk become sensitive to the toxic effect of GCV and can be eradicated in vivo by the administration of GCV.

In a series of animal experiments, tumor cells were transduced with a retroviral vector coding the HSVtk. In spite of a rather low gene transfer into tumor cells, a complete tumor eradication was obtained after GCV treatment in some animals. This unexpected finding is called the "bystander effect", a phenomenon where HSVtk-transduced tumor cells are toxic to nearby untransduced cells when GCV is injected[148, 153]. After the discovery of the bystander effect, such a strategy has been used to treat various experimental hematological[154] and non-hematological tumors. It has been shown that gap junctions may play an important role in the mechanism of the bystander effect in vitro[155-158] and in vivo[159]. Apart from a local bystander effect, a distant bystander effect has also been observed in vivo[154, 160].

Gene Transfer to Donor Lymphocytes

Infusion of donor lymphocytes has become increasingly common as treatment for relapse of hematological malignancies following allogenic bone marrow transplantation (BMT). Although such adoptive immunotherapy approaches have produced clinical benefits, they are complicated by the frequent development of severe graft versus host disease (GVHD). Several gene transfer studies in allogenic BMT are providing a means of monitoring such approaches, ablating donor lymphocytes in the event of severe GVHD[161]. Recently, patients who relapsed after T cell-depleted BMT have been treated with donor lymphocytes

transduced with a suicide gene (HSVtk). Some patients have developed severe GVHD, which has been effectively controlled by GCV-induced elimination of the transduced cells[162].

Immunotherapy

Immunotherapy is basically a tumor cell vaccination. Murine tumors were genetically altered ex vivo with one or more cytokines including IL-1β, IL-2, IL-4, IL-6, IL-7, TNF-α, IFN-gamma, and GM-CSF, and then reimplanted into mice[163, 164]. With a few exceptions, most tumor cells engineered to express cytokines are promptly rejected in the syngeneic animals. Most treated mice were systemically immune to reimplantation of the non-modified cells as well. Depending on the cytokine and tumor type, any of several mechanisms, including macrophages, neutrophils, eosinophil, and T cells, were shown to be responsible for the rejection of the tumor, but the primary target for obtaining an immune response to tumors is the T lymphocytes, specifically the major histocompatibility complex (MHC) class I-restricted, cytotoxic (CD8) T cell[165, 166]. In spite of encouraging results from cytokine gene alteration of tumors in the mice, it is difficult to translate this method to a clinical setting. First, the tumors used in these studies were quite immunogenic and therefore were not good models for human cancer. Second, live tumor cells were used as vaccine as it is known that irradiated tumor cells are less immunogenic than live tumor cells. Third, with few exceptions[167, 168], when animals with established large tumors were treated, the results were much less satisfactory. Nonetheless, many clinical protocols have been approved and initiated worldwide[112]. These studies are an attempt to immunize tumor-bearing patients against autologous tumors by modifying their tumors with a cytokine gene that is expected to raise the host immune response to the tumor. A biopsy of tumor tissue is obtained from a patient and the tumor cells are grown in culture. Once the cells are growing, a vector is used to transfer a cytokine gene into the cultured cells. After expansion of the cells with or without selection, the cells are irradiated and then injected into the patient. Injections of the cells have to be repeated several times. Melanoma, neuroblastoma, breast, colorectal, renal cell carcinoma, and small cell lung cancer are the primary targets of the tumor vaccine studies. This technique has been used for some hematological malignancies such as multiple myeloma.

The in vitro growth of primary tumor cells is not always an easy task. Therefore, in some studies, fibroblasts which are much easier to cultivate in vitro have been transduced with a vector carrying a cytokine gene[169, 170]. These cells were mixed with tumor cells and then injected into animals. The local productilon of cytokines by the fibroblasts could induce a strong immune response to the neighboring tumor cells resulting in a systemic anti-tumor immunizing response. A variation of this approach is to transfer a cytokine gene into tumor infiltrating

lymphocytes (TIL) in an attempt to make these cells more effective in killing tumor cells[3, 106]. TIL are lymphocytes that tend to localize in tumor cells. In the first gene transfer protocol in humans, these cells were used as target cells[3]. TIL were obtained from biopsies of melanoma tissues, expanded in vitro in the presence of IL-2, transduced with a retroviral vector carrying the neoR gene, and then returned to the patients by intravenous infection. The aim was to analyze the trafficking, homing, and survival of TIL. The results demonstrated that the TIL may home to metastatic melanoma tumor cell deposits. In the subsequent experiments, the neoR gene was replaced with TNF-α cytokine gene to enhance their anti-tumor efficacy[171].

Another approach of immunotherapy is the transfer of co-stimulatory molecules such as B7 into tumor cells. B7 is a membrane bound molecule which is the ligand for the CD28 receptor of lymphocytes[172]. The B7-CD28 interaction is critical for activating T lymphocytes. Some tumor cells engineered to express B7 are rejected in the syngeneic animals, and there are systemic immune responses capable of offering protection against challenges from the non-modified cells[173].

