

Molecular Therapy and Prevention of Liver Diseases

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Abstract: Molecular analyses have become an integral part of biomedical research as well as clinical medicine. The definition of the genetic basis of many human diseases has led to a better understanding of their pathogenesis and has in addition offered new perspectives for their diagnosis, therapy and prevention. Genetically, human diseases can be classified as hereditary monogenic, acquired monogenic and polygenic diseases. Based on this classification, gene therapy is based on six concepts: (1) gene repair, (2) gene substitution, (3) cell therapy, (4) block of gene expression or function, (5) DNA vaccination and (6) gene augmentation. While major advances have been made in all areas of gene therapy during the last years, various delivery, targeting and safety issues need to be addressed before these strategies will enter clinical practice. Nevertheless, gene therapy will eventually become part of the management of patients with various liver diseases, complementing or replacing existing therapeutic and preventive strategies.

Key Words: Gene therapy; Gene repair; Gene replacement; Gene expression or function; Immune therapy; Liver Diseases

Molecular biology and recombinant DNA technology increasingly contribute to the diagnosis, therapy and prevention of human diseases. Molecular methods allow the early and/ or specific detection of inherited, infectious and malignant liver diseases. In addition, such analyses increasingly lead to a better understanding of the pathogenesis of the various liver diseases which in turn had an impact on patient management, including the presymptomatic identification of patients at risk, the correct staging of the disease and the follow-up of patients undergoing therapy. Thus, molecular biology is increasingly

becoming an integral part of basic as well as clinical hepatology. In the following we will briefly review current concepts and potential applications of gene therapy for the treatment or prevention of various liver diseases.

GENETIC CLASSIFICATION OF DISEASES

Genetically, human diseases can be classified into three major categories (2, 27, 36, 74): (1) Hereditary monogenic diseases that are caused by a single gene defect and inherited by the classical Mendelian rules. There are more than 4,000 monogenic diseases described. For an increasing number of these diseases the genetic basis is being identified. (2) Acquired monogenic diseases are infections as well as malig-

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nancies that are caused by the mutation or epigenetic modification of a single gene. (3) polygenic diseases are associated with mutations of several genes that are acquired and frequently accumulated during life-time. Several common human diseases belong to this category, such as most malignancies.

Gene therapy is defined as the introduction of genetic material into human cells with a therapeutic or preventive benefit. In a broader definition, cell or organ transplantation are included. In the following we will discuss the basic concepts of gene therapy (2, 27, 36, 74, 77, 78, 88) as well as some therapeutic and preventive applications for liver diseases.

GENE THERAPY OF LIVER DISEASES

Based on the genetic classification of diseases detailed above, the principle of gene therapy involves 6 concepts (Table 1): gene repair, gene substitution and cell therapy for hereditary monogenic diseases, block of gene expression and DNA vaccination for acquired monogenic diseases and gene augmentation and DNA vaccination for polygenic diseases. For clinical applications, gene therapy is explored with the aim to either provide novel therapeutic strategies for diseases for which there is no treatment available or to replace and in some cases complement existing treatment modalities, thereby increasing therapeutic efficacy and/ or reduce adverse events.

Gene repair

Table 1. Concepts of Gene Therapy

Liver Diseases	Gene Therapy
Hereditary monogenic	Gene repair
	Gene replacement
	Cell/organ transplantation
Acquired monogenic	Block of gene expression
	DNA vaccination
Polygenic	Gene augmentation
	DNA vaccination

An increasing number liver diseases has been molecularly defined as a defect of a single gene (Table 2). In this context, one therapeutic concept is the *in vitro* or *in vivo* repair of the defective gene. Indeed, in the Gunn rat model of the Crigler Najjar syndrome type I Kren *et al.* (50, 59, 65, 92) were able to partially correct the genetic defect underlying the UDP-glucuronosyl transferase deficiency by the intravenous injection of a cyclic normal/ wild-type chimeric oligonucleotide. While these findings have not been independently confirmed or extended to other hereditary monogenic (liver) diseases, the data suggest that it is in principle possible to repair a gene defect *in vivo*. Further, it has been shown that cellular RNA species can be modified by *trans*-splicing group I ribozymes. Such ribozymes may in principle allow to treat a variety of inherited diseases at the RNA level (50, 65, 92).

