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Review Paper

ELECTROGENE THERAPY IN CANCER TREATMENT

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Summary: Gene therapy offers the prospect of efficient and highly specific therapy of cancer. Vectors for introduction of therapeutic genes into target tissue can be broadly classified into viral and non-viral vectors. Viral vectors provide highly efficient gene delivery technique, but there are some major concerns regarding their safety for patients. Non-viral techniques involve delivery of naked plasmid DNA into tissue using physical methods, such as electroporation or gene gun technique or deliveries mediated by chemical carriers, for example cationic polymers or lipids. Non-viral methods provide safer, but less efficient alternative compared to viral DNA delivery.

Electroporation is method for delivery of various molecules into the cells by transiently increasing permeability of cell membrane using application of controlled external electrical field to the cells. Electroporation-based DNA delivery or electrogene therapy involves injection of plasmid DNA into target tissue, followed by application of controlled electric pulses. In electrogene therapy of cancer, therapeutic genes are usually transferred either intratumorally or intramusculary. Until now, electrogene therapy using a variety of therapeutic genes, mostly encoding cytokines, but also antiangiogenic factors, suicidal and apoptosis inducing genes has shown promising results for effective cancer therapy in preclinical studies.

Key words: neoplasms – therapy; electroporation; gene therapy – methods; drug delivery system

Introduction

Intensive scientific research in molecular biology in the last decades significantly increased growth of knowledge of the molecular basis of carcinogenesis and therefore led into improvements in cancer therapy. Despite considerable progress, which has been made, many types of cancer remain resistant to conventional therapy. Therefore new therapeutic approaches are being explored, among which immunotherapy and gene therapy hold great promise for cancer treatment.

The concept of gene therapy involves transfer of genetic material into target cells in order to overcome a genetic defect or to provide a protective or corrective function with the goal of curing a disease or improving clinical status of a patient. In case of genetic disease, caused by mutation in a specific gene, therapeutic effect of gene therapy is usually achieved by delivery of functional gene into a target cells or tissue. Exogenous gene delivery can also be a tool for treatment of non-genetic disorders by delivery of genes, which, for example, encode proteins to modulate immune response or other therapeutic proteins with specific function (1, 2).

Gene of interest can be inserted into target cells using different vectors, which can be broadly classified into two groups, viral and non-viral vectors. After the introduction of therapeutic gene, genetically altered cells start with production of RNA or protein, encoded by the transferred gene. The goal of this strategy is to achieve stable, preferably regulated expression of transgenes in the target tissue for required period of time without significant side effects (1, 2).

The first time gene therapy was employed in treatment of human patients was in 1990. Treated was a group of patients with genetic disease ada-SCID (i.e. severe combined immunodeficiency due to adenosine deaminase deficiency) (3). One year later the first clinical trial of gene therapy for can-

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cer was performed in patients with melanoma (4), and until now, over 1000 gene therapy clinical trials have been conducted around the world for different indications, vast majority of them (over 66 %) are in cancer treatment (Figure 1) (5).

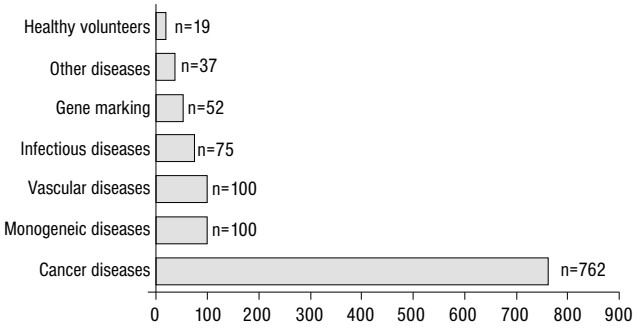

Figure 1: Distribution of ongoing gene therapy trials worldwide by indications (5)

Viral vectors are currently the most frequently used vectors in clinical trials of gene therapy worldwide (Figure 2) (5). Their main advantage is high

