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Approaches in Gene Therapy of Cancer and Cardiovascular Diseases

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1. Introduction

In this chapter we will focus on few topics. First, we summarize and discuss angiogenic gene therapy of cancer and cardiovascular diseases, with RNA interference in a separate section. Second, we briefly review delivering systems in gene therapy, their advantages and limitations and further focus on bacterial vectors. Last, we summarize antioxidant gene therapy of cardiovascular diseases and hypertension.

Considering the fact that the fundamental discoveries and new findings in medicine are being crystallized on genetic and genomic levels, gene therapy is one of the potential mechanisms for therapeutic intervention. Gene therapy in a broad sense, i.e. all the therapeutic strategies employing nucleic acids as carriers of genetic information, found its utilization in most areas of medicine, including angiogenesis research. Similarly to classical “non-gene” therapy, the research in gene therapy is happening on the preclinical level using appropriate animal models, with cancer and cardiovascular diseases being the most abundant indications. There are several different strategies known. Besides the delivery of therapeutic gene (replacement of the mutated gene by a functional one or delivery of the gene because of lack of the gene product), novel strategies are also being widely used based on blocking the function of a specific gene by application of RNA interference inducing sequences, antisense inhibition etc.

One of the key advantages of gene approaches is the endogenous production of the therapeutic molecule. Furthermore, targeted gene delivery specifically into the target tissue or only to a certain cell type can dramatically decrease the likelihood of adverse effects. Along with the development of new vectors and regulatory systems, the ability to control the expression of therapeutic gene in time and space is being improved. This is of great importance in affecting such complex and complicated processes as angiogenesis. Currently, almost three quarters of indications addressed by gene therapy clinical trials are represented by cancer and cardiovascular diseases

2. Angiogenic gene therapy

After birth, angiogenesis still contributes to organ growth. However, in adults, the majority of blood vessels remains in quiescence with the most active angiogenesis during menstruation cycle in the uterus and pregnancy in the placenta. In spite of this, endothelial cells do preserve the ability to quickly proliferate and react to physiological stimulus such as hypoxia and inflammation.

If the stimulus is too excessive, the fine balance between stimulants and inhibitors can be disrupted and thus lead to so-called “angiogenic switch”. The best described pathological states with dysregulated angiogenesis include cancer, cardiovascular disorders, inflammatory eye disorders as well as many other processes such as obesity, metabolic diseases, asthma, diabetes mellitus, cirrhosis, sclerosis multiplex, rheumatoid arthritis, macular degeneration, psoriasis, atherosclerosis, restenosis, diabetic retinopathy, endometriosis, bacterial infections and autoimmune diseases (Cao, 2010; Webb & Vande Woude, 2000).

2.1 Gene therapy of cancer

Gene therapy-based angiogenesis inhibiting strategies have gained much attention thanks to their advantages over the conventional antiangiogenic treatments. Given that effective inhibition of pathological angiogenesis requires long term treatment, gene therapy may be of importance for selective gene transfer to the affected areas and prolonged expression of therapeutic genes. Apart from that, gene therapy provides a possibility to circumvent the issues associated with recombinant proteins production, stability and solubility. Gene transfer allows for appropriate folding and stability of encoded proteins *in vivo* in the natural environment. An interesting advantage is also the ability to selectively target the gene transfer into certain tissues enabling localized expression and high regional drug concentration without increasing systemic levels. One of the key justifications for using gene therapy is also an insufficient efficiency of “non-gene” therapies based on inhibition of VEGF and other growth factors signal pathways in humans (Gridelli et al., 2007). The most commonly used gene therapy approach in cancer is so called suicide cytotoxic therapy using thymidine kinase or other chemosensitizing genes that allow the conversion of inactive prodrug (ganciclovir) into a cytotoxic product (Both, 2009).

2.1.1 Preclinical studies

Antiangiogenic gene therapy of cancer has been tested on a preclinical level in various carcinogenesis models. Most of the studies performed so far have used viral vectors (adenoviruses, retroviruses, lentiviruses, adeno-associated viruses, herpes simplex viruses) encoding endogenous angiogenesis inhibitor genes such as cytokines/chemokines (IFN- α , IFN- β , IFN- γ , CXCL10, IL-12, IL-18, TNF- α), VEGF blockers (sFlt-1, Flk-1), proteolytic fragments (angiostatin, endostatin, vasostatin, tumstatin) and others (Persano et al., 2007). For example, in a colorectal cancer model an adenovirus-based therapy using genes encoding IFN- β (Tada et al., 2001) and endostatin (Oliner et al., 2004) as well as plasmids encoding Flk-1 (W.J. Kim et al., 2006) and tumstatin (Yao et al., 2005) have been successfully applied. In a model of malignant melanoma, retrovirus vectors carrying gene encoding CXCL10 (Feldman et al., 2002) and plasmids encoding vasostatin (Jazowiecka-Rakus et al., 2006) and MCP-1 (Koga et al., 2008) genes have been successfully used, all exerting a clear antiangiogenic effect. Recently, a systemically available antiangiogenic gene therapy using

adenovirus bearing soluble VEGF receptor gene has been proven to be effective in suppressing tumor growth in various oral cancer cell line xenografts in mice (Okada et al., 2010).

Several studies have been performed using gene delivery of endogenous angiogenesis inhibitor endostatin. A liposome-encapsulated adenovirus encoding endostatin was applied in therapy of ovarian cancer (Yang et al., 2010). Systemic administration was well-tolerated and resulted in marked suppression of tumor growth, which was associated with a decreased number of micro-vessels and increased apoptosis of tumor cells. An interesting novel therapeutic approach for pancreatic cancer has been employed in a study using vaccinia virus encoding the endostatin-angiostatin fusion gene (Tysome et al., 2009). Besides high selectivity of the used vector, inhibition of angiogenesis and a clear antitumor potency has been observed. In another study, combined antiangiogenic and proapoptotic gene therapy involving endostatin and sTRIAL (soluble tumor necrosis factor-related apoptosis-inducing ligand) effectively suppressed hepatocellular carcinoma growth and angiogenesis in nude mice (Zhang et al., 2009). At last, adenovirus-mediated endostatin gene delivery combined with cisplatin treatment was effective in a lung cancer murine model (Ning et al., 2008). These studies represent a future direction in cancer research in which instead of targeting a single molecule, a combinatorial approach targeting multiple factors and/or an additional therapeutic approach is applied to cover multiple pathways of cancer progression.