Gene substitution

The targeted substitution of a defective cellular gene by the normal/ wild-type homologue with production of the physiological gene product is another approach to correct a hereditary or acquired monogenic gene defect. Indeed, in an animal model of hereditary tyrosinemia type 1 (HT1), a liver disease caused by a deficiency of fumarylacetoacetate hydrolase (FAH), multiple injections of a retroviral vector

Table 2. Hereditary Monogenic Liver Diseases (selection)

Gene	Disease
UDP-glucuronosyl transferase	Crigler Najjar syndrome type I
Alpha-1-antitrypsin deficiency	Liver cirrhosis, emphysema
CF transmembrane regulator	Mucoviscidosis, cystic fibrosis
Factor VIII	Hemophilia A
Factor IX	Hemophilia B
Fumarylacetoacetate hydrolase	Tyrosinemia type 1
LDL receptor	Hypercholesterolemia
Ornithine transcarbamylase	Hyperammonemia

carrying the FAH gene resulted in a gene transfer efficiency of >90% of hepatocytes and the restoration of a normal liver function (33, 82). In patients, examples for gene substitution are the partial correction of severe hemophilia A by the *ex vivo* transduction of autologous skin fibroblasts with the normal/wild-type factor VIII gene, followed by laparoscopic implantation of the genetically modified fibroblasts into the omentum majus (89) or of hemophilia B by adenovirus-associated vector (AAV)-based gene transfer (55).

In rare situations in which a hepatocellular carcinoma (HCC) is caused by the mutation of a tumor suppressor gene, e.g., the p53 gene, the substitution of the mutated by the normal/wild-type gene *in vitro* can reduce the number of tumor cell colonies and restore cisplatin sensitivity (115, 118).

Cell therapy

Allogeneic or *ex vivo* genetically modified autologous hepatocyte transplantation is a promising strategy to treat hereditary monogenic liver diseases. In patients with familial hypercholesterolemia (FH) that is caused by various mutations in the low density lipoprotein (LDL) receptor gene (11), apart from orthotopic liver transplantation (7, 45), liver-directed gene therapy has been performed in a pilot study in five patients (34, 35). Autologous liver cells, prepared from a surgical biopsy, were transduced *ex vivo* with a recombinant retrovirus expressing the normal LDL receptor. These *ex vivo* genetically modified hepatocytes were transplanted by portal infusion and resulted in significant and prolonged reductions in LDL cholesterol in 3/ 5 patients for at least four months, demonstrating the feasibility of engrafting a limited number of *ex vivo* transduced hepatocytes.

Also, allogeneic hepatocyte transplantation has been successfully used in patients to partially correct Crigler-Najjar syndrome type I (26) and glycogen storage disease type I (79).

Block of gene expression or function

For diseases caused by the expression of an acquired gene or the overexpression of an endogenous gene, blocking gene expression can be an effective therapeutic approach. Several strategies can be employed: interference with the transcription of genes by binding of transcription factors to nucleic acids introduced into or synthesized in the cells (decoy strategy) (42, 43), by binding of single-stranded nucleic acids to double-stranded DNA, forming a triple helix structure (42, 43), hybridization of RNA molecules possessing endonuclease activity (ribozymes) to RNA, resulting in its sequence-specific cleavage (40, 99), RNA interference (RNAi) by small inhibiting RNA (siRNA) or microRNA (miRNA) (23, 56, 98, 111) block of translation by antisense oligonucleotides (14, 42, 43, 100) and the intracellular synthesis of peptides or proteins, interfering with their normal counterpart, termed dominant negative (DN) mutant strategy (44). These different strategies have been applied to a number of malignant and infectious diseases. In particular ribozymes, siRNAs, antisense oligonucleotides and DN mutants have been experimentally explored to treat hepatitis B virus (HBV) and hepatitis C virus (HCV) infections.

Ribozymes. Ribozymes ('ribonucleic acid enzymes') were originally discovered as naturally occurring RNA molecules that catalyze the sequence-specific cleavage of RNA and RNA splicing reactions (40, 99). This catalytic activity is the major attraction of the ribozyme concept since one ribozyme can cleave

many target RNAs. Ribozymes that cleave RNA are being developed as inhibitors of gene expression and viral replication. Several studies have clearly demonstrated that hammerhead ribozymes can specifically cleave HBV RNA (6, 105) or HCV RNA (66, 90) *in vitro*. *In vivo*, however, an efficient ribozyme-mediated cleavage of HBV RNA could not be demonstrated to date. For HCV infection, the elimination of HCV RNA in infected hepatocytes by ribozymes has also been reported (66, 110).

Small interfering RNA. RNAi is a recently discovered basic intracellular mechanism (23, 56, 98, 111) that has been explored also for the inhibition of HBV and HCV infection. For HBV, inhibition of viral gene expression and replication has been shown *in vitro* (58, 97, 117) and in different mouse models *in vivo* (29, 32, 57, 71, 102). For HCV, inhibition of viral gene expression and replication has been shown *in vitro* in the replicon system (54, 87, 113). While effective in blocking viral gene expression and replication, *in vivo* oversaturation of cellular miRNA/short hairpin RNA (shRNA) pathways can result in lethal hepatotoxicity (32). For future RNAi-based strategies in animals or humans, these findings indicate that the control of intracellular shRNA expression levels through optimizing shRNA dose and sequence will be key to reduce the risk of oversaturating endogenous small RNA pathways.

