REVIEW ARTICLE

APPLICATIONS OF GENE THERAPY FOR THE TREATMENT OF CANCER

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Summary

The aim of this article is to review the latest progress concerning cancer gene therapy. By the term gene therapy it is meant the transfer of therapeutic genetic material into cells for the treatment of the causes of a particular disease. The recent advance in the field of molecular biology and the rapid development of recombinant-DNA technology have improved gene therapy. It is known that cancer arises from the genetic mutations of cells; therefore there is a possibility of causal cancer treatment by gene therapy. Key-point for the success of gene therapy is the development of gene vectors, which are able to transfer the therapeutic genes. Gene vectors can be classified as viral and non-viral vectors. The various gene therapy strategies are divided into six major categories, which are briefly analyzed. Finally, methods of cell targeting are presented.

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1. Introduction

Extensive studies have shown that genetic mutations of normal cells are responsible for their transformation into malignant cells [1]. It is considered that the intervention in the genetic material of malignant cells might alter the mutating procedure and eliminate them. This is the concept of cancer gene therapy.

Gene therapy, in general, is regarded as the transfer of therapeutic genetic material into cells for the purpose of treating or eliminating the causes of a particular disease [2].

The definition of gene therapy needs to be general because there are not only many different target-cells and many ways to transfer the therapeutic genetic material into cells, but also many molecular targets inside the cell that can have therapeutic effect.

The identification of molecular pathology for many diseases and the recent advance in biotechnology have decisively boosted gene therapy and applied it in clinical practice.

The first clinical gene therapy trial took place in 1990 and aimed at the treatment of a monogenic disease, the so-called deficiency of adenosine deaminase (ADA) [3,4]. Since then, a lot of progress has been made in the gene therapy field and its applications have been expanded.

2. Gene Vectors

In order to achieve gene therapy in cells, it is necessary to develop vectors able to carry the therapeutic material inside the cells. Vectors can be divided into two categories: viral vectors and non-viral vectors [5].

(*A) Viral vectors*

The most frequent viral vectors are adenoviruses and retroviruses [6]. In the laboratory, adenoviruses and retroviruses are modified and the therapeutic genes are incorporated in their genetic material. Moreover, some of their genes may be deleted to obtain the desired properties and to save space for the insertion of the foreign genes [2].

(A_1) Adenoviruses

Adenoviruses replicate in a wide variety of cell types including both dividing and non-dividing cells. They do not integrate into the host genome. As a result, they do not mutate the host genome [2,7].

Under natural conditions, adenoviruses usually cause minor human diseases such as upper respiratory tract infections, keratoconjunctivitis and gastroenteritis [2].

During their circulation in the blood stream, they can cause strong inflammatory and immunological responses. Consequently, they are relatively safe gene vectors as long as intravascular circulation is avoided [2].

Another viewpoint is that if adenoviruses remain limited within the tumor area, localised induction of immunological responses may enhance tumor killing [2].

(A_2) Retroviruses

On the contrary, retroviruses transduce only dividing cells and they integrate their genome into host DNA. Thus, they alter the host genetic material. Neoplastic cells are dividing and accordingly retroviruses can be used for cancer gene therapy [2].

(B) Non-viral vectors

Non-viral gene delivery systems include naked plasmid DNA, DNA complexed with cationic lipids and according to the latest studies DNA complexed with (poly)cationic lipids covered with hydrophilic polymers and equipped with external molecules that target cell receptors [8].

The molecule of DNA is polyanionic. It can be damaged when it circulates through the blood vessels and it can also be disintegrated within few seconds by nucleases [9].

To prevent its damage, the polyanionic DNA molecule can be condensed with (poly)cationic carrier molecules [10]. Thus, DNA reduces its size and is protected from nucleases. On the other hand, polycationic carrier molecules are generated with an excess of positive charge to achieve sufficient DNA compaction. The resulting positively-charged surface of the DNA-polyplexes leads to undesirable effects:

(i) When the positively charged polyplexes are inserted into the blood stream, they bind to negatively charged erythrocyte membranes and they usually end up in the lung [11].

(ii) In addition, polyplexes are opsonised with complement, albumin, immunoglobulins and proteins of coagulation. As a result, they become phagocytable [8].