2.1.2 Clinical studies

Despite a relatively high number of clinical studies using cancer gene therapy, specifically antiangiogenic gene therapy has only been exploited in a few studies. Intratumoral injection of adenovirus encoding immunostimulatory cytokine IL-12 has been tested in patients with advanced gastrointestinal cancer (liver, colorectal, pancreatic tumors) in phase I study (Sangro et al., 2004). Therapy was well tolerated, although only a moderate antitumor effect was observed. In another study, plasmid bearing IL-12 gene was applied to patients with malignant melanoma (Heinzerling et al., 2005). In two out of nine patients, the disease was stabilized for period of over three years and a complete remission was achieved in one patient. In these patients, a localized reduction in angiogenesis has been proven by immunohistochemistry. However, the rest of the patients showed only temporal response to the therapy. A recent phase I clinical trial of IL-12 plasmid/lipopolymer complexes has also shown a clinical benefit in treatment of recurrent ovarian cancer without adverse events (Anwer et al., 2010). In a different phase I study, adenovirus vector carrying IFN- β gene has been used in therapy of malignant pleural mesothelioma (Serman et al., 2007). In all the above-mentioned studies, however, inhibition of angiogenesis was not the primary goal, yet a part of the antitumor effect.

2.2 Gene therapy of cardiovascular diseases

In view of the many advances in explaining the molecular mechanisms of angiogenesis high hopes were put in gene therapy. However, in the clinical studies with genes encoding for individual proangiogenic growth factors (similar to recombinant proteins) only moderate success has been observed so far, thus creating a space for development of new vectors and alternative approaches (Vincent et al., 2007).

Progress in the field of gene therapy for cardiovascular disease has been modest; one of the key reasons for this limited progress is the lack of gene delivery systems for localizing gene

therapy to specific sites to optimize transgene expression and efficacy. However, progress toward the site-specific delivery of cardiovascular gene therapy is still ongoing and promising novel approaches are being tested (Fishbein et al., 2010).

2.2.1 Preclinical studies

A lot of studies have been performed on a preclinical level with very promising results. Angiogenic gene therapy using intramuscular injection of plasmids encoding VEGF and plasminogen activator has been applied in a mouse model of myocardial infarction (ligation of coronary artery) and hind limb ischemia (ligation of arteria femoralis) (Traktuev et al., 2007). Authors were able to detect functionally significant angiogenesis that clearly improved the pathological consequences of disease induction. The model of hind limb ischemia is actually a commonly used model to prove the efficiency of proangiogenic viral and non-viral gene delivery in ischemic disease (Schgoer et al., 2009). Bosch-Marce *et al.*, have used this model to test adenovirus vector carrying the gene encoding for constitutively active HIF-1 α , resulting in a sufficient induction of reperfusion of the affected areas even in older animals (Bosch-Marce et al., 2007). These results suggest that the HIF-1 α activity is necessary and sufficient for mobilization of angiogenic cells and that gene therapy using HIF-1 α gene can compensate the pathological effects of ageing. Gene therapy using gene encoding VEGF has further shown a clear beneficial effect in a rat model of myocardial infarction (Tang et al., 2010). In a mouse model of myocardial infarction, the ultrasound-targeted gene delivery of VEGF or stem cell factor resulted in increased vascular density and improved myocardial perfusion and ventricular function (Fujii et al., 2009). Yockman *et al.*, used an ischemia-inducible plasmid construct expressing VEGF to treat myocardial ischemia and infarction (Yockman et al., 2009). Ischemia-inducible system was superior to constitutively expressed gene construct in reducing the infarct size. A different regulation system for VEGF gene delivery based on tetracycline-inducible AAV-based vector has been employed by Tafuro *et al.*, in a hind limb ischemia model (Tafuro et al., 2009). These results clearly indicate that the fine tuning of VEGF expression is required to achieve the formation of a stable vasculature able to sustain functional neovascularization. Hypothesizing that the transient nature of VEGF gene expression provokes instability of neovasculature, Olea *et al.*, compared single vs. repeated transfection in a rabbit model of hind limb ischemia by injecting a plasmid encoding human VEGF₁₆₅ (Olea et al., 2009). Repeated, but not single, VEGF gene transfection resulted in increased microvasculature, which in turn afforded effective protection against ischemic muscle damage. Pajusola *et al.*, have compared the virus vector-mediated delivery of genes encoding VEGF and HIF-1 α into the muscle of mice (Pajusola et al., 2005). Results of this study show that HIF-1 α gene therapy is able to ensure increased expression of numerous proangiogenic molecules and, thus, can circumvent the shortcomings associated with overexpression of a single growth factor. A rationale for combination proangiogenic therapy has been provided in two studies, in which a synergistic effect of concurrent application of plasmids encoding FGF-2 and PDGF-BB by intramuscular injection was observed in a rabbit/rodent hind limb ischemia model (Li et al., 2010).