Also of current interest is the transfer of HLA genes into tumor cells. The aim is to elicit an immune response to the foreign HLA antigen in the hope that other surface antigens are also recognized and an imune response is simultaneously developed against them. Dendritic cells (DC) are the natural antigen-presenting cells which express high levels of co-stimulatory molecules such as B7.1, B7.2, CD40[174]. They have a unique function and ability to capture, process, and present antigens in peptide-HLA complexes to unsensitized T lymphocytes and to deliver the co-stimulatory signals necessary for T cell activation. DC originate from hematopoietic progenitor cells in the bone marrow. Large numbers of DC can be generated from the bone marrow or peripheral mononuclear cells in the presence of GM-CSF+TNF-α or CM-CSF+IL-4[175]. It has been demonstrated that DC pulsed with synthetic tumor peptides could induce protective and therapeutic anti-tumor immunity[176]. Another approach may be to transduce these cells with a tumor-specific antigen or tumor-associated antigen, and/or a cytokine gene. Since the antigen is produced in the transduced cells, tumor-specific CD8+ cytotoxic T lymphocytes may recognize tumor-antigen peptides presented by class 1 MHC molecules. .

Modification of Tumor Suppressor Genes

It is known that several tumor suppressor genes encode vital cell cycle regulatory or other growth-related functions. The protein of the p53 tumor suppressor gene can arrest the replication of a cell with damaged DNA until the DNA is repaired. If it is not repaired, cells go into apoptosis. Losing of both alleles of p53 may transform normal cells into malignant cells. Defects in the function of this gene represent the most common alteration in human cancer. Transfer of the wild-type p53 gene into tumors with a mutated p53 gene resulted in the elimination

of the malignant clone[177]. Recently, a retroviral vector carrying the wild-type p53 gene was directly injected into tumor deposits in nine patients with non-small cell lung cancers. Although the efficiency of transduction of retroviruses into tumor cells was low, tumor regression was noted in three patients. It has been suggested that a bystander effect may have contributed to the tumor regressions observed[178].

Antisense Strategies

Ribozymes: Ribozymes are small, catalytic RNA molecules annealing to complementary sequences of mRNA, cleaving the mRNA and thereby suppressing the translation of mRNA. They have been demonstrated to be efficacious and specific in eliminating some transcripts in cellular and in vitro systems. The hammerhead ribozymes are well known ribozymes for purposes of gene therapy[179, 180]. These ribozymes consist of three basepaired stems, I, II and III, and a core region in which 13 residues are very specific for cleaving 3' to any GUX sequence (X can be C, A, or U). In the ribozymes, specific sequences can be introduced into stem I and III that arrange complementary recognition between the ribozyme and target RNA. It has been shown that designed ribozymes can inhibit the activities of some oncogenes[181]. Viral vectors are being constructed to carry a ribozyme sequence in the development of these strategies for gene therapy.

Oligodeoxynucleotides: Oligodeoxynucleotides are short synthetic nucleotide sequences of DNA that are synthesized as exact reverse complements of the desired mRNA target nucleotide sequence[182, 183]. Oligos of more than 15 to 17 nucleotides in length would have a unique sequence relative to the entire genome and should normally be able to bind to the RNA species and interrupt the translation of mRNA into protein by the destruction of molecules with RNase H. Oligos complementary to genomic DNA can also interact with DNA and form a triple helix which would inhibit transcription. Antisense oligonucleotides can be administered systemically. The main problems of the technique are degradation of the oligonucleotides in plasma and low cellular uptake. This method could potentially be used for example in CML to block the gene product of the pathological bcr/abl gene sequence, and subsequently induce disease control. In CML, both the bcr/abl and myb genes can be targeted. Growth inhibition has been obtained in vitro with synthetic antisense oligonucleotides against bcr/abl in CML[184], myc in lymphoma[185], and myb in leukemia cell lines[186]. A K-ras gene in the antisense orientation was successfully used to transduce human lung cancer cell lines using a retroviral vector[187]. A protocol for ex vivo purging of CD34+ enriched CML marrow cells with c-myb antisense DNA followed by autologous transplantation is in progress[183].

These approaches also have limitations. An important issue is the targeted delivery of the vectors that carry antisense oncogenes or tumor suppressor genes. Another issue is whether it is necessary to deliver the genes into each malignant cell, in which case reaching them through multiple cell layers of solid tumor masses poses a major difficulty.

Conclusions and Future Perspectives

Although it has been shown by several groups that hematopoietic progenitor cells or even LTC-IC can be easily transduced with high efficiency by retroviral vectors in vitro, and that long-term expression and high transduction can be obtained in mice after transplantation of the gene manipulated cells, this has not been observed in primate models or in human clinical trials. The reason for this discrepancy is not properly known. Since there is no well defined method to evaluate the pluripotent stem cells in vitro, it is not clear whether true repopulating stem cells are transduced by retroviral vectors. A low transduction rate into true stem cells may be due to lack of cell cycling and integration, but could also be due to insufficient viral receptor density. The presence and density of viral receptors on pluripotent stem cells are unknown. New in vitro methods and animal models for evaluation of the true stem cells need to be developed.

Although more than 250 clinical gene transfer trials have been initiated[101], and more than 2.000 patients have been treated[112], very few patients have directly benefitted from them. However, they have been very important in establishing proof of principle: that it is possible to transfer genes into human cells and achieve long-term expression in vivo with very few side effects to the patients. For therapy of most single gene defects to be effective, better delivery systems are required, as well as more precise knowledge of the optimal conditions for transduction of hematopoietic cells. Low dose myeloablative conditioning could increase engraftment of gene-corrected cells in the patients. New vectors for in vivo gene transfer and targeting will possibly reduce the need for ex vivo manipulation and reinfusion of hematopoietic cells in the future.

We have far to go before the extraordinary potential of gene transfer for therapy of hematological malignancies can be fully realized. However, the promises are still great, and the problems have almost been identified. Therefore, new developments are excepted to establish gene therapy as an effective revolutionary approach.

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