To avoid non-specific interactions with blood components, it is necessary to cover the outer layer of DNA-(poly)cationic polyplexes with hydrophilic polymers, such as (poly)ethylene glycol (PEG), (poly)hydroxypropylmethacrylamide (pHPMA) or (poly)vinylpyrrolidine. The resulting gene delivery system is able to circulate in the blood stream after systemic application without non-specific interactions with blood components [11,12].

A chemical substance, which is called polyethylenimine (PEI), has been described as a carrier that not only condenses DNA, but also enables its efficient delivery into cells [13].

To achieve gene integration in the desired cell type, it is essential to set it as target. An attractive way to aim the desired cells is by using their surface receptors. For example, DNA polyplexes can be equipped with the iron-transporting plasma protein transferrin, which acts as targeting agent for a wide variety of cell types and lines [14]. Gene delivery is increased by this targeting agent in many rapidly dividing tumors, which overexpress transferrin’s receptors [15,16].

As soon as the synthetic gene-transfer particles reach the targeted cell, they are internalized by receptor mediated or absorptive endocytosis. By this procedure the gene vector is "trapped" in an endosome or lysosome. The intracellular proton pump acidifies endosomal or lysosomal compartments and lysosomal nucleases can degrade the internalized DNA up to 99 % [8].

This obstacle has already been overcome by the nature: Viral proteins and venoms of vertebrates and invertebrates contain membrane-active domains, which can mediate the delivery of the internalized DNA to the cytoplasm, since they are activated in the endosome or lysosome [17,18].

Another way to overcome this obstacle is to use PEI carrier molecules, since they offer an intrinsic mechanism that enables the release of endocytosed DNA into the cytoplasm under certain conditions. The buffering capacity of the carrier molecules can hamper the

acidification of the endosomes and it makes them burst [8,19].

(C) Advantages and disadvantages of viral and non-viral vectors

The advantages of non-viral vectors over their viral counterparts are:
(i) they have no limitation as to the size of the transferred DNA,
(ii) they are less immunogenic, and
(iii) they are easier to be produced.

However, a major drawback of non-viral vectors is that they don't transfect cells in vivo as efficiently as viral vectors [2,7,8].

3. Strategies of Gene Therapy against Malignant Cells

The various strategies of gene therapy can be divided in six categories:

(A) Firstly, there are strategies that require the expression of a therapeutic gene: Immunogene, chemogene and tumor suppressor gene therapy are included.

(A_1) Immunogene therapy

This method aims at making malignant cells produce cytocine molecules. Gene vectors can mediate the transfer of cytokine genes to malignant cells. Cytokines such as interleukin-2 (IL-2), gamma interferon (IFN-g) and alpha tumor necrosis factor (TNf-α) are important mediators of immune responses against cancer [20,21,22]. However, the systemic use of these cytokines is limited in cancer patients because of their low concentrations in tumor cells and severe side effects. These obstacles can be circumvented, if tumor cells secrete cytokines [2].

An interesting experiment was conducted by Wright et al. (1998). MCA-26 tumor cells were established in BALB/c mice. Ten days or two weeks after tumor inoculation, two intratumoral injections of recombinant adenovirus carrying TNF-α gene resulted in significant reduction of tumor weight [2].

Can really immunogene therapy give the solution to cancer treatment? It is not always effective since tumor cells resist to Cytotoxic T - Lymfocytes (CTL) killing. Progressive tumors are generally nonimmunogenic, at least in part, because they have lost the ability to

express Human Leukocyte Antigen (HLA) molecules or to provide costimulatory molecules on their surface [23,24].

Another reason why tumor cells prevent the immune system from being stimulated is that they switch their proteasome type when they are treated with gamma interferon (IFN-g). The proper process of the antigenic peptide occurs when the tumor cell harbors the standard proteasome. However, when the cells are treated with IFN-g, they produce immunoproteasomes and from that point the cells are not properly processing the antigenic peptides. Proteasome switching may occur in an environment rich in IFN-g, such as a lymph node [25,26].

(A_2) Chemogene Therapy

The concept of chemogene therapy is to transduce tumor cells with genes, which do not exist in normal cells and which convert a non-toxic substance into a toxic one. The non-toxic substance (prodrug) is not produced by the human organism. applied systemically. Therefore, chemogene therapy is also known as "enzyme/prodrug approach".The transferred genes are called suicide genes. Chemogene therapy has the advantage of avoiding side effects from systemic administration of toxic drugs. In addition, the produced toxic substances can kill neighbouring tumor cells, which may not express the incorporated genes, through a bystander effect.