2.2.2 Clinical studies

Results from several interesting clinical studies have been published recently. In a phase I study testing the safety of intramuscular injection of VEGF-encoding plasmid vector in 9 patients suffering from serious peripheral arterial disease of the lower extremities a significant improvement in most of the measured parameters has been observed regardless

of the dose (Kim et al., 2004). However, the serum VEGF levels were not elevated and the control group was missing in that study. Interestingly, no significant improvement in primary and secondary endpoints between groups was achieved in two recent multicenter, double-blind, placebo-controlled phase II trials exploring intramuscular application of plasmid encoding angiogenic factors (Grossman et al., 2007; Rajagopalan et al., 2003). Authors of Euroinject One phase II trial analyzed the effect of gene transfer using plasmid encoding VEGF₁₆₅ gene on myocardial perfusion, left ventricle function and clinical symptoms in 80 patients suffering from stable severe ischemic heart disease (Kastrup et al., 2005). A direct intramyocardial injection of plasmid did not improve the study endpoints compared to placebo, although a regional improvement in ventricle wall movement was achieved. Results thus show the safety of direct intramyocardial injection, but not the efficiency. In the REVASC trial, a total of 67 patients with ischemic heart disease and severe angina pectoris were enrolled to the study (Stewart et al., 2006). Here, the intramyocardial injection of replication-deficient adenovirus vector bearing VEGF₁₂₁ gene significantly ameliorated the primary endpoint (exercise time needed for 1mm ST segment depression) as well as overall exercise time and exercise time to moderate angina after 26 weeks of therapy in patients with exercise-induced ischemia. On the other hand, in a recent double-blind, placebo-controlled study VEGF gene therapy failed to improve perfusion of ischemic myocardium in patients with advanced coronary artery disease (Stewart et al., 2009). Further, intramuscular injection of plasmid with VEGF gene was compared against placebo (saline) in 54 diabetic patients suffering from severe peripheral arterial disease (Kusumanto et al., 2006). Although a significant improvement of some of the parameters (hemodynamic status, skin ulcerations) has been achieved, the primary endpoint of this study – amputation of lower extremity after 100 days – stayed unchanged. Moreover, long-term safety of VEGF gene therapy has been proven in an eight-year safety follow-up of coronary artery disease patients after intracoronary VEGF gene transfer (Hedman et al., 2009). Local intracoronary VEGF gene delivery is, thus, considered safe and does not increase the risk of major adverse cardiovascular events, arrhythmias, cancer, diabetes or other disease.

So far the only clinical study testing the delivery of HIF-1 α gene was a randomized, double-blind, placebo-controlled phase I trial enrolling 34 patients with peripheral arterial disease (Rajagopalan et al., 2007). Treatment based on direct intramuscular administration of adenovirus vector carrying the HIF-1 α gene was well tolerated and provided relief of rest pain one year after the therapy, supporting the necessity for more clinical trials.

The second generation of angiogenic gene therapeutics is represented by constructs enabling the expression of two or more proangiogenic cytokines. Analogous to gene therapy using the master regulatory gene HIF-1 α , these “multivalent” approaches may provide a benefit against the classical “monovalent” ones (Vincent et al., 2007).

Overall, we might conclude that only slight clinical benefit of gene therapy in cardiovascular diseases has been observed so far on the level of multicenter, randomized, placebo-controlled clinical trials. This finding, however, is in direct contrast to promising and convincing results from preclinical studies and, thus, further stimulates searching for new and alternative approaches in experimental as well as clinical settings. New viruses have been introduced and new results have been collected from preclinical and clinical studies. Recent results from preclinical developments and clinical trials have been reviewed (Karvinen & Yla-Herttuala, 2010).

2.3 RNA interference in therapeutic modulation of angiogenesis

Since the first discoveries of RNA interference mechanism (RNAi) 13 years ago, much new information has shown up and a new age of gene therapy, in broad sense, has actually begun. This molecular phenomenon found its usage, besides in functional genetic studies, also in therapy based on silencing the expression of disease-causing genes (so called gene knock-down).

One of the main obstacles in achieving *in vivo* gene silencing using RNAi is the means of delivery. The siRNA molecules are, if present outside the cell, unstable and are subject to rapid degradation. Therefore, they are being chemically modified or incorporated into vectors like in the case of classical gene delivery. Within such a vector siRNA are encoded as so called short hairpin RNA (shRNA) that are expressed inside the target cell.

One of the break-through experiments was based on intravenous administration of chemically modified siRNA against endogenous apolipoprotein B (apoB) in mice leading to apoB mRNA silencing in liver and jejunum as well as decrease in plasma levels of apoB and overall cholesterol (Soutschek et al., 2004). These siRNA even reduced the expression of human apoB in transgenic mice, and the results further extended the potential of RNAi-based therapy of not only cardiovascular diseases.

Several studies have been performed to test the efficiency of RNAi in therapy/prevention of cardiovascular diseases. It is known that activation of NFkappaB pathway can be associated with development of cardiac hypertrophy and its transition to heart failure. Intramyocardial delivery of shRNA against NFkappaB in lentiviral vector has led to regression of cardiac hypertrophy in transgenic mice, suggesting the potential role of NFkappaB as a therapeutic target in prevention of hypertrophy/heart failure (Gupta et al., 2008). Similar results have been observed in a model of pressure-induced hypertrophy using systemic administration of siRNA against focal adhesion kinase (FAK), which acts as one of the hypertrophy mediators (Clemente et al., 2007). In the work of Jiang *et al.*, it was even proven that RNAi against HIF-1 α inhibits the formation of foam cells *in vitro*, indicating that induction of HIF-1 α by atherogenic factors may be a key step in coordinating the cellular processes leading to atherosclerotic lesions (Jiang et al., 2007). An alternative approach was tested in a study of Natarajan *et al.*, that, instead of using constitutively active HIF-1 α , reduced the activity of HIF-1 α prolyl hydroxylases by RNA interference (Natarajan et al., 2006). Small interfering RNAs (siRNA) managed not only to activate HIF-1 α signaling *in vitro*, but also rescued the endangered myocardium in a mouse model of overall ischemia. Thus, the authors proved that indirect activation of HIF-1 α signaling is not only possible, but also efficient. In another study the systemic administration of siRNA against β 1-adrenergic receptors has been proven to be specific for β 1 receptors expression without influencing β 2 receptors (Arnold et al., 2007). This therapy significantly reduced the hypertrophy and the blood pressure in spontaneously hypertensive rats. Moreover, preventive application of β 1-specific siRNA 3 days before myocardial infarction improved the heart function and reduced cardiomyocyte apoptosis.

