Chapter 27

Clinical and Translational Challenges in Gene Therapy of Cardiovascular Diseases

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1. Introduction

Cardiovascular (CV) disease is the most prevalent life-threatening clinical problem and is a major cause of disability and economic burden worldwide [1]. Despite extensive pharmacotherapies, there remain many vascular conditions for which pharmacological interventions are either non-existent or largely ineffective. CV gene therapy offers the benefit of sustained and/or controlled expression of desired proteins in cell types, which makes it more beneficial in providing durable clinical benefits [2]. The therapeutic gene works by either over-expressing therapeutically beneficial proteins, replacing a deficient gene or its expression proteins, or silencing a particular gene whose expression is not beneficial in the clinical scenario [3]. In addition, success of gene therapy also depends on the choice of the vector and the delivery approach. Blood vessels are among the most feasible targets for gene therapy because of ease of access using a catheter or by systemic delivery. The new genetic material should enter the cells in the vasculature overcoming the anatomical, cellular and physiological barriers and induce the expression of the transfected gene in the target tissue. The target cells in the arteries are endothelial cells (EC), smooth muscle cells (SMC) and fibroblasts, which constitute the intimal, medial and adventitial layers, respectively [4]. In the case of atherosclerotic lesions, macrophages also become a target cell. For the treatment of cardiovascular diseases, gene therapy strategies have been designed to enhance re-endothelialization and EC function to reduce thrombosis, inhibit SMC proliferation and migration to prevent neointimal hyperplasia, and to improve therapeutic neo-vascularization to counteract ischemia.

Viral and non-viral vector systems have been evaluated for gene transfer to the vasculature. Lipoplexes, polyplexes and lipopolyplexes as well as naked DNA have been used as non-viral vectors for gene delivery to vascular tissues. Retroviruses, lentiviruses, adenoviruses

and adeno-associated viruses have been tested as viral vectors. Both systems have their own advantages and disadvantages that determine its use for a particular subset of CV diseases. Another challenge is the development of delivery approaches that are clinically viable and are capable of achieving consistent therapy for diseased arterial tissues. The efficiency of localization, restriction of systemic distribution and adequacy of permeation into the target tissue are required for the optimal delivery of the vector. It is also dependent on the requirements of a given patho-physiological situation. Systemic, intravascular and perivascular approaches are used for gene delivery to the vasculature.

In this chapter, our goal is to summarize the current understanding of gene therapy strategies used to treat CV diseases, specifically the therapies targeting thrombosis, atherogenesis, SMC proliferation and migration, modification of extracellular matrix (ECM) and regeneration of the endothelial cell layer. We will discuss various vectors and delivery approaches used in the CV gene therapy and describe, in detail, the challenges associated with each approach.

2. Vectors in vascular gene therapy

The ideal vector for clinical application would target the specific cell, offer the capacity to transfer large DNA sequences, result in therapeutic levels of transgene expression that are not attenuated by the host immune response, express transgene for a duration required to alleviate the clinical problem, pose no risk of toxicity either acutely (as a result of immunogenicity or unregulated transgene expression) or in the long-term (such as oncogenesis), and be cost-effective and easy to produce in therapeutically applicable quantity [5]. Currently, no available vector fulfils all these criteria; therefore, a perfect vector for vascular gene therapy does not exist. Nonetheless, viral and non-viral vector systems have been evaluated for gene transfer to the vasculature.

2.1. Viral vectors

Retroviruses, adenoviruses (Ad) and adeno-associated viruses (AAV) are used as viral vectors in vascular gene transfer. Recombinant retroviruses are RNA viruses that are capable of integrating transgene into the target genome. Disadvantages of this vector include instability, the requirement of cell division for gene transfer and the inability to attain high titers. Since the majority of vascular cells are not undergoing mitosis at the time of exposure to the viral vector, the efficiency of gene delivery to vascular cells by such vectors may be as low as 1% to 2% [6]. Attempts have been made to increase the transduction efficiency in endothelial cell using multiple viral exposures [7] or increasing viral titers by ultracentrifugation [8]. Murine leukemia retroviral vectors (MuLV) pseudotyped with the vesicular stomatitis virus G glycoprotein (VSV-G) have the capacity to transfect human ECs and SMCs *in vitro* with significant improvement in stability and transduction efficiency [9]. Unlike other retroviruses, lentiviruses are able to transduce non-dividing cells, which is an attractive characteristic for CV gene therapy. These vec-

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tors demonstrate significantly broadened tropism and high stability and have been used to demonstrate efficient transgene delivery *in vitro* into SMCs and ECs from human saphenous vein [10], human coronary artery SMCs and ECs [11], and cardiomyocytes [12].

Ad vectors are the most commonly used viral vectors in the CV system. They transfect non-dividing cells efficiently [Figure 1], but sustained gene expression is limited to approximately 2 weeks because the gene is kept episomal [2]. The administration of the Ad vectors is almost invariably associated with the development of systemic neutralizing antibodies directed against the vector [13]. Therefore, lowering the immunogenicity of the Ad virus is desirable and can be achieved by deleting genes that encode viral proteins [14]. Another method of reducing the inflammatory reaction to gene transfer by Ad vectors is to preserve the E3 region, which is supposed to modulate the host immune response *in vivo* [15]. When systemically administered, Ad5 poorly transduced ECs but could effectively transduce medial SMCs during endothelial denudation [5]. Efficient myocardial transduction was observed following local delivery of Ad5 vectors in porcine heart, where almost 80% of cardiomyocytes were transduced [16].

AAV vectors have emerged as versatile vehicles for gene delivery due to their efficient infection of dividing and non-dividing cells in the presence of helper virus, sustained maintenance of viral genome leading to long-term expression of the transgene, and a strong clinical safety profile [17]. AAV is non-pathogenic since it cannot replicate without the assistance of a helper virus. Recombinant AAV (rAAV) vectors have almost the entire viral genome removed, thereby yielding a delivery vehicle with enhanced safety and reduced immunogenicity [18]. The AAV *Rep* and *Cap* genes, which are required for viral replication and packaging, are supplied by a helper plasmid during the production process. Wild type AAV preferentially integrates to a specific locus of human chromosome 19. The rAAV has mechanisms for sustained episomal maintenance or semi-randomly integrates at a low rate [19]. Problems with AAV vectors include limited tissue tropism for serotypes that bind heparan sulphate, challenges with preexisting immunity due to prior exposure, and also substantially delayed onset of transgene expression compared to other vectors.