A variety of genes encoding different types of enzymes have been investigated for their potential use in cancer gene therapy [27]. One of the first suicide genes was the varicella-zoster virus thimidine kinase (VZV-TK) combined with the prodrug 6-methoxypurine arabinonucleoside (ara-M), which is monophosphorylated in the presence of VZV-TK [28].

Nowadays, promising enzyme/prodrug systems are:
(i) The Escherichia Coli enzyme nitroreductase in combination with the prodrug CB19545 [5-(aziridin-1-yl)-2,4-dinitrobenzamide].
(ii) The Escherichia Coli gtp gene codes for the enzyme xanthine/guanine phosphoribosyltransferase (XGPRT), which is combined with the prodrug 6-thioxanthine.
(iii) The Escherichia Coli enzyme purine nucleoside phosphorylase (PNP), combined with the prodrug 6-methyl purine deoxyriboside [28].

One of the most common chemogenes used for the treatment of cancer is the herpes-virus thimidine kinase (HSV-TK) with the prodrug gancyclovir (GCV) [28,29]. The prodrug gancyclovir (GCV) is not toxic

as long as it is not metabolised. Yet, as a result of HSV-TK chemogene's action, it is converted into triphosphate-GCV, which is incorporated into DNA and RNA and causes the termination of their synthesis. Subsequently, tumor cells expressing HSV-TK are killed by infusion of GCV.

Chen et al. (1994) applied chemogene therapy in nude mice. Experimental gliomas were implanted to nude mice. Eight days after the implantation, recombinant adenoviruses expressing TK were inoculated into the tumors. Afterwards, the mice were given GCV for 6 days. 20 days after tumor implantation, the tumor volumes were measured and the tumor volume in the TK/GCV–treated mice was 500 fold smaller than in control mice [30].

The bacterial and fungal gene encoding cytosine deaminase (CD) is widely used for chemogene therapy. The CD enzyme deaminates infused 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU). 5-FU is toxic, because it inhibits RNA and DNA synthesis and leads to cell death [2,28].

(A_3) Tumor suppressor gene therapy

Genes which are characterized as tumor suppressors regulate the cell cycle. Inactivation of these genes by mutation aids the emergence of neoplastic cell populations. These are inactivated by point mutations and by deletions. Normal copies are usually found to be inactivated in tumors [2,31,32]. Tumor suppressor gene therapy tries to restore the inactivated tumor suppressor genes.

(A_3)(i) The p53 tumor suppressor gene

The protein which is encoded by p53 gene plays a pivotal role in the detection of DNA damage. It is responsible for the reaction of damaged cells; that is to say whether they undergo growth arrest allowing time for repair or they undergo apoptosis because the damage is too extensive [2,33].

Mutations in p53 are the most common genetic alterations in cancer cells [34]. p53 can trigger apoptosis in DNA-damaged cells and is effective despite the presence of multiple genetic mutations in tumor cells [35].

Studies on a mouse model showed that the transfer of p53 gene from an adenoviral gene vector to human prostate cancer cells had as a result

marked tumor growth inhibition and apoptosis [36]. In vivo studies on human prostate cancer have shown that the impact of a single injection of recombinant adenovirus carrying p53 gene was primary tumor growth suppression and reduction of metastatic disease [2].

(A_3)(ii) The p16 tumor suppressor gene

The $p16^{NK4A}$ gene is also a negative cell-cycle regulator by controlling the activity of CDK4-cyclin D, a cyclin dependent kinase [37]. It is frequently deleted, mutated, or silenced by promoter methylation in many human cancers [38,39,40]. Hence, $p16^{NK4A}$ is an appealing gene for tumor suppressor gene therapy.

Xiang et al. (2001) inocubated four different breast cancer cell lines in vitro, which lacked the $p16^{NK4A}$ gene. They observed significant reduction of cells' number in three of the cell lines after their infection with recombinant adenovirus carrying $p16^{NK4A}$ gene [2].

(A_3)(iii) The FHIT tumor suppressor gene

Another promising gene for tumor suppressor gene therapy is the fragile histidine triad (FHIT) gene. It is situated in the short arm of chromosome 3. It is considered to be a tumor suppressor gene, because it is frequently inactivated in most human tumors and in several precancerous lesions. Additionally, it encompasses a translocation breakpoint associated with hereditary renal carcinoma [41,42,43,44,45,46,47].