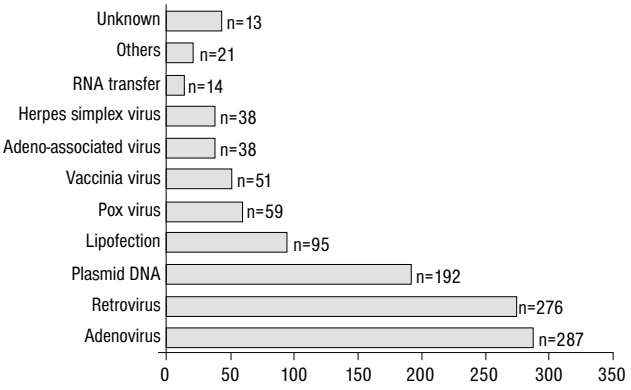

Figure 2: Distribution of ongoing gene therapy clinical trials worldwide, dependent on the vectors used (5)

Gene therapy offers the prospect of efficient and highly specific therapy of cancer, which created lots of excitement among investigators and clinicians and lead to intensive research in this field. Researchers developed several of different approaches to cancer gene therapy, which can be divided into three basic concepts (6, 7):

a.) Strategies to enhance immunological rejection of the tumor by the host;
b.) Strategies to repair the cell cycle defects caused by losses of tumor suppressor genes or inappropriate activation of oncogenes, and
c.) Suicide gene strategies.

DNA delivery systems in cancer gene therapy

The success of gene therapy largely depends on development of suitable vectors or vehicles for *in vivo* gene transfer. In order to eliminate potential risks of exogenous gene transfer, for example, evolution of new viral diseases in humans, induction of malignant transformation, systemic toxicity, etc, DNA vectors employed in gene therapy *in vivo* have to fulfill several conditions. Optimal DNA vector would have to enable high levels of stable and long-lasting exogenous gene expression without significant side effects for the patients undergoing gene therapy. In search for such vector, a number of viral- and nonviral- vector based delivery systems have been developed (1, 2, 6, 7, 8, 9).

transfection efficiency (6). Viral vectors are derived from naturally evolved viruses, which are capable of transferring their genetic material into the host cells. They are genetically modified by deleting genes, essential for viral replication, assembling or infection and replacing deleted genes with relevant therapeutic genes (1). Such viruses lose their ability to reproduce in target cells, and can be replicated only in cell lines, which provide the deleted function. This modification is necessary to prevent replication of recombinant viral vectors in the host organism in order to ensure safety of their clinical use.

Gene therapy vectors are being developed by genetic modification of retroviruses, lenitiviruses, adeno- and adeno-associated viruses, herpesviruses, poxviruses, and others. Among variety of different types of viral vectors, two of the most often used in gene therapy clinical trials are retro- and adenoviral vectors (5).

Retroviral vectors are one of the first constructed human gene therapy vectors, and have been used for DNA delivery since the early 1980s. Retroviruses are small RNA viruses, which use cellular transcription machinery to copy their own genome and integrate it into the genome of host cell. Ability for integration into host's genome is one of the most desirable features of retroviruses, since it allows long-term expression of transgenes. On the other hand, random integration is associated with risks of insertional mutagenesis. The other drawbacks for their use in clinical settings are inability to infect non-dividing

cells, problems with production of high titers and low capacity for exogenous DNA insertion (8).

Adenoviral vectors are considered among the most efficient DNA delivery methods, which are currently available for *in vivo* gene transfer to mammalian cells. They have large capacity for exogenous DNA insertion, can infect a large variety of cell types and are relatively easy to produce in high titers. However, their main limitations are the absence of expression of adenoviral receptors on certain types of cells and high prevalence of anti-adenoviral antibodies in humans, since naturally occurring adenoviruses are associated with common cold and other respiratory, intestinal and eye infections (9).

