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Abstract

Several decades ago, the first retroviral vectors were constructed. They have been proved as delivery vehicles in basic and translational research; many of them were used in clinical trials in the treatment of genetic and immunologic disorders or malignancies to deliver therapeutic genes into target tissue. Gammaretroviral and lentiviral vectors are popular viral delivery vehicles; their ability to integrate into genome of the host cell enables permanent genetic modification of the target cell and long-term expression of the transgene. Besides classical cancer gene therapy, they are used in cell-mediated cancer gene therapy in combination with mesenchymal stromal cells (MSC) or neural progenitors. Based on the promising preclinical studies, clinical trials with genetically engineered cell vehicles were initiated.

Keywords: Retroviral vector, lentiviral vector, gene therapy

1. Introduction

Besides negative and pathogenic attributes, viruses can also be beneficial when used as delivery vehicles in gene therapy. The advocates of viral vectors even claim that just viruses are the right tools for delivery of foreign genetic information into the cell because they have been evolving for this purpose for millions of years. Gene therapy can be defined as the delivery of nucleic acid into the cell for the purpose of acquiring new features or restoration of physiologic status. The idea that disorders can be treated by genes arose in the 1960s, when the mechanism of cell transformation by SV40 virus and papovaviruses was described [1]. Gene therapy enables modification of cell by the replacement of non-functional or missing gene, suppression of another gene, or induction of cell death as in the case of oncologic diseases. Monogenic diseases and age-related disorders can be treated by retrovirus-mediated gene therapy, but (retro)viral vectors are most frequently used in cancer gene therapy.
1.1. Basic terminology

- Viral vector – a synthetic construct containing given viral sequences determined to transfer genetic information into the target cell.
- Transfection – transfer of genetic information by a non-viral system.
- Transduction – transfer of genetic information by a viral vector (genetic information is packaged in the viral particle).
  - Transient – DNA is not integrated into the genome of host cell, genetic modification is temporary.
  - Stable – transgene is an integral part of the genome of the host cell, and it is transferred into daughter cells by cell division.
- Provirus – viral genome integrated into chromosome of the host cell.
- Episome – foreign genetic information in the cytoplasm or nucleus, which replicates independently from the genome of the host cell.
- Replication-competent vector – the genetic information of the virus is complete, and the realisation of the whole life cycle of the virus is facilitated in the target cells. Viral progeny infects surrounding cells.
- Replication-defective vector – some viral sequences are removed from the genome; helper cell lines are necessary for the production of virion. Replication-defective vectors are not able to replicate in target transduced cells.

2. History of gene therapy and retroviral vectors

The transfer of genetic information among bacteria by a bacteriophage was first described by Joshua Lederberg and Norton Zinder. They named this phenomenon as ‘transduction’ [2].

The work of Howard M. Temin performed on Rous sarcoma virus (RSV) is the fundamental part in the research of retroviruses and retroviral vectors. He discovered that specific genetic mutations could be inherited as a result of viral infection. Moreover, his study showed that RSV infection required cellular DNA for replication, and genetic information can also flow in the direction RNA → DNA, and he postulated the provirus hypothesis [3-6].

In the 1970s, specific viral genes involved in the transformation were discovered. The SRC and other (proto-)oncogenes with cellular origin were described. The insertional mutagenesis was revealed as another mechanism of transformation [7].

While pioneer work was performed on avian-infectious alpharetroviral Rous sarcoma virus, Moloney murine leukaemia virus (MoMLV) belonging to gammaretroviruses was initially used for the preparation of therapeutic vector [8, 9], and until now, MoMLV-derived constructs along with human immunodeficiency virus (HIV)-derived vectors are most
frequently used. The construction of mutant Moloney murine leukemia virus defective in the packaging of genomic RNA into virions represents an important step towards the development of retroviral vectors [7]. The first gene delivery systems based on HIV-1 were prepared in the early 1990s [10].

The first officially approved clinical study was conducted by Rosenberg. In this initial study, the gene for neomycin phosphotransferase was introduced into the tumour-infiltrating lymphocytes (TIL) of patients with advanced cancer. Subsequently, he performed clinical trials in which the gene for tumour necrosis factor (TNF) was inserted by retroviral vector into TIL in an effort to increase their therapeutic effectiveness [11]. During the following years, the gene therapy became a very promising approach for the treatment of genetic and oncologic diseases. But serious complication halted the progress of this therapeutic approach. In 1999, Jesse Gelsinger, suffering from a partial deficiency of ornithine transcarbamylase, took part in a gene therapy clinical trial at the University of Pennsylvania. He died due to excessive immune response to a high dose of adenoviral vector [12]. There was a study conducted in Paris, in which 20 children suffering from severe combined immunodeficiency (SCID) took part. They were treated by ex vivo–transduced autologous CD34+ haematopoietic progenitor cells. Five of these patients developed T-cell leukaemia [13], and the disease was fatal for one out of these patients [14].