(B) The second category is the antisense gene therapy.

Antisense oligonucleotides are short synthetic stretches of DNA which hybridise with specific mRNA strands that correspond to target genes. By binding to the mRNA, the antisense oligonucleotides prevent the sequence of the target gene being converted into a protein; thereby the action of the gene is being blocked. Several genes known to be important in the regulation of apoptosis, cell growth, metastasis and angiogenesis, have been validated as molecular targets for antisense therapy [48].

The inhibition of oncogene expression in malignant cells can be achieved by antisense therapy. Oncogenes are genes in which biologic activity was increased by mutation. They are classic members of growth factor signaling pathways [49,50]. Normal celular genes are turned into oncogenes by point mutation, by gene amplification, which produces

multiple copies of the gene, or by fusion to other genes and their regulatory elements [48].

Thus, some possible gene targets for antisense therapy are: the BCL-2 family (which inhibits apoptosis), protein kinase A and C (which regulate cell growth), clusterin (which possibly inhibits apoptosis), DNA methyltransferase (which can possibly downregulate tumor suppressor genes), and MDM2 gene (which is a negative feedback regulator for p53) [48].

Any sequence larger than a minimum number of bases – 13 in RNA and 17 in DNA – is unique in the human genome. Antisense oligonucleotides are manufactured large enough to aim only at a specific genomic sequence and not unrelated ones [48].

In initial experiments, antisense oligonucleotides were susceptible to degradation by cellular nucleases, whereas today several sugar, base and backbone modifications have been introduced to stabilize the molecule, allowing its clinical use [48].

(C) The third category consists of transcription factors called "zinc fingers", which can either activate or repress the targeted genes.

Zinc fingers are transcription factors. They can be transferred inside the cells by vectors such as adenoviruses. Polydactyl zinc-fingers proteins can be assembled through the combination of zinc-finger domains of predefined specificity. The combination of such domains leads to the assembly of a protein that can bind to an 18 base pair DNA sequence and either activate or repress the targeted gene. It is certain that zinc-finger protein will bind to the targeted DNA sequence, because DNA sequences which are bigger than 17 base-pairs are unique in human genome [51,52,53].

Selective gene suppression has already been achieved for the oncogenes ERBB-2 and ERBB-3 in cell culture [54].

Many new pathways are created by this strategy: oncogenes and angiogenetic genes could be suppressed and tumor suppressor genes could be overactivated in malignant cells.

(D) The inhibition of angiogenesis in tumor cells is listed in the fourth category.

Tumor growth depends on the development of new blood vessels [55]. Once the diameter of the tumor exceeds 2mm, angiogenesis is required for further growth [56]. Suppression of angiogenic inhibitors together with stimulation of angiogenic growth factors is essential for tumor growth and progression [57].

As a result, the attempt of the gene therapy targeting tumor vessels is exactly the opposite: to reduce angiogenic growth factors and to increase angiogenic inhibitors inside tumor cells [58].

Several proteins with antiangiogenic action seem to be effective in reducing tumor growth. Platelet factor 4 (PF-4) was the first to prove its efficiency in a model study [59]. Cytokine IL-12 and chemokine IP-10 reduce tumor vasculature [60,61]. The p53 protein, thrombospondin, defective VEGF (Vascular Endothelial Growth Factor) receptors, angiostatin and endostatin seem to be angiogenic inhibitors, as well. It's worth noticing that angiostatin and endostatin have completely eliminated tumors in mice [62,63]. All the above genes can be inserted in tumor cells by means of gene vectors [57].

In addition, repression of angiogenic genes could be achieved by antisense therapy and zinc-finger proteins.

(E) The fifth category refers to endonucleases.

Endonucleases can potentially create a new gene therapy strategy. The transfer of endonuclease genes via gene vectors inside malignant cells might induce their apoptosis.

Saito et al. (2003) experimented on DNase-gamma protein (DNA endonuclease) and found out that the transfer of DNase gamma gene via multimellar cationic liposomes (gene vector) induces apoptosis in human glioma cells in vitro. DNase-gamma gene transfer resulted in an overexpression of DNase-gamma protein and induced DNA fragmentation in gene-transferred cells [64].