Antiangiogenic therapy using RNAi has found its broad experimental application (Hadj-Slimane et al., 2007). Silencing of HIF-1 α by RNAi leads to transient stasis or regression of tumors *in vivo* and silencing in early stage tumors is more effective than in stable tumors (Li et al., 2005). In addition, siRNA against HIF-1 α inhibit the expression of this transcription factor in cancer cells and in endothelial progenitor cells even in hypoxic conditions, when it is naturally activated. At the same time, differentiation, proliferation and migration of these cells are also inhibited. Effectiveness of anticancer therapy using siRNA against VEGF

(naked or within a vector) has been proven to be effective *in vitro* and *in vivo* in a number of cancer models. Furthermore, a combination of oncolytic virus therapy and VEGF specific-siRNA has also been proven to be effective *in vitro* and *in vivo* (Yoo et al., 2007). Based on different physiological roles of VEGF family members, a combination therapy with siRNA vectors against VEGF-A and VEGF-C was used to suppress lymph node and metastasis in a mouse metastatic breast cancer model (Shibata et al., 2008). Another combined approach has been successfully used by Chen *et al.*, in therapy of laryngeal squamous cell carcinoma by concurrent application of plasmids encoding siRNA against VEGF, hTERT and Bcl-xl (Chen et al., 2008).

One of the most important papers in recent years was the study of Kleinman *et al.*, who have reported a sequence- and target-independent angiogenesis suppression by siRNA via toll-like receptor 3 (TLR3) (Kleinman et al., 2008). Here, the non-targeted siRNA suppressed dermal neovascularization in mice as effectively as VEGF siRNA. The effect was mediated through cell surface TLR3, its adaptor TRIF and induction of IFN γ and IL-12. These results suggest that all siRNA-based RNAi strategies activating TLR3 have to face non-specificity, which, however, does not have to be considered a disadvantage. Even though a specific silencing is desired, a different approach/vector should be used to avoid activating of TLR3 pathway. Bacterial vectors are characterized by cellular entry into the host cell, where they can mediate their therapeutic effects. Thus, bacteria can represent an ideal system for delivery of RNAi.

Apart from RNAi, another big area of small RNA-related research that is gaining much more attention these days is the microRNA research. More importantly, microRNA have been found to play a key role in regulation of angiogenesis, both in cancer and ischemic diseases, indicating that the development of clinically relevant therapies can be expected in a short time period (Fasanaro et al., 2010).

3. Delivery systems

Vectors for transfer of therapeutic sequences into target cells can be divided into three basic groups: viral, non-viral (naked DNA) and bacterial. Each of these groups has different research and therapeutic indications, and features specific pros and cons (Gardlik et al., 2005; Chailertvanitkul and Pouton, 2010). Currently, the most frequently used vectors in gene therapy clinical trials include: adenoviruses (400 clinical trials; 23,8%), retroviruses (344 clinical trials; 20,5%), naked/plasmid DNA (304 clinical trials; 17,7%), adeno-associated viruses (75 clinical trials; 4,5%) and others (John Wiley & Sons, 2010).

Adenoviruses are the most commonly used gene-delivery vectors due to the efficiency of their *in vivo* gene transfer their ability to deliver double-stranded DNA to the nucleus efficiently. In addition, their large genome allows for extensive modification and incorporation of therapeutic genes. Since 1993, about 300 protocols using an adenoviral vector have been performed, although they have yet to be proven effective in clinical trials.

By 2009 over 350 protocols had been approved for clinical trials of gene therapy using attenuated adenoviral vectors, 210 of which were open, but only 5 of which were Phase III trials (for details see the Clinical Trials Worldwide Database at <http://www.wiley.co.uk/genmed/clinical>).

The adenovirus-based vector has been continuously improved by modification of the adenoviral genome and capsid, and novel adenovirus-delivery systems have been recently proposed (Shirakawa, 2008). Since their first clinical trial 20 years ago, retroviral vectors

have now been used in more than 350 gene-therapy studies. Retroviral vectors are particularly suited for gene-correction of cells due to long-term and stable expression of the transferred transgene(s), and also because little effort is required for their cloning and production. Despite several unsuccessful attempts using retroviral therapeutics, a new generation of vectors with improved genome integration and safety characteristics are now available, making them a useful tool for several gene therapy applications (Maier et al., 2010). On contrary, adeno-associated vectors (AAV) are characterized by low frequency of random integration into the genome and moderate immune response. This makes AAV an attractive platform for vector design. Like in most other vector systems, the tropism of AAV vectors limits their utility for certain tissues especially upon systemic application. However, the tropism can be modified by targeted capsid modification and the use of different serotypes, thus making them a good cell-type specific delivery system (Michelfelder & Trepel, 2009).

Another group of delivery systems includes bacterial vectors (Gardlik et al., 2005). Owing to the specific ability of some bacterial strains to colonize hypoxic areas, bacteria found their primary application mainly in cancer therapy (Gardlik & Fruehauf, 2010). A number of experimental studies have been published to date and several clinical studies employing bacterial therapies are currently ongoing. Apart from live bacterial delivery systems, empty envelopes of Gram negative bacteria, so called bacterial ghosts are also being explored as a potential tool for gene delivery (Kudela et al., 2010). In the upcoming sections we specifically focus on modulation of angiogenesis-related events using bacterial vectors.

3.1 Bacteria – mediated gene therapy

The first attempts at using bacteria for therapeutic purposes were made more than 40 years ago. At this time, it was discovered that bacteria could predominantly replicate in solid tumors (Moese & Moese, 1964). However, the first indications of this phenomenon date back to 19th century. These findings remained largely unexplored until the turn of 20th century, when oncolytic bacteria capable of lysing host cells were first studied by various research groups (Theys et al., 2001; Yazawa et al., 2000).

Despite recent progress, only a few recent studies on bacterial tumor therapy have focused on antiangiogenic therapy. Although effects on the vasculature were observed in most of these studies, these changes seemed to be a consequence mainly of bacteria-mediated therapy. Bacteria-mediated antiangiogenesis tumor therapy, however, is a reasonable approach given that solid tumors are often characterized by increased vascularization. Herein we summarize latest research on cancer therapy using genetically modified bacteria with particular emphasis on the potential of blocking tumor angiogenesis.