The major drawbacks, associated with DNA delivery using viral vectors, which raise concerns about their safe clinical use, are especially insertional mutagenesis, stimulation of the patient's immune system, which can preclude multiple administrations and cause adverse immune reactions, and toxicity with systemic application (2, 10). Therefore, as an alternative to overcome some of the major concerns and risks of viral vectors, different nonviral gene delivery techniques have been developed, for example naked DNA delivery using physical methods such as electroporation or gene gun technique or deliveries mediated by a chemical carriers, for example cationic polymers or lipids (11).

All of these modalities use naked plasmid DNA, which is, contrary to viral vectors, noninfectous and nonimmunogenic and has low toxicity profile. Compared to production of viral vectors, large amounts of endotoxine-free plasmid DNA are relatively easy and quick to produce (12, 13). But the major limitation of these techniques is low *in vivo* transfection efficiency, compared to viral methods (11).

Chemical methods for *in vivo* gene delivery employ synthetic vectors, which protect naked DNA from degradation and improve its admission intracellulary thus facilitating transfer of naked DNA into target cells. One of such approaches is use of cationic lipids, which interact with negatively charged DNA, forming DNA-lipid complexes, called lipoplexes. These complexes are positively charged, which allows them to bind to negatively charged cell surface. Entry of lipoplexes into the cell is achieved by endocytosis, followed by release of DNA into the cytoplasm. Cationic lipoplexes are suitable for clinical use, since they are noninfectious, nonimmunogenous, well tolerated, easy to produce and can be targeted to specific cells (14).

Another chemical method, which can be utilized for *in vivo* gene delivery, is use of cationic polymers. Positively charged polymers, for example DEAE-dextran, polybren, polylysin, polyethilenimine, spontaneously interact with DNA molecules to form complexes, called polyplexes. The potential for clinical use of polyplexes is in inhalation gene therapy, which is non-invasive and relatively effective gene transfer into respiratory tract, with permanent gene expression without adverse expression in other tissues (15).

One of the physical methods, which can dramatically enhance transfection efficiency of plasmid DNA application into tissue alone, is electroporation (13, 16). Electroporation is a method for delivery of various molecules into the cells by transiently increasing permeability of cell membrane using application of controlled external electric field to the cells (17, 18).

Electroporation is already well established as in vitro method for increasing delivery of various molecules (e.g. RNA, DNA, oligonucleotides, dyes, ions, chemotherapeutic drugs, etc) into different types of cells. *In vivo* it is gaining much interest as a tool for two prospective therapeutic modalities, electrochemotherapy (i.e. application of controlled electric pulses to tumor cells in order to increase uptake and cytotoxicity of chemotherapeutic drugs (19, 20, 21, 22) and electrogene therapy (i.e. enhancing transfection efficiency of plasmid DNA application into different tissues (13, 16, 23, 24).

Other physical method for introduction of plasmid DNA into cells is gene-gun technique or DNA-coated particle bombardment (25). This technique utilizes heavy metal (gold or tungsten) microparticles, covered with DNA, which are accelerated to the sufficient speed using compressed helium to penetrate the target cells. Clinical application of the technology remains limited because of relatively low efficiency of the method and the potential tissue damage created by impact of the particles.

One of the newest nonviral physical methods for gene delivery is use of ultrasound or so called sonoporation, which increases permeability of cell membrane to different macromolecules, including DNA (26, 27). The efficacy of this method can be improved by use of microbubbles, or ultrasound contrast agents. The use of ultrasound-enhanced gene delivery has potential for clinical use, because it allows safe and focused delivery of DNA to target tissue (26).

Another physical method is hydrodynamic delivery, which employs the force, generated by the rapid

injection of a large volume of DNA solution in the circulation to overcome the physical barriers of endothelium and cell membranes and enable gene delivery to parenchymal cells, e.g. liver or muscle cells (27, 28).

