Somatic Gene Therapy

Current Status and Future Prospects

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Introduction

The great power of recombinant DNA technology to provide pure, cloned genes in unlimited quantities has inevitably led to proposals for the genetic treatment of inherited disorders. Before substantial research into gene structure and function, these scenarios were highly speculative. Recent developments in molecular biology permit the formation of more focused and realistic goals. Here we review current concepts relating to gene therapy in the light of recent research.

Gene therapy: potential strategies

The term "gene therapy" potentially encompasses many different approaches to the management of inherited disease. In principle, genes could be introduced either during embryonic or fetal life (germline therapy) or only into specific somatic cells of an individual (somatic gene therapy). In the latter instance, genetically modified DNA would not be passed on to subsequent generations. Ideally, investigators would choose an approach that might correct a defective gene in situ. This necessitates, of course, precise knowledge of the gene involved and, possibly, its particular mutation. In situ correction would ensure appropriate regulation of the corrected gene with no damage to the remaining DNA. Although experimentally feasible in yeast (1), directed insertion (or recombination) in the mammalian genome appears to occur rather infrequently relative to nonspecific, random integration of exogenous genes (2). Therefore, this method of gene therapy is precluded for the foreseeable future.

A second alternative would be the introduction of appropriately regulated, intact genes into the germline without replacement of the abnormal gene(s) that resides in the body. This strategy is particularly effective in Drosophila using a special plasmid vehicle (3). Although several genes have been introduced into the germline of mice and been shown to be appropriately regulated in resulting progeny (transgenic mice) (4), insertional mutagenesis due to the random integration of foreign genetic material into critical regions of the genome and current ethical considerations precludes this approach to the treatment of human disorders.

Therefore, as currently envisioned, potential gene therapy in man will be restricted, if not by technical obstacles alone, to random introduction of normal gene copies into somatic cells that already possess mutant alleles of the gene. To a large extent, this will be "makeshift" therapy as the introduced genes will not be regulated as faithfully and precisely as the normal in situ gene. These distinctions are especially relevant to consideration of some genetic diseases which might realistically benefit from somatic therapy. Disorders which are most frequent worldwide, such as the thalassemias, may be least suitable candidates.

Of somatic cells of the body, bone marrow stem cells represent a particularly attractive target as bone marrow is readily accessible for sampling, in vitro manipulation, and reinfusion. In addition, stem cells have the potential to differentiate and, thereby, repopulate hematopoietic tissue completely in a suitable host environment.

Several techniques have been developed for the introduction of foreign genetic material into somatic cells. In principle, each could be applied to bone marrow stem cells. These include the use of microinjection (5), coprecipitation of DNA and calcium phosphate (6) or DEAE-dextran (7), electric shock (8), and modified DNA (SV40 or adenovirus) (9) and RNA (retroviruses) (10) viruses. However, since stem cells are present in bone marrow at such a low concentration ($10–30/10^5$ nucleated marrow cells), most of these methods are too inefficient or cumbersome to be utilized. Attention of late, therefore, has focused on the potential of defective retroviruses to provide highly efficient transfer and integration of foreign genetic material into such cells.

Retrovirus-mediated gene transfer

Retroviruses have been tailored by evolution for efficient delivery of their genomes to cells, subsequent integration within the host genome, and high level expression of their internal sequences. Defective retroviral vectors in which sequences essential for virus production have been removed to permit insertion of foreign DNA sequences have been constructed in various laboratories to take advantage of many of these attractive features. The life-cycle of a wild type retrovirus, such as Moloney leukemia virus (MuLV)[1] (Fig. 1), illustrates salient features which can be exploited for gene transfer. The virion particle contains a dimer of the viral RNA within a coat of core proteins surrounded by a lipid bilayer which contains viral-specific glycoproteins that mediate attachment to cells upon infection. Several molecules of the virus-encoded enzyme, reverse transcriptase, are present in the virion. Upon entry into the cytoplasm of an infected cell, the virion coat is removed and the RNA genome is copied into an exact DNA copy by reverse transcriptase. A double-stranded DNA closed circle is formed and retroviral sequences in the

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1. *Abbreviations used in this paper:* ADA, adenosine deaminase; DHFR, dihydrofolate reductase; HPRT, hypoxanthine phosphoribosyltransferase; LTR, long-terminal repeats; MuLV, Moloney leukemia virus.