3.2 Bacteria affecting angiogenesis

Pronounced angiogenesis is one of the hallmarks of solid tumors. Therefore, in order to search for more efficient anticancer drugs efforts are being made to block tumor angiogenesis. Despite notable successes achieved in studies using oncolytic bacteria for cancer treatment, bacteriolytic therapy in and of itself is often insufficient for complete eradication of experimental tumors (Agrawal et al., 2004). The idea of using combined therapy with bacteria and angiogenesis inhibition has therefore been suggested.

Below are reviewed four different approaches for using modified bacteria as anticancer therapeutics - bactofection, DNA vaccination, alternative gene therapy and bactoference - with a focus on angiogenesis suppression (Figure 1).

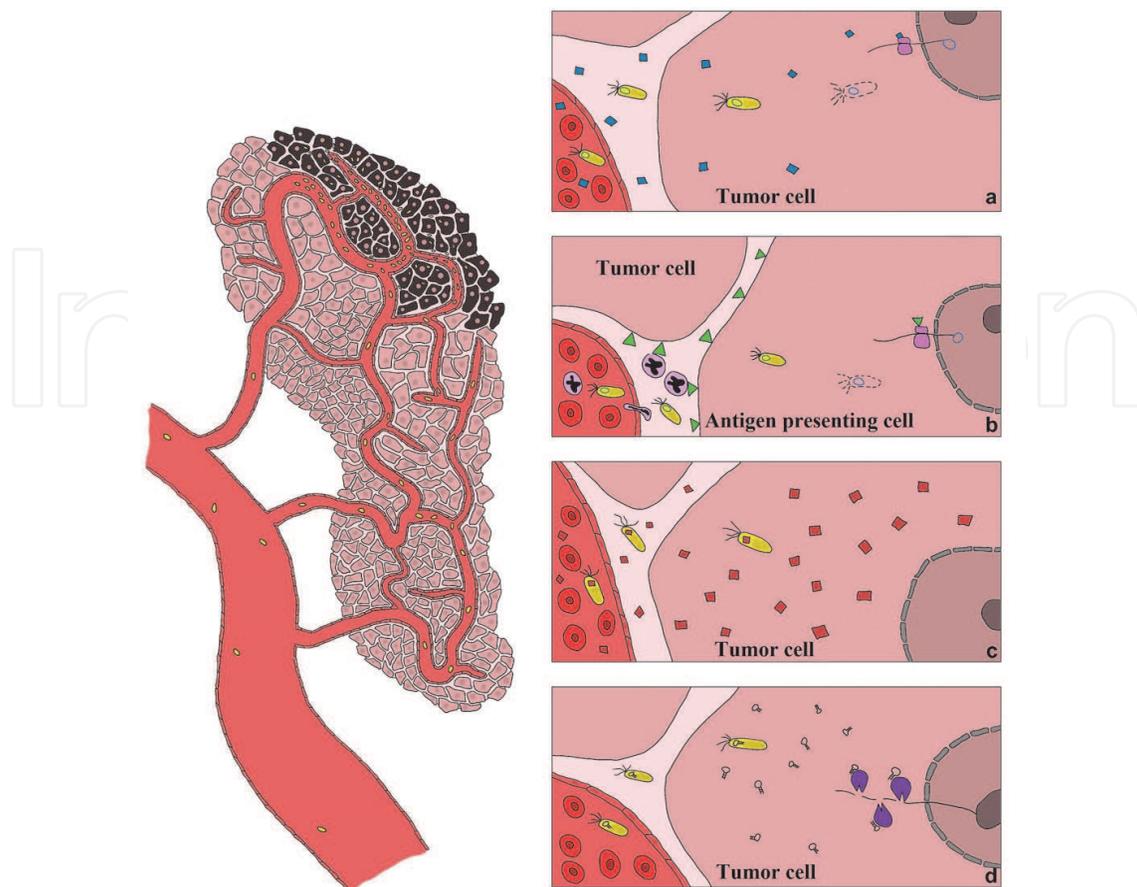


Fig. 1. Antitumor effect of bacteria colonizing tumor tissue. Auxotrophic bacteria specifically colonize tumors with necrotic and hypoxic areas (panel on the left). The anticancer effect of bacteria can be exerted in four different ways: (a) Bactofection – after escaping the vessel and entering the target cell, bacteria disrupt and release a plasmid vector encoding the therapeutic gene. The plasmid is transferred into the cell nucleus and the therapeutic protein is expressed by the host cell's expression system (blue square symbol). (b) DNA vaccination – bacteria deliver therapeutic plasmid into the host cell in a similar way as in bactofection. The host cell may in this case be an antigen presenting cell. The plasmid encodes a tumor cell-expressed antigen to help prime a T-cell response against the tumor antigen which is present on the surface of tumor cells leading to induction of humoral and cellular immune response against the tumor. (c) Bacterial protein delivery – bacteria, either in extracellular environment or inside tumor cells, express the therapeutic gene directly and serve as protein delivery vehicles (red square symbol). (d) Bacterial delivery of RNA interference – bacteria deliver plasmid encoding shRNAs or express the shRNAs to induce RNA interference against an oncogene or a tumor-expressed factor.

3.3 Bactofection

The use of bacteria as a vector for the delivery of therapeutic genes to target cells is known as bactofection, and several studies have used this approach to deliver genes encoding antiangiogenic molecules to tumor cells *in vivo*. A study was made using *Salmonella choleraesuis* strain bearing gene encoding endostatin on eukaryotic expression plasmid (Lee et al., 2004). The expression of endostatin was targeted exclusively to tumor tissues colonized by bacteria and significant inhibition of tumor growth (40-70%) with decreased intratumoral microvessel

density and reduced expression of vascular endothelial growth factor (VEGF) was observed. Bactofection using auxotrophic salmonellae as vectors has also been effectively employed for therapy of experimental tumor models using expression plasmids carrying various cytokine genes such as IL-12 and GM-CSF (Yuhua et al., 2001), IL-4 and IL-18 (Agorio et al., 2007) or other molecules playing role in angiogenesis, such as Flt3 ligand (Yoon et al., 2007). Further, a dual tumoricidal and antiangiogenic effect of *S. choleraesuis* carrying an expression plasmid containing the thrombospondin-1 gene under control of eukaryotic promoter was observed in a murine model of malignant melanoma (Lee et al., 2005). A potential limitation of bactofection for cancer therapy is the fact that the effector molecule will likely be expressed exclusively in cells infected by bacteria, leaving a potentially large population of tumor cells untreated. However, if the product of the transgene is secreted outside the target cell, it may still have a therapeutic effect on non-infected tumor cells.