Electrogene therapy

Electroporation-based gene transfer *in vivo* or electrogene therapy involves injection of plasmid DNA into target tissue, followed by application of appropriate electric pulses, which facilitate transport of DNA molecules through the destabilized cell membrane into the cells (29).

In vivo gene delivery using electroporation was first performed in the 1990's (30) and since then a number of different types of tissue have been successfully transfected using this approach, for instance tumors (13, 24), skeletal muscle (16), skin (31, 32) and liver (33). Transfection efficiency of this method is still low compared to viral vectors (34); yet its advantages, mostly lack of pathogenicity and immunogenicity, make it promising new gene therapy technique which can in the future become well established in clinical work.

Potential for use of electrogene therapy in treatment of several different diseases, including muscle disorders, blood disorders, arthritis and cancer, was demonstrated in number of preclinical studies (23, 35).

Types of tissue, targeted for transfection with therapeutic genes in cancer electrogene therapy

Gene therapy in cancer patients can be instituted using two different approaches. The first one is *ex vivo* gene therapy, where cells are removed from patient, transfected *in vitro* with the plasmid or viral vector, selected, amplified, and then reinjected back into the patient. The other approach is *in vivo* gene therapy, where exogenous DNA is delivered directly into host's target tissue (e.g. tumor, peritumorally or into skeletal muscle) (11).

Among variety of tissues, which have already been successfully transfected using electrically-assisted plasmid DNA delivery, the most interesting target tissues for electrogene therapy in cancer patients, are tumor tissue and skeletal muscle.

Electrically-assisted gene delivery into tumors

Electrically-assisted delivery of therapeutic genes into tumors facilitates local intratumoral production of high concentrations of encoded proteins, which enables sufficient therapeutic concentrations without the need for systemic delivery of high concentrations of therapeutic genes or proteins. This is especially important in case of cytokines, where high systemic concentration is associated with severe toxicity (36). This approach can be used as a single therapy or in combination with other modalities for cancer treatment, for example electrochemotherapy.

The first evaluation of intratumoral electrogene therapy for cancer treatment was performed on murine melanoma tumor model in 1999 by Niu et al (37). Since then, a variety of therapeutic genes, mostly encoding cytokines, but also antiangiogenic genes, p53 gene, HSV-TK gene, etc, have been introduced to a number of animal tumor models, e.g. melanoma, squamous cell carcinoma and hepatocellular carcinoma (35).

Results of preclinical studies indicate, that electrically-assisted intratumoral delivery of therapeutic genes enables efficient transgene expression with sufficient production of therapeutic proteins, which can lead to pronounced antitumor effect on treated tumor (for example suppression of tumor growth, partial or complete reduction of tumor nodule) and even induces long-term antitumor immunity in treated animals (38, 39, 40).

Electrically-assisted gene delivery into skeletal muscle

Skeletal muscle is an attractive target tissue for delivery of therapeutic genes, since it is usually large mass of well vascularized and easily accessible tissue with high capacity for synthesis of proteins, which can be secreted either locally or systemically (41). Electrically-assisted gene delivery into skeletal muscle can be applied for therapy of different muscle diseases, for local secretion of angiogenic or neurotrophic factors or for systemic secretion of different therapeutic proteins, such as erythropoetin, coagulation factors, cytokines, monoclonal antibodies, etc. (16, 34, 42, 43).

The transfection efficiency of electrically-assisted gene delivery is the highest in skeletal muscle, compared to all other types of tissue (41). Electroporation significantly enhances expression of plasmid DNA, even up to 2000-times, and reduces variability of gene expression compared to application of plasmid DNA into skeletal muscle without electroporation (16, 44). Owing to the postmitotic status and slow

turnover of skeletal muscle fibers, which ensures that transfected DNA isn't readily lost, it is possible to achieve long-term expression of exogenous DNA, which can last up to 1 year (16, 41). This is due to the dynamics of naked DNA transfer, since plasmid does not integrate into genome of transfected cell and thus duration of exogenous DNA expression in part depends on lack of cell division. In contrast to muscle cells, in tissues, where cell turnover is much higher, plasmid DNA is rapidly lost from the cells (41).