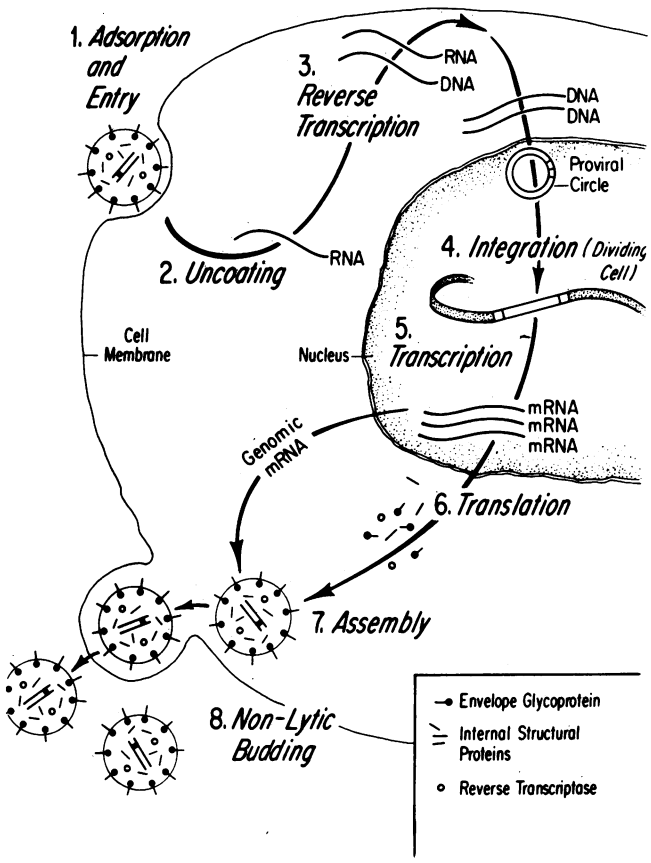

Figure 1. Life cycle of wild type retrovirus. Steps in the life cycle include (*1*) adsorption and entry, (*2*) the uncoating of the virion particle, (*3*) production of a DNA copy of the viral genome by viral-encoded reverse transcriptase, (*4*) integration of provirus after entry of provirus into the nucleus, (*5*) transcription of new genome with the subsequent production of messenger RNA, (*6*) translation of messenger RNA into new glycoproteins, internal structural proteins, and reverse transcriptase, and (*7*) nonlytic budding of new particles after assembly.

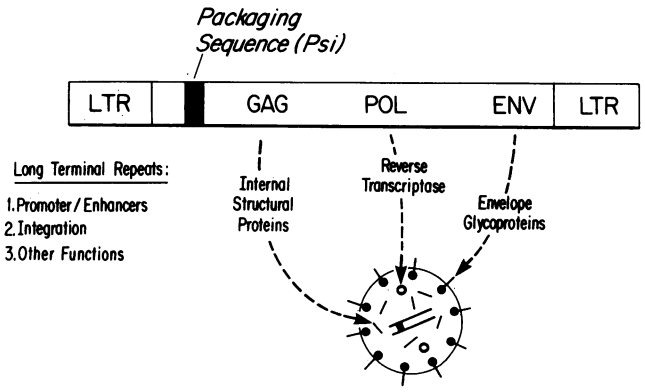

Figure 2. Genome of wild type retrovirus. LTR including the viral promoter, enhancers, and sequences important for integration. Psi, packaging sequence required in *cis* for packaging of genomic RNA into virion particles; *GAG*, gene encoding internal structural proteins of virion core; *POL*, gene encoding reverse transcriptase; *ENV*, gene encoding envelope glycoproteins present on virion membrane.

long-terminal repeats (LTR) (Fig. 2) direct integration into the host genome. After integration, promoter sequences of the LTR transcribe full length (genomic) and spliced (subgenomic) RNAs. Subgenomic RNAs are translated to generate glycoproteins, while genomic RNA is either translated into core (gag) proteins and reverse transcriptase or packaged into virion particles as new genomic RNA. The subsequent assembly and budding of virion particles from the infected cells is nonlytic.

For their use as gene transfer vectors, the following features of this life cycle are important: (*a*) the integration of the DNA copy of the viral genome is highly efficient and is directed by sequences within the retroviral genome and preserves the structure of this genome; (*b*) essential virion proteins (gag, pol, and env) can be supplied effectively in *trans;* (*c*) the integrated viral genome behaves subsequently as a cellular gene and is transferred as such to all progeny of the originally infected cells; and (*d*) the budding of virus is nonlytic, which allows maintenance of permanent cell lines that continuously produce recombinant retroviruses.

Recombinant retrovirus vectors maintain those sequences needed in *cis* for the infection, integration, and transcriptional control of the genome (contained in the LTRs). Viral sequences whose functions can be supplied in *trans* are deleted. Thus, *gag, pol,* and *env* sequences are replaced in most vectors by a dominant selectable marker—*Escherichia coli.* xanthaine-guanine phosphoribosyl transferase (11): G418-resistance [*neo*] (12); hypoxanthine phosphoribosyltransferase (HPRT) (13); or dihydrofolate reductase (DHFR-methotrexate-resistance) (14, 15)—and a restriction site into which the sequence to be transferred (cDNA or genomic DNA) may be cloned.