3.4 DNA vaccination

It is known that bactofection of plasmids encoding a tumor-expressed antigens can lead to induction of humoral and cellular immune response in the host thereby providing protective defense against tumors (R. Xiang et al., 2000). This approach, termed DNA vaccination, has been successfully implemented for antiangiogenic therapy. Oral antiangiogenic bacterial vaccines directed against VEGFR-2 were proven to be efficacious in animal models of malignant melanoma, colorectal carcinoma and lung cancer (Niethammer et al., 2002) as well as non-cancer diseases like stromal keratitis (Kim et al., 2006) and atherosclerosis (Petrovan et al., 2007). Furthermore, salmonella-mediated vaccination against murine VEGFR-2 has been successfully combined with classical gene therapy for the treatment of malignant melanoma (Lu et al., 2008). Bacterial vaccines directed against the apoptosis inhibitor survivin (Xiang et al., 2005) and the TGF- β 1 co-receptor endoglin (Lee et al., 2006) also proved to be effective in inhibition of tumor angiogenesis. Taken together, these findings underscore the key role of angiogenesis in cancer as well as other diseases and, at the same time, highlight the complexity of this essential process.

3.5 Alternative gene therapy

Another means of using bacteria for gene therapy is the so-called alternative gene therapy (AGT) approach, which is also known as bacterial protein delivery (Palffy et al., 2006). It is based on transfer of bacterially expressed therapeutic proteins to the host organism using genetically modified (transformed) bacteria. As with bactofection, AGT is mostly used for treatment of tumors and employs primarily oncolytic and tumor-colonizing bacterial strains of *Clostridia*, *Bifidobacteria* or *Salmonellae* (Theys et al., 2001; Zheng et al., 2000). Li et al., successfully used *Bifidobacterium adolescentis* as a vector for the expression of endostatin within tumors (Li et al., 2003). These authors showed a strong inhibition of angiogenesis was able to significantly to inhibit local tumor growth. In another study, *Bifidobacterium longum* was shown to efficiently deliver the antiangiogenic protein endostatin to murine liver tumors, and induce antitumor activity (Fu et al., 2005). Furthermore, the antitumor effect was enhanced by co-administration of the same bacterial strain expressing tumor necrosis factor-related apoptosis inducing ligand (TRAIL) (Hu et al., 2009). These findings emphasize the need for combination therapy, in which multiple antitumor pathways are inhibited. *In situ* production of cytokines by bacteria represents a cost-effective and safe alternative mainly to systemic administration which can be associated with unwanted side-effects. However, in recent study employing

Listeria monocytogenes as a vehicle for tumor specific gene and protein delivery, bactofection proved to be more efficient than AGT (Stritzker et al., 2008).

Interestingly, previously unknown antiangiogenic effects have recently been discovered for a variety of molecules involved in the immune response and cellular apoptosis mainly due to extensive ongoing research in the field of cancer therapy. Bacteria in gene therapy, differences between bactofection and AGT, advantages and disadvantages as well as specific application of both approaches are discussed in detail in our review article (Palffy et al., 2006).

In spite of great success in a preclinical setting, the application of bacteria for human tumor therapy has not been particularly efficacious, although this approach was well tolerated in most studies (Cunningham & Nemunaitis, 2001; Nemunaitis et al., 2003; Toso et al., 2002). In light of above-mentioned findings, however, we would suggest that angiogenesis would be a meaningful target for further experimental and clinical studies of bacteria-mediated anticancer therapy, particularly if used in conjunction with oncolytic strains of bacteria.

3.6 Bactoference – bacteria-mediated RNA interference

A promising new approach for bacteria-mediated anticancer therapy is the combination of two distinct methodologies: bacteriotherapy and RNA interference. Bacteria that have engineered to produce and deliver short interfering RNA (siRNA) represent a novel tool for the efficient induction of RNA interference (RNAi) in host cells. This concept herein termed “bactoference” was first tested at the *in vitro* level in 2005 by Zhao *et al.*, (Zhao et al., 2005). These authors showed that siRNAs produced by invasive *E. coli* strain could induce RNAi against in mammalian cell cultures. The first *in vivo* demonstration of bactoference was provided by Xiang *et al.*, who showed that invasive *E. coli* capable of producing beta-catenin shRNA could induce RNAi in colonic epithelial cells and colonic tumors xenografted into nude mice (Xiang et al., 2006). To date, however, bactoference has not been used to directly target angiogenesis, although some of the targets explored (beta-catenin, STAT3 and bcl-2) are known to augment angiogenesis via their effects on the expression of various proangiogenic factors. Thus, antitumor effects of knocking down these targets may, in part, be related to suppression of blood vessel formation. However, a number of studies using “non-bacterial” application of siRNA against VEGF (Jia et al., 2007), HIF-1 α (Jiang et al., 2006) and other angiogenesis-related molecules indicates that inhibition of endothelial cell proliferation and new blood vessel formation is a viable target for RNAi-based treatment of cancer as well as other diseases.

4. Antioxidant gene therapy

Reactive oxygen and nitrogen species may remodel the extracellular matrix and blood vessels, cause endothelial dysfunction, induce apoptosis, exacerbate inflammatory reaction, regulate cell proliferation and key signal transduction pathways, and inhibit histone deacetylase activity involved in hypertension (Cohen and Tong, 2010; Fostermann, 2010).