Since then, many steps were taken to improve the safety and efficacy of the gene therapy, and until now, many clinical studies have been conducted.

3. Comparison between retroviral and other viral and non-viral systems

There are two systems for the delivery of transgene into the cell – viral and non-viral. The non-viral approaches are represented by polymer nanoparticles, lipids, calcium phosphate, electroporation/nucleofection or biolistic delivery of DNA-coated microparticles. The safety is mentioned as the major advantage of non-viral approaches. In general, non-viral delivery of transgene is less effective in comparison to viral systems.

There are several categories of viral vectors. We distinguish two main types of vectors depending on whether the DNA is integrated into chromatin of the host cell or not. Retroviral vectors derived from gammaretroviruses or lentiviruses persist in the nucleus as integrated provirus and reproduce with cell division. Other types of vectors (e.g. those derived from herpesviruses or adenoviruses) remain in cell in the episomal form.

The overview of viral vectors is depicted in Figure 1. Adenoviral and retro/lentiviral vectors are most frequently used in research and gene therapy clinical trials.

As stated above, adenoviral vectors are very popular. They have been used for several decades. Since adenoviruses are non-enveloped dsDNA viruses, they are relatively resistant to chemical and physical agents, which enable them to persist out of host cells and make the work in laboratory easier in comparison to enveloped RNA viruses. They are often used in cancer gene therapy as replication-defective or replication-competent vectors. They infect proliferating as
well as non-dividing cells. In general, adenoviral vectors are considered safe. Since they do not integrate into host DNA, the transduction is transient. The drawback is their immunogenicity [15-17].

Adeno-associated vectors share with retroviral vectors the ability to integrate into host DNA. Wild type adeno-associated virus integrates into specific site of the chromosome 19 (19q13.3-qter). Recombinant vectors lack this characteristic and the risk of insertional mutagenesis exists. These vectors transduce dividing and non-dividing cells, and the transgene expression is long term. Transduced cells are minimally immunogenic [18].

Herpetic viruses are relatively complex enveloped dsDNA viruses. The vectors have been prepared from Herpes simplex type 1 virus, Epstein–Barr virus or cytomegalovirus. They are less immunogenic in comparison to adenoviruses. The transduction is transient; the drawback of HSV 1-derived vector is the short-term expression of the transgene. Herpes virus-derived vectors are preferentially used in vaccination [19, 20].

Poxviruses are the most complex viruses. Their major advantage lies in the huge cloning capacity. Up to 25 kbp of foreign DNA can be cloned into vaccinia-derived vectors. Similarly to herpes virus-derived vectors, they are popular in the preparation of vaccines including cancer immunotherapy [21, 22].

Baculoviruses, the viruses specific for invertebrates, are not competitors of retroviruses in gene therapy. They have been used for more than 30 years for transduction of insect cells for expression of recombinant proteins. Pseudotyping enables the transduction of mammalian cells [23].

Vectors derived from alfaviruses (ssRNA viruses) are also used in cancer gene therapy and immunotherapy [24, 25].
4. Retroviral vectors

Retroviruses are relatively complex enveloped RNA viruses with diploid ssRNA genome. Typical feature of retroviruses and retroviral vectors is their ability to integrate into host DNA. Viral RNA is reversibly transcribed and integrated in the form of provirus. They very effectively cooperate with enzymes of the host cell, and they use it for their own replication and long-term expression of viral proteins. The entry of virus into the host cell is receptor-dependent [26].

Many types of retroviruses (bovine leukaemia virus, Rous sarcoma virus, lentiviruses and spumaviruses) were used for preparation of vectors. The most popular vectors are constructs based on MoMLV and HIV.

4.1. Gammaretroviral vectors

The first MoMLV-based vectors were prepared more than 30 years ago [8, 9], and they are still very popular. The construct is relatively small, and it is possible to achieve high titres in inoculum. The diagram of MoMLV provirus and MoMLV-derived vector is depicted in Figure 2.

Contrary to lentiviruses, the packaging system of gammaretroviruses does not require incorporation of sequences overlapping coding region gag, pol or additional genes.

Since MoMLV lacks elements necessary for active transport of genetic information through nuclear membrane, integration of viral DNA is possible only during the mitosis. MoMLV-derived vectors transduce only dividing cells. Integration of viral genome is mediated by the pre-integration complex (PIC) consisting of integrase, capsid, p12, proviral DNA and host cell proteins. Proviral DNA is surrounded by two long terminal repeats (LTR), which are com-
posed of U3, R and U5 regions. Transcription of proviral DNA starts from enhancer/promotor in 5’ U3 region [27].