The recombinant defective retroviral genome (as a plasmid DNA) is introduced by standard transfection into murine fibroblasts to generate cell lines that produce the recombinant retrovirus. Although simultaneous infection with a wild type virus, such as MuLV, can provide the required packaging proteins, this leads to generation of mixed wild type/recombinant retrovirus stocks, and most investigators now use specialized "packaging" cell lines, called $\psi 2$ (16) and ψam (17), which permit generation of pure, helper-free recombinant retrovirus. This approach is particularly useful for studies of intact animals since the presence of helper-virus may be deleterious to the animal and may also lead to the continued spread of recombinant virus throughout the organism once the infected cells are placed back in the animal. Pure recombinant retroviruses are, therefore, designed for "single" hit infections of cells and do not lead to viral dissemination.

Gene transfer into hematopoietic cells

Hematopoietic cells are the most accessible primary cells for possible genetic manipulation. These cells can be removed easily, manipulated in vitro, and then reintroduced into an intact organism. Hematopoiesis is a well-characterized and carefully orchestrated developmental pathway. Both in vivo and in vitro assays have been developed to study cells at various stages of maturation (18). Several severe genetic disorders are manifested primarily in bone marrow-derived cells.

Several studies have demonstrated gene transfer into primary hematopoietic cells. Although Cline and co-workers (19) reported introduction of foreign genes into murine bone marrow by calcium phosphate coprecipitation, the low and variable efficiency of transfer by this approach severely limits its applicability. To achieve transfer into pluripotent hematopoietic cells, most research groups have turned to recombinant retroviruses. Murine bone marrow is cocultivated with packaging cell lines that produce recombinant retrovirus, harvested, and injected into lethally

irradiated, syngeneic recipient mice (or into the murine mutant strain W/W^v without irradiation). Since the spleen is a major site of hematopoiesis in the mouse, especially post-transplantation, these cells can be used to monitor gene transfer by standard Southern blot analysis of DNA. Use of the spleen colony assay of Till and McCulloch (20) has permitted demonstration that gene transfer into a self-renewing, pluripotent stem cell has occurred (21). More recent work by Dick et al. (22) and Keller et al. (23) showed that the stem cell containing the inserted gene is long-lived (present up to 4 mo after transplant) and totipotent (giving rise to both lymphoid and myeloid progeny). Particularly efficient gene transfer has also been observed by Eglitis et al. (24). Recently, Gruber et al. (25) have described retroviral vector-mediated gene transfer into human hematopoietic progenitor cells. Genes that have been transferred into intact mice by retroviruses include G418 (neomycin-resistance gene), HPRT, DHFR (methotrexate-resistance), and human adenosine deaminase (ADA). At present, gene transfer into a significant proportion of hematopoietic stem cells with retroviruses appears to be feasible with the available technology.

Gene expression following transfer

Although retrovirus-mediated gene transfer generally leads to high-level expression of the transferred genome in tissue culture cells, less data exist on the subsequent expression of integrated sequences in vivo, that is within the stem cell-derived progeny. The success of somatic gene therapy naturally will depend on the regularity and level of this expression. Although experience is limited to date, it appears that potential difficulties remain in this area.

Miller et al. (13) first reported data on human HPRT expression in mice which received marrow infected with a recombinant virus carrying human HPRT and helper virus. Although most spleens contained human HPRT gene sequences, HPRT expression was observed in only few mice and at a very low level. Recently, Dick et al. (22) reported the recovery of G418-resistant bone marrow progenitor cells (CFU-GM) from animals transplanted with marrow infected with a G418-resistance gene. Although all hematopoietic stem cells (as measured by the CFU-S assay) contained the transferred gene, only a fraction (3%) expressed G418 resistance in vitro upon selection of primary cells in progenitor assay. Similarly, Keller et al. (23) have also demonstrated G418 resistance of progenitors recovered from transplanted mice. In two studies (23, 24), the phosphotransferase activity encoded by the G418-resistance gene has been demonstrated in CFU-S after retroviral infection.

Although expression of G418-resistance (23, 24) and DHFR (15) genes is demonstrable in hematopoietic cells in vivo, the precise level of expression appears substantially below that seen in cell culture and desired for genetic therapy. In our own work with ADA, we have not yet observed substantial human enzyme activity upon infection of murine CFU-S with retroviruses containing human ADA (26). We have observed that only about 50% of retrovirus DNA positive spleens or spleen foci of transplant recipients contain retrovirus-directed messenger RNA transcripts. Where present, the level of such RNAs is variable and on average much lower than that seen in tissue culture cells infected in vitro (26).