2.2. Non-viral vectors

Even though the transfection efficiency of non-viral vectors are lower than that of their viral counterparts, they are associated with many advantages such as low immunogenic response, the capacity to carry large inserts of DNA (52Kb), the possibility of selective modification using ligand and large scale manufacture [20]. Ideal non-viral vectors should be degradable into low molecular weight components in response to biological stimuli for lower toxicity and effective systemic clearance. They should also be efficient in overcoming extracellular and intracellular barriers and tissue/cell-targeted for specific accumulations [21]. In this group of vectors, naked DNA, cationic liposomes and cationic polymers have been used for vascular gene transfer.

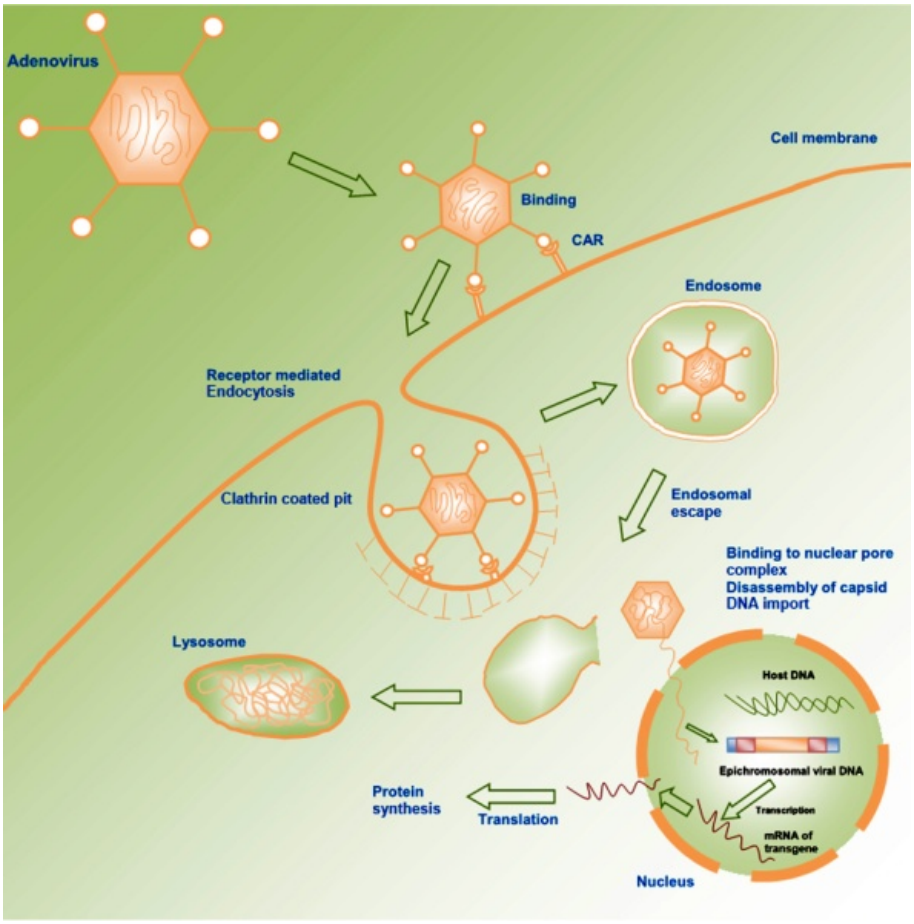

Figure 1. Transduction using adenoviral vectors. Recombinant adenovirus enters cells via CAR-mediated binding allowing internalization via receptor-mediated endocytosis through clathrin-coated vesicles. Inside the cytoplasm, the endocytosed adenoviral vector escapes from the endosomes, disassembles the capsid and the viral DNA enter into the nucleus through the nuclear envelope pore complex. The viral DNA is not incorporated into the host cell genome, but rather assumes an epichromosomal location, where it can still use the transcriptional and translational machinery of the host cell to synthesize recombinant protein. [CAR; Coxsackievirus and adenovirus receptor]

Gene transfer with naked DNA is attractive because of its simplicity and lack of toxicity [22]. However, the efficiency of gene transfer with naked DNA is low due to its negative charge conferred by the phosphate groups, making cellular uptake difficult by the negatively charged cell surface, rapid degradation by nucleases in the serum and clearance by the mononuclear phagocyte system in the systemic circulation. However, site-specific arterial gene transfer of vascular endothelial growth factor (VEGF)-165 could yield efficient gene transfection resulting in accelerated re-endothelialization, inhibition of neointimal

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thickening, reduced thrombogenicity, and restoration of endothelium-dependent vasomotor reactivity after injury due to balloon angioplasty in a rabbit model [23]. Physical approaches have been explored for plasmid gene transfer into vascular cells *in vitro* and *in vivo*. Ultrasound exposure can induce transient pore formation in the cell membrane, thereby increasing the plasmid DNA uptake. Indeed, microbubble-enhanced ultrasound can achieve transgene expression levels *in vitro* at approximately 300-fold than that of naked plasmid DNA alone in porcine VSMCs [24]. The non-invasive nature of this technique makes it more feasible for clinical use. Local administration of plasmid DNA, coupled with application of brief electric pulses to cells or tissues to increase cellular permeability-- also called electroporation--yields high levels of transgene expression in the arteries [25]. However this technique is limited by its invasive nature and tissue damage associated with high voltages applied [26].

To increase the efficiency of gene transfer by naked DNA, they are complexed with cationic lipids (liposomes or lipoplexes) or polymers (polyplexes). The resulting net positive charge of the cationic lipid/polymer DNA complexes facilitates fusion with the negatively charged cell membrane and also reduces susceptibility to circulating nucleases. Transfection efficiency of cationic lipoplexes varies dramatically depending on the structure of the cationic lipids (the overall geometric shape, the number of charged groups per molecules, the nature of lipid anchors, and linker bonds), the charge ratio used to form DNA–lipid complexes, and the properties of the co-lipid [22]. Although transfection efficiencies of liposomes are generally seen lower in vascular cells [22], the LID vector system, consisting of a liposome (L), an integrin targeting peptide (I), and plasmid DNA (D), transfects primary porcine vascular SMCs and porcine aortic ECs with efficiency levels of 40% and 35%, respectively, under *in vitro* conditions [27]. Some of the cationic lipids have been found to negatively affect cell function. Cationic lipid-mediated transfection of bovine aortic ECs inhibits their attachment [28].