MoMLV constructs can be prepared as replication-competent or replication-deficient. Gag and env genes are removed in replication-deficient vectors. Gene of interest is cloned in the free space. A typical replication-deficient vector contains packaging signal (ψ), primer binding site (PBS) and LTRs. Viral genes gag, pol and env are cloned on separate expression helper plasmids, and helper cell lines are co-transfected with more plasmids. Stably transfected helper cell lines expressing gag pol (GP) and env were prepared.

Packaging cell lines GP+E-86 and GP-envAM-12 were derived from NIH-3T3 cell line by electroporation of two plasmids. One plasmid contained the gag and pol regions of MoMLV, and the other contained the env region. GP+E-86 cells are used for the production of ecotropic viral particles, and line GP+envAM-12 is used for amphotropic viral particles (explained later) [29]. Expression cassette with gene of interest should not contain introns, internal polyadenylation signals and large secondary structures, which could interfere with reverse transcriptase. The cloning capacity of retroviral vectors is up to 10 kbps, but the size of transgene significantly influences its expression and viral titre [27].

Envelope protein coded by gene env is responsible for the tropism of MoMLV. Three natural variants of MoMLV were described. They differ in their envelope proteins. Ecotropic MoMLV infects only mouse and rats cells; amphotropic is also able to infect the cells derived from other mammals including human cells. Xenotropic MoMLV was supposed to infect many types of cells excluding murine, but recent studies indicate that it also infects mice and it has the widest tropism among MoMLV [27, 30].

Tropism of the viral vector can be modulated by pseudotyping using a particular envelope protein. The glycoprotein of vesicular stomatitis virus (VSVg) is very popular. It enables broad host range (mammalian and non-mammalian cells). It also has stabilisation properties, and viral particles can be purified by ultracentrifugation. On the other hand, VSVg is recognised by complement, and this fact can decrease the transduction efficacy in vivo [31].

4.2. Lentiviral vectors

In comparison to MoMLV-derived vectors, lentiviral vectors are more complex. Three generations of lentiviral vectors were prepared in order to increase the safety and efficacy of the gene transfer. The viral genome was split into packaging and transfer vectors. The first and second generation are composed of three plasmids; the third generation consists of four plasmids [31]. The main difference between lentiviral vectors and vectors derived from other retroviruses is their ability to infect/transduce quiescent non-dividing cells [32]. They are able to pass through nucleopores into the intact nucleus. The mechanism of this phenomenon has not been completely clarified; yet, it is known that both viral and cellular proteins participate in this process. In addition to HIV vectors, vectors based on feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV) or equine infectious anaemia virus (EIAV) have also been prepared. Pseudotyping (VSVg is the most common) is typical for lentiviral vectors [31].
4.3. Self-inactivating vectors

The risk of insertional mutagenesis is a drawback of retroviral vectors. With the purpose of increasing the safety of gene therapy, self-inactivating (SIN) vectors were prepared in the 1980s. SIN vectors have a deletion in the 3' U3 region, where promoter and enhancer sequences occur. This deletion is copied into 5' LTR during the reverse transcription, and virus becomes free of LTR-bound promoter activity. Transcription control is under the chosen cloned promotor [33].

SIN vectors are characterised by decreased risk of insertional mutagenesis; the vector is not activated via infection by another retrovirus, and the internal promotor is autonomous. Controlled/inducible or cell-specific expression of transgene can be achieved based on the chosen promotor [33]. The use of tetracycline (Tet)-inducible system was published in the 1990s [34], and until now it is being used for enhanced expression of an exogenous gene in a cell-type-specific manner.

4.4. Replication-competent retroviral vectors

Retroviral vectors can be constructed as replication-defective to transduce target cells and enable long-term expression of transgene (immunology disorders, genetic diseases), or they carry transgenes inducing cell death (cancer gene therapy). On the other hand, the replication-competent vectors (RCV) are prepared in order to replicate in the target (tumour) cells. Their progeny infects surrounding malignant cells. Since the targeting is an inevitable characteristic of RCV, they can be engineered to express ligands to tumour cell-specific markers. The advantage of MoMLV-derived vectors is their natural preference to tumour cells [35]. MoMLV is unable to infect quiescent cells, making them suitable vehicles for the treatment of brain tumours. RCV with suicide gene mediate synchronised cell killing after prodrug administration, and due to their stable integration into DNA of infected cells, residual cancer cells serve as a reservoir for long-term viral persistence even when they migrate to new sites. Multiple cycles of prodrug administration to achieve improved treatment efficacy are possible [36].