Antisense oligonucleotides. Antisense nucleic acids are designed to specifically bind to RNA or mRNA, resulting in the formation of RNA-DNA (antisense oligodeoxynucleotides) or RNA-RNA hybrids (antisense oligoribonucleotides) with an arrest of RNA replication, reverse transcription or mRNA translation (14, 42, 43, 100, 107). Antisense effects can be

potentiated by degradation of RNA in RNA-DNA hybrids by cellular RNases H. While conceptually simple, it is clear now that not all desired as well as undesired effects are caused by the target sequence specific antisense action of the oligonucleotides or the cellular enzymes mentioned above (10, 22).

The antisense strategy has been successfully applied *in vitro* to HBV infection (9, 30, 80, 114) and to HCV infection (1, 39, 75, 96, 103, 108). In addition, studies in nude mice (116), in the duck hepatitis B virus (DHBV) (81) and the woodchuck hepatitis virus (WHV) model of HBV infection (5) demonstrated the *in vivo* applicability of this approach. While no toxic effects have been observed in these experiments, the contribution of non-antisense effects to the inhibition of viral replication or gene expression has not been systematically assessed in most studies. Independent of the antisense or non-antisense mechanism of the biological effects, an *in vitro* screening procedure for the identification of functionally active oligonucleotides (10, 101) should greatly facilitate the design of oligonucleotide based antiviral therapies.

Interfering peptides or proteins. The intracellular synthesis of interfering peptides or proteins, including single chain or whole non-secreted antibodies, is aimed at the specific interference with the assembly or function of viral structural or non-structural proteins and represents a type of intracellular immunization (3). This approach has been shown for block mammalian and avian hepadnavirus gene expression and replication *in vitro*. For example, the fusion of different polypeptides of various lengths to the carboxy-terminus of the viral core protein yields DN mutants (21, 93, 94, 106). These DN mutants are species-specific and suppress viral replication by at least 90 %

at an effector to target ratio of 1:10. Moreover, the non-secretory form of the hepatitis B e antigen (HBeAg) was shown to effectively inhibit viral replication and may indeed act as a natural regulator of HBV propagation (14, 42, 43, 100). The potential advantage of DN mutants over ribozymes or antisense oligonucleotides is their relative independence from viral sequence variations, minimizing the risk of selecting or accumulating 'therapy escape' mutants.

DNA vaccination

A novel approach is DNA vaccination resulting in the manipulation of the immune system by introduction of expression vectors into muscle cells or dendritic cells and long lasting cellular and humoral immune responses. The direct gene transfer into muscle (8) represents an exciting new development and elegant application of gene therapy (72, 83). The therapeutic DNA vaccine acts by the intracellular plasmid-derived synthesis of a viral protein which enters the cell's MHC class I pathway (72). Only proteins that originate within the cell can be processed by MHC class I molecules that carry fragments of the protein to the cell surface. There they stimulate CD8+ cytotoxic T cells, resulting in cell-mediated immunity. In principle, this strategy is applicable to the treatment of acquired genetic diseases, associated with the expression of disease-specific antigens serving as targets for CD8+ cytotoxic T cells.

Therapeutic DNA vaccination has been experimentally explored for HBV (20, 62, 70, 95, 104) as well as HCV infection (64, 69) and holds great promise as an effective molecular therapy for these viral diseases. In this context, the coexpression of HBsAg and interleukin-2 was shown to greatly increase humoral as well as cellular immune response

(16).

Further, DNA-based tumor vaccination against HCC may be possible, for example, by intramuscular introduction of a plasmid expressing HCC-specific antigens or antigens that are highly overexpressed in HCC cells, such as AF-20 antigen, insulin receptor substrate-1 (109) alpha-fetoprotein (31), aspartyl asparaginyl hydroxylase, mutated p53 protein and others. Potential limitations of this strategy include the regulation of the immune response as well as the low level expression of the targeted antigen in non-malignant cells (28), rendering them susceptible to immune mediated elimination as well.

Gene augmentation

Polygenic diseases are among the most prevalent clinical problems. In this situation, gene augmentation is aimed at the local expression of a therapeutic gene product that is physiologically not expressed or expressed at therapeutically insufficient levels. This strategy is explored among others for the treatment of hepatocellular carcinoma (HCC). Gene augmentation is aimed at the local expression of a therapeutic gene product that is physiologically not expressed or expressed at therapeutically insufficient levels.