It was established in different studies, that electrically-assisted gene delivery into skeletal muscle enables sufficient systemic expression of transgene products to ensure antitumor therapeutic effect. Therapeutic genes, which manifested encouraging antitumor effect after electrically-assisted delivery into skeletal muscle, are for example genes, encoding interleukin-12 (45), interleukin-24 (46), interferon-α (47) and different antiangiogenic factors (48).

Therapeutic genes used in electrogene therapy of cancer

A number of different therapeutic genes were employed in successful electroporation-mediated gene therapy of cancer in preclinical studies.

One of the major classes of genes of interest are immunostimulatory genes. The concept of stimulation of host's immune system to attack tumor cells has long been investigated as an alternative to conventional cancer therapies, since specificity of the immune system could provide means to target tumor cells while leaving normal cells intact (49). Unfortunately, many early attempts to employ immunotherapy for cancer treatment showed only modest benefits or were even highly toxic. Recently, gene therapy offered new possibilities to develop clinically applicable immunotherapy of cancer.

Some of the most significant clinical responses in cancer immunotherapy to date have been achieved with employment of active nonspecific immunotherapy, i.e. use of cytokines. However, wide-spread use of recombinant cytokines in clinical work is limited by short half-life of recombinant cytokine proteins. In order to obtain sufficient therapeutic effect, repetitive systemic applications of high dosages of cytokine proteins are required, which can lead to severe side effects (36, 49). Therefore new application strategies have been developed to improve therapeutic efficiency and alleviate side effects. One of such alternatives to application of recombinant cytokine proteins is immunogene therapy - transfer of genes, which encode production of different cytokines into target tissue, e.g. tumor or muscle. Electrically-assisted therapeutic gene delivery into tumor nodule has a direct therapeutic effect on tumor cells, since high concentrations of encoded proteins are produced locally in the tumor tissue. This local approach has an obvious disadvantage that it can not be employed for nodules, which are not easily accessible (e.g. nodules in internal organs) or are not visible (e.g. microresidues of tumor tissue after surgical removal or micrometastases). Delivery of therapeutic genes into skeletal muscle cells will have therapeutic effects on distal tissue targets, which can be both primary tumor nodules and metastases, via secretion of transgene products in the systemic circulation.

Electrogene therapy with genes, encoding different cytokines, has already shown promising results in preclinical trials on different animal tumor models. Cytokine genes, which showed the most potential for cancer therapy, are interleukin (IL)-2, IL-12, IL-18, interferon (IFN) α, and GM-CSF (23).

Currently, one of the hot topics in cancer immunotherapy is use of IL-12 (49), which plays important role in the induction of cellular immune response through stimulation of T-lymphocyte differentiation and production of IFN-γ and activation of natural killer cells (50). Antitumor effect of electrically-assisted delivery of gene, encoding IL-12, has already been established on various tumor models, e.g. melanoma, lymphoma, squamous cell carcinoma, urinary bladder carcinoma, mammary adenocarcinoma and hepatocellular carcinoma (45, 51, 52, 53, 54). Results of these preclinical studies show that beside regression of tumor at primary and distant sites, electrogene therapy with IL-12 also promotes induction of long-term antitumor memory and therapeutic immunity, suppresses metastatic spread and increases survival time of experimental animals (40, 45, 51, 52, 53, 54, 55).

Electrically-assisted gene delivery was also employed in suicide gene therapy of cancer. The concept of suicide gene therapy is intratumoral transfer of a prodrug-activating gene, which selectively (intratumorally) activates otherwise non-toxic drugs (6). The most often used strategy in suicide gene therapy is the delivery of gene, encoding herpes simplex virus thymidine kinase (HSV-TK) and prodrug ganciclovir (GCV) (56, 57). HSV-TK activates GCV, which blocks extensions of DNA strands, leading to cell death by apoptosis (56). Results of several studies show that electroporation-based HSV-TK/GCV gene therapy

may provide potentially effective gene therapy for cancer (57, 58, 59).