Although gammaretroviral and lentiviral vectors are derived from the same viral family, they differ in some characteristics. Advantages of gammaretroviral vectors reside in the complete absence of viral gene remnants in the transfer vector, efficient pseudotyping and the lack of mobilisation by human-infectious viruses. In comparison to HIV-derived vectors, there are also minor concerns related to potential seroconversion in vivo. Lentiviral vectors are clearly superior for the ability to transduce non-dividing cells. Both types of vectors are equally potent in terms of expression properties when containing similar internal expression cassettes [37]. Compared to vectors derived from non-integrating viruses, retroviral vectors possess the risk of insertional mutagenesis. Gammaretroviral vectors preferentially integrate close to transcription start sites and CpG islands, which are enriched in gene-regulatory elements. Lentiviral vectors prefer integration inside of the transcription units of actively transcribed genes [38].

Despite the promise for success in the clinic, one major drawback of the retrovirus-based vector is that any unintended insertion events from the therapy can potentially lead to deleterious
effects or it can cause an abnormal expression of nearby host genes driven by the enhancer of the inserted viral DNA in patients, as demonstrated by the development of malignancies in both animal and human studies. The better prediction of the integration sites by elucidation of this mechanism might lead to the development of retroviral vectors capable of selective integration. This understanding could provide the ultimate solution to the problems of insertional mutagenesis [39]. The definition of the precise mechanism of the retroviral pre-integration complex is required. Many efforts have been made in designing modified integrases with sequence-specific integration capability, which can be accomplished by rational modification of the protein or by using the directed evolution approach [40]. One approach to directing integration into predetermined DNA sites is fusing integrase to a sequence-specific DNA-binding protein, which results in a bias of integration near the recognition site of the fusion partner [41]. Efforts to engineer integrase to recognise specific target DNA sequences within the host genome may lead to development of effective retroviral vectors that can safely deliver gene-based therapeutics in a clinical setting. Insertion of a lentiviral vector via virion-associated Cre protein, capable of directing site-specific insertion of a gene in the vector, into a defined loxP site in the host genome was described [42].

A detailed study of the vector integration sites performed on haematopoietic stem cells by Aiuti et al. [43] concluded that lentiviral gene therapy was safer than retroviral gene therapy, and lentiviral gene therapy did not induce selection of integrations near oncogenes, and no aberrant clonal expansion was observed after 20 to 32 months follow-up. Also, the so-called integration-deficient lentiviral vectors (IDLVs) can be produced through the use of integrase mutations that specifically prevent proviral integration. These lentiviral episomes lack replication signals and are gradually lost by dilution in dividing cells, but are stable in quiescent cells. Compared to integrating lentivectors, IDLVs have a greatly reduced risk of causing insertional mutagenesis [44].

4.5. Overview of preparation of the gammaretroviral vector

Recently, many viral cloning systems are available. Transgene is cloned either directly or via a bacterial intermediate. Circular dsDNA is found at the beginning of the process. It contains viral sequences including LTR necessary for the integration, genes coding for antibiotic resistance and target sequences for different restriction endonucleases (multicloning sites). The first part of vector construction takes place in bacteria; therefore, the bacterial origin of replication (ORI) is a necessary component. The gene of interest is cloned into the vector, then propagated in bacteria and verified by sequencing. Subsequently, the packaging cell line is transected, and the sequences between LTRs are integrated into host-cell DNA. Vector-containing cells are selected via antibiotic resistance. In order to increase the titre of the viral vector, ecotropic and amphotropic packaging cell lines can be used. First, the ecotropic cell line is transfected, and then the amphotropic packaging cells are transduced by virus-containing cultivation supernatant from ecotropic cells. The ‘ping-pong’ method – mutual exchange of virus-containing medium between ecotropic and amphotropic cells – is performed to further increase the viral titre. The viral titre is determined and cultivation supernatant from transduced cells is collected for transduction of target cells. Transgene-containing cells are selected via antibiotic resistance (Figure 3).
5. Gene therapy

Treatment of genetic diseases and cancer gene therapy are the main targets of recent gene therapy. They belong to serious diseases, which are difficult to treat or are incurable using conventional treatment, or the treatment is accompanied by severe adverse effects.

Over 60% of ongoing gene therapy clinical trials represent cancer treatment followed by monogenetic and cardiovascular diseases [11]. Two approaches of gene therapy are defined: (i) The \textit{ex vivo} method is characterised by the collection of target cells from the organism, genetic modification and subsequent administration to the patient. (ii) The \textit{in vivo} method is characterised by the direct administration of therapeutic gene to the patient.