Overproduction of reactive oxygen species plays an important role in a number of cardiovascular pathologies, including hypertension, atherosclerosis, myocardial infarction, ischemia/reperfusion injury, and restenosis after angioplasty.

In this section we focus on gene therapy research using experimental models of cardiovascular diseases and hypertension.

4.1 NO levels and the superoxide dismutases

There are at least two major strategies of modulation of nitric oxide (NO) levels in hypertension and cardiovascular diseases: (i) modulation of NO levels by NO synthase (NOS) stimulation, increase in NO bioavailability, administration of NO donors or precursors, and NOS gene incorporation; (ii) scavenging of superoxide and suppression of oxidative stress by activation of antioxidant gene expression or by suppression of selected genes via knock-out or RNA silencing (Dovinova, Gardlik et al., 2009).

NO reacts with superoxide at a rate three times faster than the dismutation of superoxide by superoxide dismutase (SOD). Because of the efficiency of the reaction, the local concentration of SOD is a key determinant of bioactivity (the biological half-life) of NO. Individual SOD isoform in different cell compartments (cytosol, mitochondria, extracellular space) protects against superoxide-mediated cytotoxicity and functioning as a signaling molecule (Mendez, 2005). SOD enzymes therefore play an important role in cardiovascular tissue by protecting NO against oxidative inactivation by superoxide and they are important in vasodilatation and in the protection of NO bioactivity in blood vessel walls (Gongora & Harrison, 2008).

Gene transduction of individual SOD genes (Chu et al., 2003; Zimmerman et al., 2004; Dovinova et al., 2008; Kamezaki et al., 2008) or combination of both SOD and NOS transgenes (Kung et al., 2008,) has positive influence on experimental hypertension. This protective effect is the end results of an increase in tissue level of NO and the decreases in oxidative stress (Chan et al., 2006) and peroxynitrite production (a cytotoxic molecule generated by reaction between superoxide and NO) (Kishi et al., 2004). The SOD genes are the first natural antioxidant defenses of an aerobic cellular system. The following subsection deals with gene therapies by individual SOD isoforms (alone or combined with other genes) and about their influence on the mechanisms of the cardiovascular disease.

4.2 Antioxidant gene therapy of the cardiovascular system with the SOD 1 gene

The connection between the influence of angiotensin II (Ang II) and the increased superoxide production by activation of NADPH oxidase has been observed in animal studies (Paravicini and Touyz, 2004; Zimmerman et al., 2004, Chan et al., 2005) and patients with cardiovascular complications (Yokoyama et al., 2000). Peripheral angiotensin II exerts potent effects on blood pressure and cardiovascular function through its actions on neurons located in specialized brain regions called the circumventricular organs, in particular the subfornical organ (SFO). Zimmerman et al., (2004) reported that following peripheral infusion of Ang II at initially subpressor doses, there was a gradually developing hypertension paralleled by an increase in superoxide production in the SFO. Using the adenoviral vectors for *in vivo* gene delivery, they found that both the superoxide production and the hypertension were prevented by an overexpression of CuZnSOD (also known as SOD1) in the SFO, whereas ECSOD (also known as SOD3) was ineffective. Ang II receptors are also highly expressed in brain stem cardiovascular centers, including the rostral ventrolateral medulla (RVLM) and nucleus tractus solitarii (NTS). Activation of Ang II receptors in the RVLM (Gao et al., 2004; Kishi et al., 2004; Chan et al., 2005) or NTS (Hirooka, 2008) promotes neurogenic hypertension. This induced hypertension can also be prevented by protection against oxidative stress via treatment with *in situ* gene transduction of SOD1 (Chan et al., 2006). Using a gene therapy based on SOD1 gene transduction by a bacterial gene delivery system it was found that such application decreases the blood pressure, regulates SOD and NOS activities, and decreases oxidative stress response (Dovinova et al., 2008).

The overexpression of cytoplasm-targeted superoxide dismutase via an adenoviral vector (AdSOD1) efficiently scavenges angiotensin-II-induced increases in intracellular superoxide, markedly attenuates the increase in $[Ca^{2+}]_i$ and suggests a potential intracellular signaling mechanism involved in Ang II-mediated oxidant regulation of central neural control of blood pressure (Zimmerman et al., 2005). Genetically-altered mice and rats have been generated which overexpress SOD1. Compared with nontransgenic controls, mRNA for CuZn-SOD1 and SOD activity are increased several-fold in the vascular and non-vascular tissues, decreased vascular superoxide levels in atherosclerosis and diabetes to improve endothelial function and to protect in a model of fluid percussion injury that produces impairment of autoregulation (Faraci & Didion, 2004). Downregulation of antioxidant gene expression and enzyme activity may underlie the augmented levels of superoxide and hydrogen peroxide in the RVLM, leading to oxidative stress and hypertension in the spontaneously hypertensive rats (SHR). A causative relationship between biochemical correlates of oxidative stress and neurological hypertension was established after a gene transfer by microinjection of adenovirus encoding SOD1 and MnSOD (also known as SOD2) or catalase (CAT) into brain (RVLM), which promoted a long-term reduction of blood pressure in SHR (Chan et al., 2006).

4.3 Antioxidant gene therapy of the mitochondrial dysfunction with the SOD 2 gene

Mitochondrial dysfunction is a prominent feature of most cardiovascular diseases and hypertension and is associated with the deterioration of mitochondrial energy production in several organs such as the liver, the heart and the brain (Chan et al., 2009a, b). In the myocardium of the SHR, the evidence that points to the occurrence of mitochondrial dysfunction includes the decrease of cytochrome oxidase activity, ATP production, and inorganic phosphate translocator activity (de Cavanagh et al., 2006). Attenuated intracellular ATP content, results in a long-term maintenance of elevated blood pressure by increasing in sympathetic outflow, whereas augmented ROS production following mitochondrial dysfunction lowers the capacity of the NO-dependent vascular relaxation. The stationary elevated blood pressure in chronic arterial hypertension should be regarded as a compensatory response to decreased mitochondrial ATP synthesis (Postnov et al., 2007). Depletion of p22^{phox} subunit of NADPH oxidase with small interfering RNA inhibited Ang II-mediated mitochondrial ROS production. Ang II depletes mitochondrial glutathione, increases state 4 and decreased state 3 respirations, and diminishes the mitochondrial respiratory control ratio. It also prevents the Ang II-induced decrease in endothelial NO and mitochondrial membrane potential. Therefore, Ang II induces mitochondrial dysfunction via a protein kinase C-dependent pathway by activating the endothelial cell NADPH oxidase and formation of peroxynitrite. Furthermore, mitochondrial dysfunction in response to Ang II modulates endothelial NO and superoxide generation, which in turn has ramifications for the development of an endothelial dysfunction (Doughan et al., 2008). In the brain stem cardiovascular neurons in the RVLM, mitochondrial superoxide mediates the rebound hypertension induced by the eNOS transgene in SHR (Kung et al., 2008).