The DNA packaging efficiency and *in vivo* stability are higher for cationic polymers compared to cationic lipids. Furthermore, these complexes can be surface-modified with antibodies or other targeting ligands to deliver nucleic acids to specific cells [29]. Several cationic polymers have been evaluated for their ability to form complexes with DNA, the most significant being poly-lysine (PLL) and polyethylene-imine (PEI) [30]. PEI affects EC function [31]; however, when conjugated with fractured polyamidoamine (PAMAM) dendrimers, less toxic effects were observed on vascular cells in addition to the enhanced transfection efficiencies [32]. Brito *et al*. [33] developed lipo-polyplex nanovector systems that can transfect EC and SMCs with reasonably high efficiency. They used a combination of a cationic biodegradable polymer, poly(beta-amino ester) (PBAE), and a cationic phospholipid, 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and obtained 20% and 33% transfection efficiencies *in vitro* in SMC and ECs, respectively. Molecular tuning of non-viral vectors via stimuli responsive degradation is another novel approach that can be adopted in vascular gene transfer [21]. Schematic representation of non-viral gene delivery is given in Figure 2.

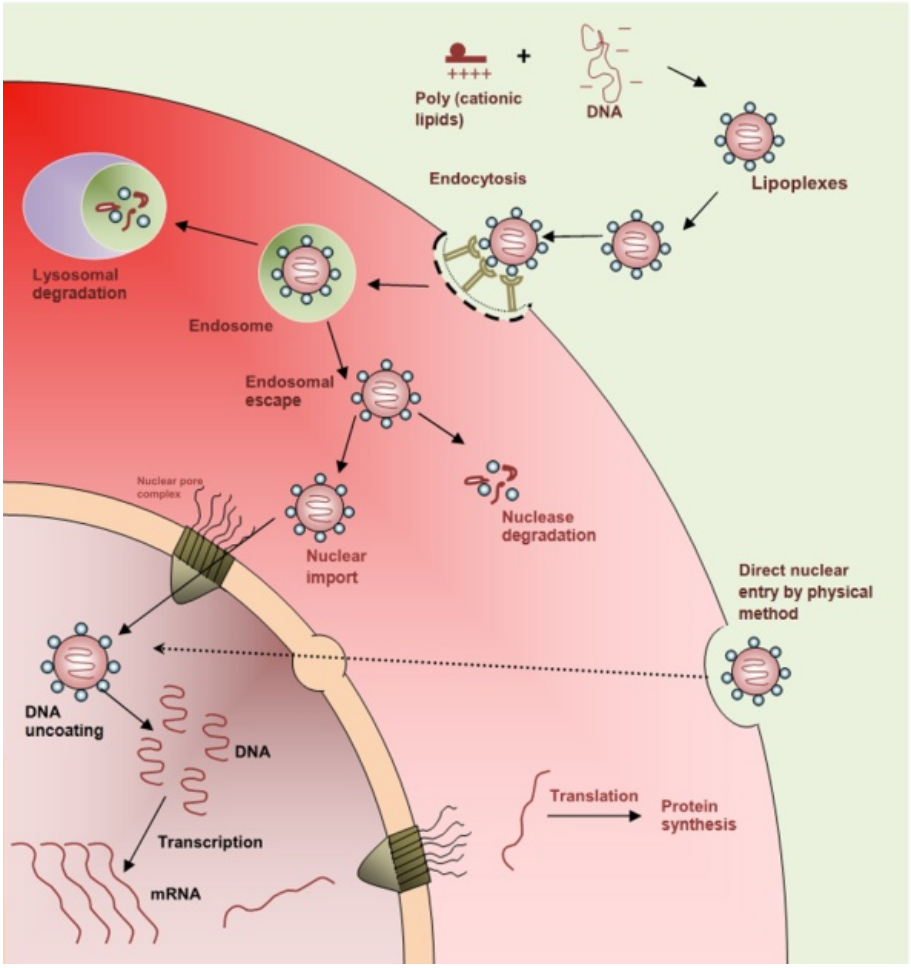

Figure 2. Non-viral gene delivery using lipoplexes: DNA is complexed with cationic liposomes and is internalized through receptor mediated endocytosis. After their internalization large amounts of complexes are degraded in the endolysosomal compartments. Only a small fraction enters into the nucleus and elicits desired gene expression.

2.3. Stem cells

One of the recent approaches is to use stem cells as gene delivery vehicles. Stem cell-based gene therapy approaches are currently being employed in recent studies as an alternative strategy to promote myocardial angiogenesis and regeneration. Indeed, the injection of genetically modified bone marrow-derived mesenchymal stem cells to express angiopoietin-1 improved arteriogenesis and increased collateral blood flow in porcine model of chronic myocardial ischemia [34]. Nanofiber-expanded hematopoietic stem cells over-expressing

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VEGF and platelet-derived growth factor (PDGF) had a favorable impact on the improvement of rat myocardial function accompanied by upregulation of tissue connexin 43 and pro-angiogenic molecules after infarction [35].

3. Major targets in vascular gene therapy

3.1. Promotion of re-endothelialization

EC loss because of vascular injury is a major contributing factor to the local activation of patho-physiological events leading to the development of neo-intimal hyperplasia [36]. Previous reports have shown that transplantation of autologous endothelial progenitor cells (EPCs) onto balloon-injured carotid artery leads to rapid re-endothelialization of the denuded vessels [37]. EPCs can be genetically manipulated *ex vivo*, expanded, and reintroduced *in vivo*, where at least a proportion will contribute to a long-lasting pool that can provide therapeutically relevant levels of transgene expression. Chemokine receptor, CXCR4, is a key molecule in regulating EPC homing [38]. Chen *et al*. [38] reported that CXCR4 gene transfer to EPCs contributes to their enhanced *in vivo* re-endothelialization capacity. In another study, Ohno and colleagues over-expressed C-type natriuretic peptide by gene transfer in rabbit jugular vein grafts and observed accelerated re-endothelialization [39]. EPCs over-expressing endothelial nitric oxide synthase (eNOS) further enhance the vasculo-protective properties of these cells [40]. Local intravascular and extra-vascular expression of VEGF, using plasmid DNA, accelerated re-endothelialization and decreased intimal thickening after arterial injury in rabbit models [23, 41].