5.1. Treatment of genetic, immunologic and other non-oncologic diseases

Genetic as well as age-related diseases can be treated by gene therapy. They are caused by deficiency or aberrant expression of one or more gene(s). Patients suffering from severe combined immunodeficiency (SCID) – devastating disorder of adapted immunity – are not able to defend against infections. The term SCID covers several genetic defects. Adenosine deaminase (ADA) deficiency was the first SCID condition for which a genetic and molecular cause was identified [45]. The patients suffering from ADA-SCID are ideal candidates for gene therapy, when haematopoietic cells are transduced by a gene encoding adenosine deaminase. A retroviral vector carrying ADA was the first construct to contain a therapeutic gene (Rosen-
berg transduced cells with neomycin resistance gene to track them in vivo) used in an FDA-approved clinical trial. Two children were treated [46]. SCID-X1 is characterised by various mutations in the gene encoding interleukin 2 receptor-γ (IL2RG) [47, 48]. Transduction of functional IL2RG restores expression of functional interleukin 2 receptor-γ. It was demonstrated that gene therapy for primary immunodeficiencies is an effective treatment modality providing long-term clinical benefit for patients. Lentiviral vectors contributed significantly to this achievement [49]. Haematopoietic stem cells were engineered ex vivo and administered to the patient. Long-term T-cell reconstitution was achieved in patients suffering from ADA-SCID and SCID-X [50].

Age-related macular degeneration is accompanied with excessive vascularisation in which vascular endothelial growth factor (VEGF) takes place. Its function can be inhibited by retrovirally delivered antiangiogenic factors such as angiostatin, endostatin or extracellular domain of VEGF receptor. Vectors are administered into vitreous body or under retina. Antiangiogenic genes are also used in cancer gene therapy [51-53].

Epidermolysis bullosa is a group of devastating skin disorders. Mutations in the COL7A1 gene result in the absence or dysfunction of type VII collagen protein and cause recessive dystrophic epidermolysis bullosa (RDEB). Collagen VII expression has been restored by retroviral and lentiviral vectors carrying COL7A1 gene. Long-term expression of transgene in keratinocytes, fibroblasts or epidermal stem cells was achieved [54]. Mutations in genes encoding the basement membrane component lamin 5 (LAM5) are the cause of junctional epidermolysis bullosa. Retrovirally transduced epidermal stem cells have been used for preparation of epidermal grafts. Analysis revealed that synthesis and proper assembly of normal levels of functional LAM5 were observed, together with the development of a firmly adherent epidermis that remained stable at least for 1 year [55].

The treatment of congenital disorders of liver metabolism is currently limited, and prognosis of patients suffering from Crigler–Najjar syndrome, urea cycle disorders, familial hypercholesterolemia and primary hyperoxaluria type 1 is unfavourable. In comparison to the liver transplantation, ex vivo gene therapy offers a less invasive method without the need for lifelong immunosuppression [56].

Homozygous mutation in LDL receptor causes familial hypercholesterolemia. The gene therapy approach for treatment of this lethal disease was developed. Grossman et al. [57] described the treatment of a 29-year-old woman by ex vivo gene therapy. Autologous hepatocytes isolated from the patient were genetically corrected with recombinant retroviruses carrying the LDL receptor and subsequently reimplanted. The patient’s LDL/HDL ratio improvements have remained stable for the duration of the treatment (18 months). Ex vivo gene therapy using modified haematopoietic stem cells has generated encouraging results for treatment of multisystemic lysosomal storage disorders [58]. Retroviral and lentiviral vector-transduced bone marrow-derived cells overexpressing lysosomal enzymes can migrate into the central nervous system (CNS) and mediate cross-correction of the neighbouring brain cells. This approach has resulted in excellent outcomes, preventing the development of clinical manifestations in metachromatic leukodystrophy. According to a study by Cartier et al. [59],
patients with peroxisomal disorder X-linked adrenoleukodystrophy treated with ex vivo lentiviral vector-mediated gene therapy have also exhibited clinical benefits.

Based on the significant progress made to date, in spite of the expected setbacks of all drug development efforts, gene therapy for liver metabolic disorders is becoming a viable option for treatment in future clinical trials.

5.2. Cancer gene therapy

Cancer is a complex disease accompanied by progressive accumulation of genetic and epigenetic alterations, which enables the cell to escape from cell and environmental control. Conventional treatment consists of surgery, chemotherapy and radiotherapy. Despite progress in the treatment, it is ineffective or accompanied by severe adverse effects in many cases; therefore, novel approaches are needed. Recently, research has been focusing on targeted therapy. Gene therapy – classical or mediated by cellular vehicles – is the promising approach, primarily for patients suffering from glioblastoma multiforme (GBM), neuroblastoma, metastatic melanoma and other metastatic cancers. As stated above, cancer treatment is recently the most elaborated area of gene therapy.