The precise reasons for the apparently variable and low level of expression of retroviral constructs in hematopoietic cells in vivo are unknown, but very likely reflects biological differences between cultured cell lines and hematopoietic stem cells and their progenitors. The failure of several strong promoter and enhancer sequences to function in teratocarcinoma cells provides a precedent for such apparently contradictory findings. Recent findings by Gorman et al. (27) demonstrate that sequences within the retrovirus LTR may be targets for transcriptional down-regulation in these teratocarcinoma stem cells. Whether these results are relevant to the problems encountered in hematopoietic stem cells is unknown. Systematic examination of various enhancer/promoter combinations and their subsequent expression following retroviral transfer into hematopoietic cells is warranted.

Additional considerations

Another important issue relates to the nature of the stem cell into which a foreign gene is inserted by retroviral transfer. As noted above, initial work showed the cell to be a self-renewing, multipotential stem cell capable of giving rise to hematopoietic colonies in the spleen of recipient animals (CFU-S) (21). More recently, at least some of the cells have been shown to give rise to both lymphoid and myeloid hematopoietic cells (22, 23). The vast majority of the most primitive of stem cells (those with most self-renewal capacity) are in the G0 state. As a result these cells are least likely to integrate foreign DNA via the retrovirus life cycle. In fact, some investigators now believe that the CFU-S stem cells, and certainly those scored on day 8–10 in the spleen following transplantation, may bear little relevance to long-term reconstitution of the animal after transplant (28). This may explain why the number of progeny cells containing a foreign gene some months after transplant is uniformly low (either by functional assay or by DNA analysis of stem cells remaining within bone marrow) irrespective of how efficient the gene transfer into CFU-S is initially.

These concepts have added significance within the context of "gene therapy." It should be anticipated that initial attempts at therapy might achieve gene transfer into only a limited number of bone marrow stem cells. Once infused back into the recipient, those cells will be required to repopulate the deficient hematopoietic compartment. Added to the stress of expanding a limited number of stem cells containing a foreign gene into a vast hematopoietic compartment are the potential effects on self-renewal and differentiation of in vivo selective pressures. For instance, in ADA deficiency leading to combined immunodeficiency, where lymphoid precursors seem to be the cytotoxic target of enzyme deficiency, one would presume some selection of these cells in vivo. However, these cells may possess little self-renewal capacity and those progenitors containing a functional gene might be lost rapidly by differentiation. The benefits of introducing the foreign gene into cells with the highest capacity for self-renewal are apparent. Considerable research is required to understand in detail the behavior of stem cells, both with and without selective pressures in vivo, into which foreign gene sequences have been inserted.

Disorders suitable for initial attempts at therapy

Inadequacies in our understanding of gene regulation and limitations in those somatic cells which are accessible for gene introduction severely restrict those disorders which might be amenable to genetic therapy. As a first approximation, only those conditions for which bone marrow transplantation is beneficial stand to profit from gene transfer into hematopoietic cells. Immunodeficiency states due to either ADA or purine nucleoside phosphorylase (PNP) deficiency would appear to be among the best candidates (29). Both diseases are the result of enzyme deficiency within bone marrow-derived cells. Both enzymes are single-chain polypeptides that undergo essentially no intracellular

processing and for which the respective human genes have been cloned. Tissue-specific regulation of gene expression is likely not to be critical in these instances and subnormal amounts of enzyme (perhaps 10–15% of normal) may be sufficient to correct the disorders. Those inherited conditions in which major effects are manifest outside the bone marrow, such as Lesch-Nyhan disease or aminoacidopathies, are probably less optimal candidates for gene transfer. In these situations where central nervous system disturbances are seen, clinical benefit would rely on the efficiency with which marrow cells could metabolize toxic substances that act at a distant site. For example, high-level expression of the argininosuccinate synthetase gene in marrow cells might remove the diffusible substrate that accumulates in citrillinemia (30).

A logical first step in evaluating the potential for gene therapy in these disorders is treatment with bone marrow transplantation. If clinical improvement occurs, then gene therapy might be of benefit. If not, it is unrealistic to expect that introduction of a gene into marrow would effect a positive clinical result. Whether gene introduction into other somatic cells, e.g., hepatic cells or skin, will be useful in selected diseases or deficiency states needs additional study.

The above considerations imply that gene therapy is likely to be applied only in rare, specifically chosen genetic disorders for the foreseeable future. Until our understanding of gene regulation permits high-level and regulated expression of eukaryotic genes upon gene transfer into somatic cells, the treatment of more common conditions, such as thalassemia or sickle cell anemia, cannot be rationally approached.

Nevertheless, given the current pace of progress in the understanding of eucaryotic regulatory sequences and factors, application of these technologies to seemingly more complex diseases will hopefully develop in the not too distant future.

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