3.2. Promotion of endothelial cell function

Antithrombotic and anticoagulation therapy generally involves the systemic administration of agents that target a small region of the vasculature. Localized and controlled delivery of specific genes could allow sustained antithrombotic or anticoagulant treatment when prolonged systemic administration is undesirable. Antithrombotic gene therapy strategies could include inhibition of coagulation factors, over-expression of anticoagulant factors, or modulation of EC biology to make thrombus formation or propagation unfavorable [42]. Ad gene transfer of thrombomodulin decreased arterial thrombosis to 28% compared to 86% in control rabbit model [43]. Hemagglutinating virus of Japan (HVJ)-liposome-mediated gene transfer of tissue factor pathway inhibitor (TFPI), a primary inhibitor of TF-induced coagulation, significantly reduced/inhibited thrombosis after angioplasty in atherosclerotic arteries without any significant adverse effects [44]. Ad gene transfer of many mediators, including hirudin to inhibit thrombin [45], tissue plasminogen activator (tPA) to enhance fibrinolysis [43], cyclo-oxygenase to augment prostacyclin synthesis [46], prevents arterial thrombosis and promotes local thromboresistance. Vascular gene delivery of anticoagulants by local infusion of retrovirally-transduced EPCs with tPA and hirudin genes has also been attempted [37].

3.3. Inhibition of atherogenesis

The extensive cross-talk between the immune system and vasculature leading to the infiltration of immune cells into the vascular wall is a major step in atherogenesis. In this process, reactive oxygen species play a crucial role, by inducing the oxidation of low-density lipoprotein (LDL) and the formation of foam cells, and by activating a number of redox-sensitive transcriptional factors, such as nuclear factor kappa B (NFκB), Nuclear factor E2-related factor-2 (Nrf2) [47], or activating protein 1 (AP1) that regulate the expression of multiple pro-and anti-inflammatory genes involved in atherogenesis [48]. Delivery of genes encoding antioxidant defense enzymes, like extracellular superoxide dismutase [49, 50], catalase [51], glutathione peroxidase [51] or heme oxygenase-1 [52], suppresses atherogenesis in animal models.

Apolipoprotein E (ApoE), a blood circulating protein with pleiotropic atheroprotective properties, has emerged as a strong candidate for treating hypercholesterolemia and CV disease. The gene transfer of ApoE Ad vectors produced substantial amounts of plasma ApoE following intravenous injection into ApoE-/- mice, which lowered plasma cholesterol, and after 1 month, slowed aortic atherogenesis [53]. Hepatic expression of human ApoE3 using a second-generation recombinant Ad vector directly induced regression of pre-existing atherosclerotic lesions without reducing plasma cholesterol or altering lipoprotein distribution [54]. High concentrations of atherogenic apolipoprotein (apo) B100 could also be lowered by hepatic gene transfer with the catalytic subunit of apoB mRNA editing enzyme [55].

3.4. Inhibition of SMC proliferation and migration

SMC migration and proliferation as well as deposition and turnover of ECM proteins contribute to the process of Intimal hyperplasia. Several different approaches were introduced to inhibit SMC proliferation during restenosis. Most of the approaches targeted inhibition of cell cycle, where cell cycle inhibitor genes are over-expressed. Non-phosphorylated retinoblastoma gene (Rb) [56]; p21 [57, 58]; p27-p16 fusion gene [59, 60] ; cyclin-dependent kinase inhibitor p57Kip2 [61]; and the growth-arrest homeobox gene gax [62] are few of the genes over-expressed to inhibit cell proliferation and neo-intimal formation. Genes that have a beneficial influence on various aspects of vessel wall physiology also inhibit SMC proliferation. Nitric oxide generation by endothelial nitric oxide synthase inhibits SMC proliferation *in vitro* and modulates vascular tone locally *in vivo* [63].

Another approach was to inhibit growth factor signaling by the introduction of nucleic acid constructs that interfere with mRNA stability, such as antisense oligonucleotides, hammer head ribozymes and siRNA [64]. Gene transfer of a truncated form of fibroblast growth factor (FGF) receptor using Ad vector suppressed SMC proliferation *in vitro* [65]. Hammerhead ribozymes directed against PDGF-A chain [66] and transforming growth factor-β [67] inhibited SMC proliferation and neointima formation in rat carotid artery after balloon injury.

The regulation of a target gene can influence the level of transcription, either by decoy oligonucleotides, which are either short double-stranded oligonucleotides or dumb-bell shaped circular oligonucleotides that represent transcription factor binding sites, and thus compete

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for binding of a specific transcription factor that is relevant for the respective gene [64]. Administration of AP-1 decoy ODNs *in vivo* using HVJ-liposome method virtually abolished neointimal formation after balloon injury to the rat carotid artery [68]. Transfection of vein grafts with a decoy antisense oligonucleotide to block transcription factor E2F imparted long-term resistance to neointimal hyperplasia and atherosclerosis in rabbits on a cholesterol diet [69]. Another approach was to drive SMC into apoptosis during the process of proliferation and migration. Transduction of rabbit iliac arteries with recombinant Ad vectors for Fas ligand (L) reduced neointima formation, which occurred through the killing of Fas expressing neighboring SMC by FasL-transduced cells [70].

The regulation of SMC migration is mediated partly through the action of matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs) [71]. AAV-mediated TIMP1 transduction in SMCs of injured rat carotid arteries significantly reduced the ratio of intima to media (52.4%) after two months of treatment [72]. Overexpression of TIMP-2 [73], TIMP-3 [74] and TIMP-4 [75] has also been demonstrated to inhibit SMC migration and neo-intimal proliferation in human vein grafts and porcine vascular injury models. Gurjar *et al.* [76] demonstrated that eNOS gene transfer inhibits SMC migration and MMP-2 and MMP-9 activities in SMCs *in vitro*. A combination approach of TIMP-1 and plasminogen activator system inhibited vein graft thickening in hypercholesterolemic mice, when plasmids encoding TIMP-1-ATF (amino terminal fragment of urokinase) were incorporated to the vein graft by intravascular electroporation [77].