The treatment is focused on suppression of activated oncogenes, restoration of expression of tumour-suppressor genes, activation of anti-tumour immunity and inhibition of angiogenesis-or metastatic potential-suppressing genes. The separate group is represented by genes inducing autodestruction of tumour – ‘toxic’ genes or genes coding for enzymes converting non-toxic prodrug to toxic product [60].

Prodrug-converting genes are also known as ‘suicide genes’. They are of viral, bacterial or yeast origin, and they do not have the equivalent in mammalian cells. Mammalian cells obtain the ability to utilise a new substrate after transduction – an inactive compound is converted to chemotherapeutics. The apoptosis is induced in transduced cells; therefore, this approach is called suicide gene therapy. Bystander effect is the phenomenon of suicide gene therapy. Toxic metabolites released from transduced cells are received by the surrounding bystander cells and mediate toxic effect in them [61]. This targeted chemotherapy is considerable to the organism because chemotherapeutic agent is present only in the tumour vicinity – the site where (systemically administered) prodrug and therapeutic enzyme meet together.

Herpes simplex virus thymidine kinase (HSVtk) is one of the most frequently used therapeutic genes. Its affinity to nucleotide analogue ganciclovir (GCV) is approximately 1000 times higher than mammalian thymidine kinase. HSVtk phosphorylates GCV to GCV-monophosphate (GCV-P), which is subsequently phosphorylated by cellular kinases and incorporated into replicating DNA instead of guanosine triphosphate. GCV lacks deoxyribose at 3’OH and bound between carbons 2’ and 3’, which are necessary for the elongation of DNA chain. Incorporation of GCV-3P yields into termination of DNA synthesis and subsequent cell death – preferentially apoptosis, advisable cell death in cancer treatment. GCV becomes a charged molecule after phosphorylation, and it is not able to diffuse across the membrane. As stated above, the bystander effect is an important phenomenon in contributing to the efficacy of gene therapy. Gap junctional intercellular communication (GJIC) is necessary for transport of
phosphorylated GCV. GJIC is often a limiting factor of HSVtk/GCV system because many cells are defective in expression of the connexins, which are the major components of gap junctions. Transduction of connexin gene can improve the efficacy of the treatment [62].

The second most frequently used system in suicide gene therapy is cytosine deaminase (CD) derived from bacteria (bCD) or yeasts (yCD) combined with prodrug 5-fluorocytosine (5-FC), which is used in conventional antimyotic therapy. Yeast CD is 15 times more efficient in comparison to its bacterial counterpart [63].

Transduced cell is able to convert non-toxic 5-FC to conventionally used chemotherapeutic 5-fluorouracil (5-FU). More active molecules arise by the metabolism of 5-FU, and the synthesis of both DNA and RNA is impaired. Thymidylate synthase (TS) is the key target enzyme in 5-FC/5-FU treatment. 5-fluorouridine monophosphate, one of the active metabolites of 5-FU, binds irreversibly to TS, and starvation for thymine leads to inhibition of DNA synthesis. 5-fluorothymidine triphosphate, another metabolite, impairs RNA by incorporation instead of UTP [64].

The efficacy of the CD/5-FC approach can be increased by the addition of another enzyme, bacterial or yeast-derived uracil phosphoribosyl transferase (UPRT), which supplements low expression of mammalian orotate phosphoribosyl transferase important for the activation of 5-FU. It also continuously utilises 5-FU supporting its synthesis [65]. CD and UPRT can be cloned separately or as a synthetic fusion gene CD::UPRT.

Purine nucleoside phosphatase (PNP) is an E. coli-derived enzyme, which activates fludarabine. PNP-transduced cells convert fludarabine into metabolites, which are highly toxic for proliferating as well as for quiescent cells, because they inhibit ATP-dependent reactions. The metabolism of nucleic acid and proteins is impaired.

When the therapeutic gene is inserted into a replication-defective vector, it is expressed only in the transduced cell, and surrounding cells are affected only via the bystander effect. On the other hand, the replication-competent vectors replicate in target cells, and despite the bystander killing, they are able to infect their neighbours and spread the transgene. RCV have been constructed for treatment of aggressive tumours.