Additionally, Spalletti-Cernia et al. (2003) showed that bovine seminal ribonuclease (BS-RNase), [a natural dimeric homolog of bovine pancreatic RNase (RNase-A)], and HHP2-RNase, [an engineered dimeric form of human pancreatic RNase (HP-RNase)], induced apoptosis of

human thyroid carcinoma cell lines, which were implanted in nude mice. RNase-induced apoptosis is associated with the activation of initiation caspase-8 and -9 [65].

(F) The sixth category includes the rest of the strategies: use of oncolytic viruses, targeting of normal cells to increase their resistance to chemotherapeutic agents.

(F_1) Oncolytic viruses

Adenoviruses need the viral early regulatory protein E1B (55 kDa) for efficient transcription of the other viral genes. E1B also binds to and inactivates the tumor suppressor p53 protein. This promotes the viral DNA replication [2].

Adenoviruses missing the E1B gene are not able to produce the E1B protein and can not efficiently replicate in normal cells, whereas they can efficiently replicate in cells lacking functional p53 protein and lyse these cells [2,66,67].

As mentioned earlier, many malignant cell types lack the functional p53 protein. As a result, "handicapped" adenoviruses missing the E1B gene can selectively replicate and kill tumor cells but not normal cells [2].

ONYX-015 is an adenovirus misssing the E1B gene and it was extensively used in the treatment of p53-negative human head and neck carcinomas in which 45 to 70 % have a p53 mutation [68]. The initial study showed that ONYX-015 led to anti-tumoral activity in these patients. However, the tumors recurred rapidly [69].

(F_2) Gene Therapy with chemoprotection genes

The transfer of drug resistance genes into hematopoietic stem cells increases their resistance to myelosuppressive chemotherapeutic agents. Autologous transplantation with CD34(+) stem cells has been proposed for the treatment of patients suffering from breast cancer or ovarian cancer. These cells are isolated from bone marrow and peripheral blood cells, and transduced with multiple drug resistance (MDR-1). Afterwards, they are reinfused back into the patients: subsequently, high doses of chemotherapeutic agents are possible [70,71].

4. Targeted Cancer Gene Therapy

The specific targeting of malignant cells and not any normal cells is a challenge for gene therapy. Nowadays, there are three ways of targeting cells: transductional targeting, transcriptional targeting and transgene targeting.

(A) Transductional targeting

As far as transductional targeting is concerned, the targeting of cells is achieved through their cell-surface proteins. Many cell-specific cell-surface proteins have been defined by monoclonal antibodies. Gene vectors can be equipped with surface monoclonal antibodies to bind to the cells expressing the targeted surface antigens [72].

Haisma et al., (1999), used a neutralizing anti-fiber antibody, which was conjugated to an antibody against the Epithelial Cell Adhesion Molecule (EGP-2), in order to bind the adenovirus vector to the EGP-2 antigen present on tumor cells. Gene transfer was dramatically reduced in EGP-2 negative cell lines [73,74].

(B) Transcriptional targeting

Target-cell specific gene expression may be accomplished through restriction of gene expression by tumor or tissue specific promoters. Several tumor or tissue specific promoters have been reported and tested for specificity in gene therapy approaches [75].

(C) Transgene targeting

Transgene targeting is a method to enhance the bystander effect of chemogene therapy.

In theory, complete tumor regression would occur, only if all tumor cells were transfected with the transgene enzyme. In practice, gene vectors infect only a small portion of tumor cells. However, complete tumor regression in animal models occurs when 10 % of tumor cells express the transgene enzyme. This phenomenon is called the bystander effect and is considered to be partly due to transport of toxic activated prodrug from the transfected cells into neighbouring non-transfected cells through the cellular junctions [72,76].

A fusion protein of thimidine kinase (HSV-TK) and the HIV-1 TAT protein transduction domain (Tat PTD) have been used for transgene targeting. Tat-PTD has the ability to migrate rapidly and indiscriminately across cell membranes and it has been shown to mediate the uptake of various proteins [72,77].

5. Conclusions and Future Developments

Great progress has been made in cancer gene therapy. Clever strategies have been developed for "fighting" malignant cells. The research tends to invent evoluted vectors, which would be able to transduce only target cells and would carry effective genes for cancer treatment. The fact that, at the moment, no gene therapy can cure cancer strengthens the need to improve the existing gene therapy strategies and to discover new strategies and more inspired molecular targets.

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