3.5. Enhancement of therapeutic angiogenesis

Ischemic diseases, including acute myocardial infarction and chronic cardiac ischemia, are characterized by an impaired supply of blood resulting from narrowed or blocked arteries that starve tissues of needed nutrients and oxygen [78]. Delivery of genes encoding angiogenic factors or the whole protein has been shown to induce angiogenesis in numerous animal models with the expression of a functioning product [79]. The successful application of recombinant protein and gene transfer for the treatment of myocardial ischemia was reported by Losordo and colleagues [80] by direct intra-myocardial gene transfer of naked plasmid DNA encoding VEGF-165 in porcine model. These results were confirmed in phase 1 assessment of direct intra-myocardial administration of Ad vector expressing VEGF-121 cDNA in patients with severe coronary artery disease [81]. Ad-mediated FGF-4 gene transfer improved cardiac contractile function and regional blood flow in the ischemic region during stress in pig model [82]. Placebo-controlled trials in humans with chronic stable angina indicate that Ad5FGF-4 increased treadmill exercise duration and improved stress-related ischemia [82]. In another study, following coronary artery occlusion, rabbits treated with Ad vector containing acidic FGF showed a 50% reduction in the risk region for myocardial infarction [83].

4. Challenges in gene therapy

4.1. Cellular and extracellular barriers in gene delivery

Viruses have highly evolved mechanisms for obtaining optimized receptor-mediated internalization, efficient cytosolic release, directed and fast intracellular transport towards compartments and readily disassemble. In contrast, non-viral vectors must overcome multiple extracellular and intracellular barriers [21]. These barriers include binding to the cell surface, traversing the plasma membrane, escaping lysosomal degradation, and overcoming the nuclear envelope. To overcome the delivery barriers in non-viral gene transfer, various strategies have been employed to enhance the circulation time, improve intracellular delivery, and enhance endosomal escape and nuclear import. Lipoplexes have shown rapid hepatic clearance during systemic administration. Modification of lipoplexes with hydrophilic molecules like polyethylene glycol (PEG) and polyethyleneimine (PEI) causes steric hinderance between opsonins and the delivery vectors, increasing their circulation time in the blood. PEGylation of PLL decreases interparticle aggregation, resulting in high transfection efficiency in the presence of serum [29]. One study has demonstrated that when artery wall binding peptide (AWBP), a core peptide of apo B100 -- a major protein component of LDL -- was conjugated to PLL with PEG as the linker, the PLL-PEG-AWBP protected the plasmid DNA from nucleases for more than 120 min in circulation and also showed 100 times higher transfection efficiency when compared to PLL and PLL-g-PEG in bovine aortic ECs and SMCs [84]. In an innovative approach, micellar nanovectors made of PEG-block-polycation, carrying ethylenediamine units in the side chain [PEG-PAsp(DET)], complexed with plasmid DNA to form polyplex micelle effectively transfected vascular smooth muscle cells in vascular lesions without any vessel occlusion by thrombus [85] in rabbit carotid arteries. However, PEI-mediated gene delivery can affect EC function and viability [31].

The size and charge of the lipoplex/polyplex play an important role in their intracellular delivery. Lipoplexes and polyplexes are generally formulated into particles with net positive charges to trigger endocytosis by non-specific electrostatic interaction between the positively charged complexes and negatively charged cell surface [29]. Since drug carriers with a smaller particle size have resulted in higher arterial uptake compared to carriers with larger size, the size of the complexes was expected to be a dominating factor in the arterial wall lesions because of the rapid blood flow which could wash out most of the drugs or therapeutic chemical agents from the arterial wall lesions within 20–30 min. Song *et al*. [86] reported a potentially useful particle size of 70~160 nm for local intraluminal therapy of restenosis.

By taking advantage of high expression levels of receptors or antigens in diseased conditions, gene complexes can be targeted using specific ligands, such as antibodies, peptides and proteins. Cyclic RGD (cRGD) peptide recognizes α(v)β(3) and α(v)β(5) integrins, which are abundantly expressed in vascular lesions. When cRGD was conjugated to PEG-PAsp(DET) to form polyplex micelles through complexing with plasmid DNA, the micelles achieved significantly more efficient gene expression and cellular uptake as compared to PEG-PAsp(DET) micelles in ECs and SMCs [87]. PAMAM dendrimers with E/P-selectin an-

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tibody was used for gene targeting to activated vascular ECs [88]. The lectin-like oxidized LDL receptor (LOX-1) is expressed selectively at low levels on ECs but is strongly upregulated in dysfunctional ECs associated with hypertension and atherogenesis. White and colleagues [89] confirmed the selectivity to LOX-1 for peptides LSIPPKA, FQTPPQL, and LTPATAI, which could be potential targets to dysfunctional ECs expressing LOX-1 receptor. Another approach to increase intracellular delivery is to use cell penetrating peptides (CPPs). CPPs consist of short peptide sequences that are able to translocate large molecules into the cells and increase the transfection efficiency [90].

Following internalization of lipoplexes and polyplexes via endocytosis, endosomal entrapment and subsequent lysosomal degradation are the major hurdles that limit transfection efficiency [29]. Lipoplexes are modified with dioleoylphosphatidylethanolamine (DOPE) or other helper lipids due to its fusogenic functionality and its ability to destabilize endosomal membranes. Small PLLs with cationic lipid DOCSPER [1,3-dioleoyloxy-2-(N(5)-carbamoyl-spermine)-propane] enhanced gene transfer in primary porcine SMCs *in vitro* and *in vivo* in porcine femoral arteries [91]. Polyplexes, PEI and PAMAM are cationic polymers of high efficiency partly because of their ability to burst the endosomal membrane due to 'proton sponge effect'.

A promising new delivery strategy is to use synthetic peptide carriers containing a nuclear localization signal to facilitate nuclear uptake of plasmid DNA. Nuclear import of plasmid DNA is more challenging for transfecting non-dividing cells. Strategies to increase the nuclear import of genes involve tagging the nuclear localization sequence (NLS) with DNA vectors. NLS is a major player that shuttles protein-plasmid complexes through the nuclear pore by interaction with importins and transportin [92, 93]. Incorporation of DNA nuclear targeting sequence SV40 into expression plasmids results in 10-40 fold increases in vascular gene expression in rat mesenteric arteries [94], confirming the function of DNA nuclear targeting sequences *in vivo*.