Another approach to antitumor therapy, which is currently being widely investigated, is based on inhibition of angiogenesis of tumor nodules. The basic concept of antiangiogenic gene therapy is transfection of cells with genes, encoding inhibitors of tumor angiogenesis, which prevent formation of new tumor vessels within growing tumor and thus block tumor growth or even lead to regression of tumors. Electrically-assisted delivery of genes, encoding antiangiogenic factors (angiostatin and endostanin) was demonstrated to be effective in inhibition of tumor growth and metastatic spread of different tumors (39, 60, 61).

Other gene therapy strategies, based on *in vivo* electroporation, which show potential for effective cancer treatment, include introduction of apoptosis inducing genes (62) and p53 gene (63).

Conclusions

The use of naked plasmid DNA as a DNA delivery system for *in vivo* gene therapy is an attractive alternative to viral gene delivery techniques due to its safety and simplicity. Relative poor efficiency of this gene transfer approach can be dramatically increased using *in vivo* electroporation.

Safety and efficiency of electroporation-based DNA delivery in treatment of cancer is already well established on preclinical level in numerous studies on different tumor models, which have made the potential of electrogene therapy in cancer quite clear. Even though research on this topic is still relatively new, the amount of gained knowledge already allowed electrically-assisted delivery of plasmid DNA intratumorally to reach clinical level. If future research continues to produce encouraging results, electrogene therapy will probably become promising alternative to other strategies of *in vivo* gene therapy for successful treatment of cancer patients.

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ELEKTROGENSKA TERAPIJA PRI ZDRAVLJENJU RAKA

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Povzetek: Genska terapija je nova oblika zdravljenja, ki je v zadnjih nekaj desetletjih deležna velike pozornosti raziskovalcev na področju onkologije, saj obljublja možnost učinkovite in visoko specifične terapije rakavih obolenj. Vektorje, s pomočjo katerih se v ciljno tkivo vnašajo terapevtski geni, delimo v dve glavni skupini, na virusne in nevirusne vektorske sisteme. Glavna prednost virusnih vektorjev je učinkovit vnos genskega materiala v celice, vendar pa je lahko njihova uporaba povezana s hudimi stranskimi učinki, kar vzbuja pomisleke glede varne klinične uporabe. Med nevirusne metode vnosa DNK v celice prištevamo vnos gole plazmidne DNK s pomočjo fizikalnih metod, kot sta na primer elektroporacija in genska puška, ter kemijski načini vnosa, na primer uporaba kationskih polimerov in lipidov. Te metode omogočajo varnejši, vendar manj učinkovit način vnosa DNK v primerjavi z virusnimi vektorji.

Elektroporacija je postopek, pri katerem z uporabo zunanjega električnega polja začasno povečamo prepustnost celične membrane in omogočimo vnos različnih vrst molekul v celice. Uporaba elektroporacije za izboljšanje prehoda DNK preko celične membrane intracelularno se imanuje električno posredovani vnos DNK ali elektrogenska terapija. Izvede se z injiciranjem plazmidne DNK v ciljno tkivo, ki mu sledi aplikacija ustreznih električnih pulzov. Pri elektrogenski terapiji raka se na ta način najpogosteje vnašajo terapevtski geni v tumorsko tkivo ali v skeletno mišičnino. Do sedaj je bila v številnih predkliničnih raziskavah ugotovljena protitumorska učinkovitost takega načina vnosa različnih terapevtskih genov, zlasti genov, ki nosijo zapis za citokine, pa tudi antiangiogene faktorje in gene, ki izzovejo apoptozo celic.

Ključne besede: novotvorbe – zdravljenje; elektroporacija; genska terapija – metode; zdravilo, sproščanje, sistemi