Promising results by MoMLV-derived replication-competent vector carrying cytosine deaminase were achieved on orthotopic glioblastoma model. Considerable infection of target cells, virus spread and significant bystander effect were demonstrated [66]. Based on promising preclinical results, a phase I/II clinical study is ongoing using the replication-competent cytosine deaminase-expressing retroviral vector to patients with recurrent or progressive grade III or grade IV gliomas (NCT01985256) [67].

It is important to note that despite of very promising preclinical data, many clinical studies failed because of low efficiency of the transfer of genetic information into target cells, insufficient infiltration of target tissue by the vector or low expression of transgene in tumour. The identification of mesenchymal stromal cells (MSC) and the discovery of their high affinity to tumour tissue facilitate important improvement in the cancer gene therapy.
MSC were originally isolated from bone marrow and characterised as rare non-haematopoietic population with clonogenic capacity and plastic adherence [68]. They have the self-renewal potential and are able to differentiate into specialised progeny [69-72]. Bone marrow is the most popular source of MSC, but adipose tissue is also very suitable because of its accessibility. Moreover, the frequency of MSC in the adipose tissue is 500 times higher in comparison to bone marrow. MSC can also be isolated from umbilical cord, dental pulp and different connective tissues. They serve as a source of regenerative cells in fractures, inflammation and necrosis. The injured tissue produces chemotactic signals which attract MSC [73]. Tumour can be compared to a wound that never heals [74]. Many tumours produce chemotactic signals which attract MSC [75-77]. It was demonstrated that MSC-derived cells are a component of tumour stroma, and they can support proliferation and vascularisation of malignant tissue [78]. The natural affinity of MSC to malignant tissue can be used for targeted therapy. They can be used as delivery vehicles in cancer gene therapy [28, 79]. The approach can be compared to the Trojan horse. MSC are able to pass across the endothelium, enter the blood stream and engraft in the tumour. Therefore, genetically engineered MSC can be administered intravenously, and they reach the target site. This enables to treat the disseminated tumours and metastases. MSC even cross the blood-brain barrier [80, 81]. In this regard, the MSC-mediated cancer gene therapy is superior to the ‘classic’ cancer gene therapy. On the other hand, it is also necessary to note that the therapy by genetically engineered MSC is limited by the fact that tumours differ in the attractiveness for MSC. Many paracrine factors are involved in MSC – tumour cell signalling. The SDF-1α and CXCR4 (CXCL12) signalling seem to play an important role in homing of stromal cells [76, 77].

Besides MSC, other cellular vehicles can be transduced and used in cancer gene therapy. Neural progenitors or neural stem cells isolated from brain tissue are used as delivery vehicles in targeted therapy of aggressive tumours of central nervous system [82].

The ideal candidates of cellular therapy for clinical use are the cells harvested without difficulty, which can be processed ex vivo very efficiently and afterwards transplanted. The unique biological features of MSC predetermine them as valuable gene carriers for therapeutic approaches. MSC can be easily transduced with retroviral and lentiviral vectors, which is a key prerequisite for the introduction and durable expression of marker and/or therapeutic genes within the tumour environment after homing to target tissues [83, 84].

The immunophenotype and the ability to differentiate are not affected by transduction. In order to address the safety of retrovirally transduced MSC, many studies have been performed. Particular transgene can give a proliferative advantage, but it does not preclude the entering of cells to senescence and has no impact on the safety of cancer gene therapy mediated by MSC [85].

The combination of cellular and gene therapy provides a unique opportunity to bypass the obstacles connected with direct viral delivery of the transgene. Cell vehicle protects the vector from the immune surveillance and supports targeting of a therapeutic molecule to the tumour [86].

Cell-mediated gene therapy is based on the bystander effect. The suicide effect is not the main goal; neighbouring bystander cells should be impaired at first. Therefore, it is more appropriate to use the term ‘prodrug-converting gene’ instead of ‘the suicide gene’.
The simple retroviral plasmid pJZ308 derived from Moloney murine leukaemia virus [87] was used for delivery of yeast cytosine deaminase:uracil phosphoribosyltransferase (CD:UPRT) and Herpes simplex virus-thymidine kinase (HSVtk) prodrug-converting genes into adipose tissue-derived MSC. The retroviral transduction of AT-MSC by CD gene was published for the first time in 2007. In this pilot study, the capability of AT-MSC expressing fusion yeast CD:UPRT gene in combination with prodrug 5-fluorocytosine (5-FC) to eradicate human colon carcinoma cells HT-29 in vitro, and their significant role in inhibition of tumour growth in a therapeutic paradigm in vivo were demonstrated [88]. A number of published papers reported the cytotoxic efficiency of CD:UPRT-MSC/5-FC enzyme/prodrug therapeutic system, both in vitro and in vivo, in the treatment of experimental prostate tumour [89, 90], melanoma [91, 92] and medullary thyroid carcinoma [93, 94]. The 3D multicellular culture conditions for better prediction of the therapeutic outcome in mouse xenograft models are suggested to be used according to the study performed on melanoma model [95]. Contrary to 5-FU, 5-FC is able to cross the blood-brain barrier, thus making this enzyme/prodrug approach suitable for the treatment of CNS tumours [96], which was proved on malignant glioma model [97]. The complete regression of glioblastoma simulating clinical therapeutic scenario was demonstrated by Altaner et al. [98].