4.2. Challenges associated with the vectors

4.2.1. Insertional mutagenesis

Insertional mutagenesis is a major concern in gene therapy involving viral vectors. These vectors integrate randomly or quasi-randomly into the host cell's genome, to stably transfect the target cell. The variable site and frequency of integration of the transgene can induce mutagenesis in the host genome, resulting in devastating consequences for the cell and for the organism. [95, 96]. Another disadvantage of the random integration of a transgene is the unpredictability of its stability and its expression. The genomic locus in which the vector integrates can have profound effects on the level of transgene expression, as it can completely silence the transgene, or it can increase or decrease its expression. These effects could not be avoided by sophisticated vector design or inclusion of the gene's own promoter and/or enhancer region in the transgenic vector construct, as the surrounding chromatin can override the activity of the original regulatory regions. Gene targeting by homologous recombination, however, lacks many of these shortcomings [96]. In this process, the transgene recombines

with its natural locus in the host genome, thereby ensuring correct transcription. Also, after homologous recombination, the targeted modification of the chromosomal locus is stable, whereas randomly integrated sequences might be lost over time. In their seminal paper, Russel and Hirata [97] reported that DNA vectors based on the AAV could target homologous chromosomal DNA sequences and allow high-fidelity, non-mutagenic gene repair in a host cell. Although the laborious vector design and low transfection efficiencies of AAV vectors compared to the other viral vectors still remains a concern, statistical information neatly outlines the advantage of rAAV gene replacement system over standard viral vectors, which induce strong immune response.

4.2.2. Tissue-specific targeting

The promiscuous tropism of vectors resulting in high-level transgene expression in multiple tissues is another major challenge in vascular gene therapy. After systemic application, most viral vectors are trapped by the liver, hampering delivery to target CV tissues. Approaches to restrict gene delivery to desired cell types *in vivo* relied mostly on cell surface targeting or cell-specific promoters.

The *cis*-acting regulatory elements of the SM (smooth muscle)22α [98-100], telokin [101], smooth muscle myosin heavy chain [102], smooth muscle α- [100] and γ-actin [103], and desmin [104] genes have been shown to direct reporter gene expression to smooth muscle tissues in transgenic mice. In our studies, specific gene transfer to the SMC layer was achieved in swine coronary and peripheral arteries using SM22α promoter in AAV [17]. Although the efficiency of transduction was low when compared to a similar study using AAV vectors with cytomegalovirus (CMV) promoter [105], the use of SM22α promoter caused specific transduction of SMCs *in vivo*. An interesting approach to enhance the transduction efficiency of SM22α -containing plasmid was to incorporate chimeric transcriptional cassettes containing a SM-myosin heavy chain enhancer element combined with the SM22α promoter [106]. The transfection levels obtained using these chimeric constructs in Ad vector were similar to that with CMV promoter when tested in rat carotid arteries. Certain DNA nuclear targeting sequences can be used to restrict DNA nuclear import to specific cell types. Young *et al.* [107] improved the efficiency of transduction in SMCs of rat vasculature using a SMC-specific DNA nuclear targeting sequence.

EC specific gene expression was obtained when promoters of *fms*-like tyrosine kinase-1 (FLT-1) [108], intercellular adhesion molecule (ICAM) -2 [109], angiopoietin-2 [110], eNOS [111], vascular cell adhesion molecule-1 (VCAM-1) [112], von Willebrand factor [113], tyrosine kinase with immunoglobulin and epidermal growth factor homology domains (Tie) [114], kinase-like domain receptor [115] were used in transgenic mouse models. Other EC-specific promoters include the oxidized LDL receptor LOX-1 [116] and ICAM-1 [117], which exhibit upregulation upon cytokine stimulation, a possible advantage depending on the application in inflammatory conditions [118]. With the possible exception of the mouse Tie-2 and human ICAM-2 genes, most of EC–specific promoters tested to-date have been shown to direct expression in distinct and restricted sites of the vascular tree [119]. A combination

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approach of the Tie2 promoter and enhancer (Tshort) by Minami and collegues [119] directed widespread EC expression *in vivo.*

Another challenge was in generating an EC-specific promoter with comparable efficiency as the CMV promoter. White *et al.* [120] examined several novel Ad expression cassettes for EC-specific gene transfer with CMV, Tshort, ICAM-2, ICAM-1, FLT-1 promoters, respectively and found that LOX-1 promoter elements significantly increased reporter gene expression in carotid arteries compared to other promoters. The efficacy of these novel expression cassettes in large animal models have yet to be established.

An increasingly important area to in-tissue specific targeting is to engineer viral vectors Ads and AAVs with altered cell tropisms to narrow or broaden its efficiency in tissues refractory to infection [19, 121]. Non-genetic approaches typically utilize bispecific antibodies that both neutralize wild-type virus tropism and provide a new cell binding capacity [122]. For genetic targeting strategies, the virus capsid are engineered to express foreign ligands that target selected receptors in the absence or presence of additional modification to ablate the natural tropism of the virus [122, 123]. Ad homing to target endothelial cells at specific sites of the body can be achieved by deleting the ability of the virus to interact with its natural receptor, Coxsackievirus and adenovirus receptor (CAR), and a simultaneous addition of a ligand that directs the virus to the angiotensin converting enzyme on the ECs. Retargeting of AAV-2 with novel peptides could increase both transduction efficiency and selectivity [124] in vascular ECs [125] and SMCs [126] *in vitro.*

4.3. Challenges associated with the mode and route of gene delivery

4.3.1. Systemic gene delivery

The vascular system represents an ideal route of substance transport for reaching a specific site for therapeutic intervention. However, in the case of non-viral vectors, which are cationic polymers in most cases, it has been found that electrostatic interactions between the sulphated glycosaminoglycans in the serum as well as those expressed on the cell surface cause premature release of plasmid DNA leading to its inactivation and extracellular degradation by serum DNAses [21]. Also, after systemic vascular application, non-specific distribution of plasmid DNA throughout the vasculature would result in undesired side effects because of accumulation at non-specific sites. Intravenous administration of cationic polymers resulted in their localization to liver, lung, kidney, and spleen in pigs and rabbits [127-129]. Other barriers to systemic delivery include rapid clearance of the lipoplexes by the reticulo-endothelial system and target specificity.