Suicide gene therapy. An interesting strategy to treat HCCs is genetic prodrug activation therapy *via* the introduction of a 'suicide gene' into malignant cells followed by the administration of the prodrug. This concept has been experimentally explored in HCC cells *in vitro* and *in vivo*, e.g., for the HSV-tk gene (47, 52, 53, 86, 112) the gene encoding cytosine deaminase (CD) that converts the prodrug 5-fluorocytosine to 5-fluorouracil which inhibits RNA and DNA synthesis during the S-phase of the cell cycle (51), the gene encoding purine nucleoside phosphorylase that con-

verts purine analogs into freely diffusible toxic metabolites (61, 76) as well as the gene encoding cytochrome p450 4B1 (76). A significant bystander effect of cell killing caused by suicide gene expression could be demonstrated *in vitro* and *in vivo*, based on cell-cell contact rather than release of cytotoxic substances from the transduced cells (63). At the same time, the bystander effect may also affect non-malignant dividing cells in the target tissue, potentially limiting the application of this strategy.

Immune therapy. In the process of malignant transformation new antigenic surface proteins can be expressed (tumor antigens) or oncofetal proteins can be re-expressed, e.g., alpha fetoprotein (AFP).

AFP-specific immune therapy has been explored in mice and humans. Vaccination with an AFP-expressing DNA construct resulted in tumor rejection and prolonged survival in a mouse model (31). Also in patients AFP-specific T cells could be detected (12, 13). Since AFP is not only expressed by tumor cells but also by regenerating liver cells and in liver cirrhosis immunization against AFP carries the risk of autoimmune hepatitis, as has been experimentally shown in mice (28).

Immune therapy with antigen presenting cells (APC) is another strategy that has been explored using dendritic cells (DC) exposed to tumor lysates, peptides or *ex vivo* transduced with tumor antigen expressing DNA constructs. While this strategy is conceptually very interesting, to date there are no data available that demonstrate its clinical efficacy (49).

Cytokine gene therapy has been explored using tumor necrosis factor (TNF)-alpha, GM-CSF, interferon-alpha or -gamma, interleukin (IL)-2, -4, -6, -7,

-12 and -18, B7-1 as well as CD40 ligand. Complete regression of a HCC was demonstrated *in vivo* by TNF-alpha (15), IL-2 (46), IL-12 (4) and an activatable interferon regulatory factor-1 in mice (60). Gene transfer was achieved *in vivo* by delivering retroviral (15) or adenoviral vectors (46) systemically, directly into the tumor or into the peritoneal cavity. A pilot study in patients with gastrointestinal tumors exploring the intratumoral injection of an adenoviral IL-12 expression construct showed only marginal efficacy, however (91).

Antiangiogenic gene therapy. This concept has been experimentally explored in a HCC mouse model using the angiostatin gene. Angiostatin gene transfer resulted in reduced tumor volume and vascular density (48).

Oncolytic viruses. Such a new and elegant approach uses p53 mutations for selective, adenovirus-mediated lysis of tumor cells. Thus, an adenovirus mutant was engineered that replicates selectively in p53-deficient human tumor cells (37, 41, 68). Other examples are the adenoviral introduction of Smac antagonizes the inhibitor of apoptosis proteins in HCC tumor cells and enhances tumor cell death (85) and tumor-specific replication-restricted adenoviral vectors (38). Further, the intravascular administration of a replication-competent genetically engineered herpes simplex virus (HSV)-1 resulted in oncolysis of a diffuse HCC (84). More efficient HSV-1-based vectors have been developed (67).

MOLECULAR PREVENTION OF LIVER DISEASES

DNA-based prophylactic vaccination against HBV

infection, for example, is possible by intramuscular introduction of a plasmid expressing hepatitis B surface antigen (HBsAg). HBsAg is taken up by cells *via* phagocytosis or endocytosis, processed through the major histocompatibility complex (MHC) class II system and primarily stimulates an antibody response through CD4⁺ helper T cells with the production of anti-HBs (17-19, 72, 73, 83). While the DNA-based vaccination against HBV infection induces anti-HBs antibodies and prevents HBV infection, DNA-based vaccination against HCV infection of chimpanzees has been shown not to prevent infection but to result in the resolution of acute HCV infection through an effective vaccine-induced cellular immune response (24, 25).

SUMMARY AND PERSPECTIVES

Molecular analyses have become an integral part of biomedical research as well as clinical medicine. The definition of the genetic basis of many human diseases has led to a better understanding of their pathogenesis and has in addition offered new perspectives for their diagnosis, therapy and prevention. Genetically, human diseases can be classified as hereditary monogenic, acquired monogenic and polygenic diseases. Based on this classification, gene therapy is based on four concepts: gene repair, gene substitution, cell or organ transplantation, block of gene expression or function, gene augmentation and DNA vaccination. While the recent developments in gene therapy for liver diseases are promising, various delivery, targeting and safety issues need to be addressed before these strategies will enter clinical practice. Nevertheless, gene therapy will become part of the management of patients with liver

diseases, complementing existing diagnostic, therapeutic and preventive strategies.

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