Mice lacking iNOS^(-/-) exhibits extensive cytoplasmic swelling and degeneration of mitochondria, decrease in the resting indices of cardiac function as well as an impairment in the positive inotropic actions of isoproterenol following treatment with adriamycin compared to nTg mice. Cardiac troponin, creatine phosphokinase, and lactate dehydrogenase levels are significantly increased after adriamycin treatment in iNOS^(-/-) mice. However, when iNOS^(-/-) mice are crossed with SOD2 overexpressing animals,

mitochondrial injury is ameliorated to the level of the wild type (Cole, 2006). Mice completely deficient in Mn-SOD die within a few weeks after birth and exhibit a variety of phenotypes (depending on the genetic background) including neurodegeneration, cardiac abnormalities, and extensive mitochondrial damage (Faraci & Didion, 2004)

In Ang II-induced neurogenic hypertension or in SHR, mitochondrial electron transport capacity in the RVLM is reduced, accompanied by an increase in generation of mitochondrial superoxide and hydrogen peroxide (Chan et al., 2009a). Overexpression of SOD2 ameliorates mitochondrial oxidative stress and the induced antihypertension.

4.4 Antioxidant gene therapy of the cardiovascular system with the SOD 3 gene and RNA silencing

The degree of hypertrophy, ventricular dilatation, and myocardial fibrosis was markedly increased in mice lacking extracellular SOD (SOD3) and gene transfer of cDNA encoding membrane-bound SOD3 reduces vascular superoxide levels as well as arterial pressure in SHR (Chu et al., 2009). A deficiency in SOD3 does not alter the baseline blood pressure but increases the arterial pressure in models of hypertension that are greater in SOD3-deficient mice than in controls. Studies using overexpression strategies have revealed protective effects of SOD3 on blood vessels. Gene transfer of SOD3 reduced vascular superoxide levels during atherosclerosis in SHR. Effects of overexpression of SOD3 using this approach on endothelial function have varied (Faraci & Didion, 2004).

Depletion of NADPH oxidase subunits with small interfering RNAs - another approach in gene therapy - inhibits ROS production and, thus, has the potential to reduce blood pressure. Silencing of p22phox component of NADPH oxidase *in vivo* by RNAi resulted in reduced ROS and mean atrial pressure in angiotensin II-induced hypertension in rats (Modlinger et al., 2006). Knock-down of several molecular targets by silencing of matrix metalloproteinase-7 (Wang et al., 2009), angiotensin-converting enzyme (He et al., 2009) or β 1-adrenergic receptor (Arnold et al., 2007) using systemic RNAi results in attenuation of hypertension and stops the development of cardiac hypertrophy in SHR.

5. Conclusion

Modulation of angiogenesis is of great importance from experimental, pathophysiological and especially clinical point of view. However, further research is needed to fully delineate the most effective way to target angiogenesis for treatment of cancer and cardiovascular diseases. The application of new approaches using bacteria for the transfer of therapeutic genes or the production of therapeutic protein or small RNAs has the potential to significantly advance cancer gene therapy. Recent studies indicate that treatments targeting a single molecule/pathway, even if it has pleiotropic effects, are unlikely to be completely effective; however, if the natural anti-tumor activity inherent to some anaerobic bacteria strains can be successfully combined with their ability to deliver agents targeting tumor angiogenesis, apoptosis or the immune system, this will represent a significant step toward reaching this important goal.

This chapter summarizes the preclinical and clinical studies and the use of animal models to provide evidence for potential benefit from angiogenic and antioxidant gene therapy. Gene therapy with the use of antioxidant genes may offer a promising approach for treatment of cancer and cardiovascular diseases in patients not suitable for conventional therapies.

However, one must realize that in animal models before the experimental data can be translated to clinical trials, shortcomings of antioxidant gene therapy, for example the

limited duration of transgene expression, low efficacy of the transgene expression in the target organs or tissues, and potential immune responses to the transgenes, must be resolved. Specifically related to the use of antioxidant enzymes in gene therapy is the fact that reactive oxygen species not only mediate pathological events but are also required for normal cell signaling. This double-edged sword nature of the reactive oxygen species in regulation of cellular phenotypes under physiological and pathological conditions poses challenges to the application of gene therapy for early treatment of disease condition.

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7. References

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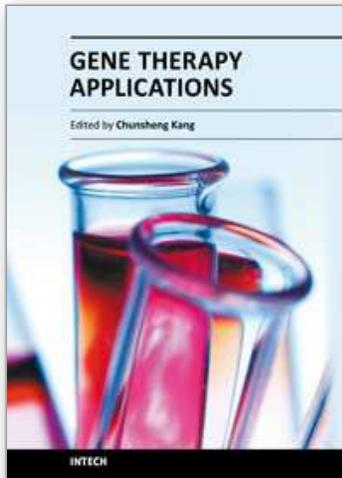
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The aim of our book is to provide a detailed discussion of gene therapy application in human diseases. The book brings together major approaches: (1) Gene therapy in blood and vascular system, (2) Gene therapy in orthopedics, (3) Gene therapy in genitourinary system, (4) Gene therapy in other diseases. This source will make clinicians and researchers comfortable with the potential and problems of gene therapy application.

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