Most Ad vectors are trapped by the liver, hampering delivery to target CV tissues after systemic application. Systemic tail vein injection of Ad vector in mice resulted in virus DNA deposition liver, lung, kidney and testis [130]. Furthermore, the use of a heterologous viral promoter CMV in the majority of vascular gene transfers causes systemic organ toxicity resulting from unrestricted transgene expression [131]. Retargeting of vectors and use of tissue specific promoters offers an enhanced safety profile by reducing ectopic expression in vital organs including the liver and lungs.

4.3.2. Endovascular gene delivery

Endovascular catheter-based gene delivery allows localization of vectors to the vessel wall and has the advantage that smaller quantities of viral vectors can be used when compared to those used in systemic delivery. The localized delivery minimizes widespread bio-distribution of vectors and simultaneously increases the local vector concentration. Several catheters are used for vascular gene delivery [132], and the efficiency of gene transfer depends on multiple physical parameters during the delivery process, including balloon pressure, vessel wall exposure time, concentration, and injection force [133]. Diffusive balloon catheters that include double balloon, channel, microporous and hydrogel balloons, facilitate passive diffusion of the vector to reach only the innermost layers of the artery (intima and inner media) [134]. Although this system has the advantage of causing relatively minor damage to the vessel media and intima, the major drawbacks include tissue ischemia caused due to blood flow blockage following balloon inflation and relatively low gene transfection rates owing to the short exposure time to the vessel wall. The pressure-driven balloon catheters [135], like the circumferential needle injection balloon catheter and the porous balloon catheter, are thought to efficiently delivery vectors to the deeper medial and adventitial layers of the artery compared to passive diffusion catheters, but they increase the risk of vascular injury. Damage to the endothelial lining promotes SMC proliferation and may lead to restenosis. The localized vascular injury can also cause increased inflammatory response. Iontophoretic catheters, a mechanically assisted injection catheter, enhance the vector penetration across the EC lining by generating an electrical current gradient to drive charged or hydrophilic molecules as deep as the adventitial layer of the artery wall, but depends on the charge, size, and concentration of the delivered compound [136]. Despite the theoretical aspects, in most cases of catheter-based gene transfer the vector is not distributed to the target vessels but to the region of tissue surrounding the target vessel or into the systemic circulation.

Gene eluting stents are attractive alternatives for localized gene delivery as they provide a platform for prolonged gene elution and efficient transduction of opposed arterial walls, especially in the treatment of in stent restenosis [132]. Local delivery of naked plasmid DNA encoding for human VEGF-2 via gene-eluting stent could decrease neointima formation while accelerating re-endothelialization in rabbit model [137]. Stents coated with lipoplexes containing eNOS plasmid accelerated re-endothelialization in hypercholesterolemic rabbits [138]. The same research group also demonstrated successful Ad and AAV delivery to the vessel wall by gene eluting stents with no systemic dissemination of the viral vectors [139]. Stents are often coated with synthetic or naturally occurring biopolymers for prolonged release of the gene to the vessel wall [140]. Recently, fully biodegradable stents have shown great promise in the treatment of peripheral arterial disease [141]. A combination approach of therapeutic gene delivery and fully biodegradable stents would be a novel approach to gene therapy.

4.3.3. Perivascular gene delivery

In endovascular approach, most catheters require prolonged total vascular occlusion for efficient gene delivery to the vasculature increasing the risk of ischemia. Delivery of genes di-

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rectly into the adventitia bypassing intima and media may facilitate relatively rapid and efficient delivery compared to endovascular approaches [132]. The advantages of perivascular gene transfer are that the blood flow and endothelium are not disrupted and the placement of vector particles within tissues will result in enhanced local transduction efficiency compared to that achievable by endoluminal delivery [142]. Moreover, the local gene delivery through this 'outside in' approach has received increased attention due to important findings on the capacity of adventitia to influence neointima formation and vascular remodeling [143]. Localized adventitial delivery of a replication-deficient Ad construct containing a fibroblast-active promoter with the gp19ds portion of NADPH inhibitor was effective in reducing overall vascular superoxide anion O_2^- and neointima formation after angioplasty in rat common carotid artery [144]. Shneider *et al.* [145] showed that the infusion of Ad vectors into the carotid artery adventitia achieved recombinant gene expression at a level equivalent to that achieved by means of intraluminal vector infusion. Further, perivascular approach has been reported to minimize the pro-inflammatory effects of Ad vectors [145]. Adventitial gene delivery are also reported to be performed with silastic or biodegradable collars [146] which act as reservoirs of the vector.

The endovascular access is comparatively difficult in the case of coronary arteries, and the numerous side branches will also permit the run-off of the infused volume. An alternative delivery approach for coronary arteries is the expression of diffusible gene products into the pericardial space surrounding the heart and coronary arteries [147]. Transvascular needle injections of Ad vectors to the adventitia and perivascular tissue of coronary arteries have also been reported [148].

4.4. Immunological barriers to gene transfer

The immune system has evolved to eliminate foreign material and therefore, constrains the successful use of gene-replacement therapy based on viral vectors. There are several reports that suggest innate and adaptive immune responses to gene transfer [149, 150]. The vector dose, the route of administration, the nature of the transgene, and host-related factors responsible for inter-individual variability influence the immune response [151]. The early responses involve mechanisms that include the detection of pathogen-associated molecular patterns (PAMPs) present on the viral structural proteins containing the transgene by pattern recognition receptors (PRRs) on cells of the innate immune system (i.e., macrophages and dendritic cells) and the subsequent elaboration of pro-inflammatory cytokines that can up-regulate later adaptive immune responses [152]. The most studied family of PRRs are the toll-like receptors (TLRs), of which TLR2, TLR3, TLR4, TLR7, TLR8 and TLR9 have been implicated in initiating inflammatory responses to viruses [153]. The adaptive responses can include: the generation of antibodies to the transgene delivery vehicle compromising vector administration, or the generation of antibodies to the transgene product which nullifies transgene expression, or cytotoxicity to vector and/or transgene product which leads to the loss of transduced cells. It also results in a $CD8^+$ memory T cell response that thwarts further efforts to use the same vector or transgene.