AT-MSC were shown to form gap junctional intercellular communication with glioblastoma cell lines, thus rendering them suitable vehicles for the enzyme/prodrug therapy system HSVtk/GCV relying on transport of polar metabolites [99]. AT-MSC transduced by this suicide gene HSVtk also via lentiviral vector proved strong candidates of gene therapy for U-87-derived model of glioblastoma multiforme [100].

Efficacy of gene-directed enzyme/prodrug therapy can be improved by the combination of individual systems. Matuskova et al. [101] demonstrated various levels of synergy depending on tested cell line and experimental set-up. Systemic administration of CD:UPRT-MSC and HSVtk-MSC in combination with both prodrugs, 5-FC and GCV, inhibited growth of experimental lung metastases derived from human breast adenocarcinoma cells.

MSC were also retrovirally transduced to stably express an exogenous gene encoding the therapeutic agent hTNFα whose effect was tested on tumour cell lines of different origins. Co-injection of such therapeutic cells with melanoma cells inhibited the tumour mass growth up to 97.5% in vivo [102].

MSC isolated from the Wharton’s jelly of the human umbilical cord were lentivirally transduced by gene carrying the soluble human tumour necrosis factor-related apoptosis-inducing ligand (sTRAIL). The specific expression of the transgene in the tumour was ensured by alpha-fetoprotein promoter. Significant therapeutic effect was observed on orthotopic hepatocarcinoma model established on athymic mice, and the treatment was even more efficient in combination with 5-fluorouracil [103].

The effectiveness of therapeutic system using TRAIL expression from bone marrow-derived MSC with significantly increased survival of nude mice was noted as suitable for use in the prevention of the recurrence of hepatocellular carcinoma after radiofrequency ablation [104].
A different therapeutic strategy comprises carboxyl esterase (CE), an enzyme hydrolysing prodrug Irinotecan. Hong et al. [105] transduced neural stem cells by this gene, which led to the development of a novel strategy for delivering therapeutic genes to brain tumours. The significant inhibition of the growth of human non-small-cell lung adenocarcinoma cells was achieved for these lung cancer brain metastases also \textit{in vivo}.

PNP-transduced AT-MSC were tested for treatment of ovarian cancer in immunodeficient mice model (unpublished data). Cell vehicles were also retrovirally transduced by interleukin-coding genes (IL-2, IL-4, IL-12, IL-23) and interferon-β in order to treat primary or metastatic brain tumours [96].

As stated above, MSC are the long-term reservoir for tissue regeneration. They are naturally radio- and chemo-resistant [73, 106, 107]. Despite being equipped by enzymes and efflux mechanisms enabling resistance to chemotherapeutics, their resistance is not absolute. The transgene or metabolite activated by prodrug-converting gene also affects MSC, which after a certain time undergo cell death [108]. In the context of tumour-promoting potential of MSC [76], this fact should be considered as the advantage improving the safety of MSC-mediated gene therapy. It was shown that expression of yCD::UPRT transgene sensitises MSC to 5-FC, and its expression as well as the expression of HSVtk lead to suicide effect of therapeutic cells in the presence of GCV [108]. As shown on neural stem cells, if they are co-expressed together, the effect is even stronger [109].

Promising preclinical data enabled the approval of clinical trials mediated by engineered cellular vehicles. Patients suffering from aggressive, by conventional approaches incurable tumours can be included. The protocol for the first clinical study utilising genetically engineered MSC was published in 2015. Patients suffering from advanced, recurrent or metastatic gastrointestinal or hepatopancreatobiliary adenocarcinoma will be treated by autologous retrovirally transduced bone marrow-derived MSC. The gammaretroviral self-inactivating vector carrying HSVtk will be used [110].

To conclude, it is important to note that despite many clinical studies, the gene therapy is in the early stage of clinical use. For now, it presents an experimental approach. Besides clinical efficacy, safety is the crucial criterion of gene therapy. It is undisputed that retroviral vectors are indispensable tools for genetic modification, and they have the potential to significantly contribute to the improvement in targeted treatment of immunologic, oncologic and genetic disorders.

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