Ad vector particles can elicit strong innate and adaptive immune responses. The interplay of both systems activates CD4+ and CD8+ T cells and B cells as well as facilitates the induction of transgene-specific immune responses. The innate immune responses after systemic administration of Ad vectors are due to several processes: complement system activation, anaphylotoxin release, macrophage activation, release of cytokines and chemokines, including Interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, macrophage inhibitory protein-2, and RANTES (regulated and normal T cell expressed and secreted); EC activation, generalized transcriptome dysregulation in multiple tissues, activation of macrophages and dendritic cells, mobilization of granulocyte and mast cells, and thrombocytopenia [154]. These responses are due to activation of multiple PRRs including RIG-I-like receptors and Toll-like receptors: TLR-2, TLR-4 and TLR-9 [155]. *In vivo* administration of higher doses of Ad vectors can result in one or all of these innate responses or may even lead to mortality in small animal models [156]. Ad infection of ECs is followed by expression of adhesion molecules such as ICAM-1 and VCAM-1 leading to increased leukocyte infiltration within transduced tissues [157]. Kupffer cells, the resident macrophages of the liver, rapidly scavenge and eliminate Ad5-based vectors from the circulation in mice [158], and this interaction contributes to the induction of pro-inflammatory cytokines and chemokines [159]. It has been reported that increasing the dose of Ad vector would probably fail to increase transgene expression, as the CAR adenoviral receptors would become saturated; in addition, the higher dose would induce a stronger inflammatory response responsible for increased elimination of the infected cells expressing the transgene [151].

Ad-based gene transfers can be hindered due to adaptive immune responses to the virus or the transgene it encodes. Ad viruses can induce a cytotoxic T-cell response as well as infiltration by CD4+ and CD8+ T cells. The mechanism involves internalization and priming by dendritic cells of capsid antigens associated with Class II Major histocompatibility complex (MHC) antigens, presentation of these antigens to CD4+ T cells, which become activated, and in turn CD8+ T cell activation by these CD4+ T cells [151]. These adaptive immune responses can limit the duration of transgene expression, and/or limit the ability to re-administer the vector.

Development of new large capacity or gutless (devoid of all viral genes) vectors [160] or modification of capsid sequences [161] are a few of the various strategies devised to reduce the immunogenicity of the Ad viral vectors. Adaptive immunity against these vectors has been substantially reduced through the development of helper-dependent Ad vectors that contain no Ad genes. However, these gutless Ad vectors can efficiently transduce antigen presenting cells (APCs) [162], which readily triggered innate immune responses and further augmented the induction of adaptive immune responses to the transgene product. This problem led to the introduction of tissue-specific promoters in gutless Ad vectors to restrict transgene expression in target cells but not in APCs [162]. Genome modification, capsid modification by Ad capsid-display of immuno-evasive proteins, chimeric Ad vectors and Ad vectors derived from alternative Ad serotypes are few techniques adopted for eluding Ad vector immunity [161]. The tropism modification strategies for targeted gene delivery using Ad vectors have been extensively reviewed [163]. Another method to decrease the im-

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mune response is to modify the route of delivery of the vector. In the adventitial delivery of Ad vectors to rabbit carotid arteries, recombinant gene expression was achieved at a level equivalent to that achieved by intraluminal vector infusion. Despite the generation of a systemic immune response, adventitial infusion had no detectable pathologic effects on the vascular intima or media [145]

Pre-existing immunity due to neutralizing antibodies against endemic Ad serotypes in human populations can contribute to pre-existing Ad specific adaptive immune responses [154]. These cellular responses may be more challenging than humoral immune responses, as these cellular adaptive immune responses to Ads have been shown to recognize multiple diverse, cross-clade Ad serotypes subsequent to exposure to only a single Ad serotype [154]. Arterial gene transfer with type 5 Ad vectors did not cause significant levels of gene expression in the majority of humans. Both immune-suppression and further engineering of the vector genome to decrease expression of viral genes show promise in circumventing barriers to Ad-mediated arterial gene transfer [164].

The innate immune response to the AAV capsid has received limited attention due to the minimal responses that AAV2 elicits [162]. According to recent reports by Herzog and others [165], innate immune system also plays important roles in activation of immunity by AAV mediated gene transfer, both in inducing the initial response to the vector and in promoting a deleterious adaptive immune responses. The initial innate immune responses were mediated by the TLR9-MyD88 pathway via a traditional NF-κB pathway to induce type 1 interferon production. Subsequently, alternative NF-κB pathway is triggered, prompting adaptive immune responses [166]. *In vivo*, intravenous injection of AAV-lacZ rapidly induces the expression of messenger RNAs (mRNAs) for the cytokines TNF-α, RANTES, interferon-γ-induced protein 10, macrophage inflammatory protein(MIP)-1β, monocyte chemotactic protein-1, and MIP-2. However, this effect lasts only 6 h, compared to more than 24 h with Ad infection [151]. The adaptive cell-mediated response is far weaker with AAV vectors than with adenoviral vectors probably due to the inability of AAVs to efficiently infect APC, including dendritic cells and macrophages. AAV vectors may be capable of infecting immature dendritic cells, but only when large doses of vector are used. In addition, even though a modest amount of dendritic cells are present at sites of AAV infection *in vivo*, they usually fail to induce a T-cell response of sufficient magnitude to eliminate the infected cells and, therefore, to decrease the duration of transgene expression [151].

Cytotoxic T-cell responses to AAV capsid antigen especially in patients with pre-existing neutralizing antibodies against AAV remain a major road block to achieve persistent therapeutic correction for clinical application. Natural, asymptomatic AAV infection in humans is common, and it estimates that up to 80% of humans possess neutralizing antibodies to some AAV serotypes, especially AAV-2 [167]. Recently, multiple serotypes of AAV in addition to AAV2 have been developed; these serotypes carry different capsid proteins and exhibit different tropism towards different organs [18]. However, changing serotypes may only lead to partial success due to the strong conservation of immune-dominant capsid epitopes in AAVs. In patients with high titers of neutralizing antibodies to gene therapy vectors such as AAV and Ad vectors, IgGs can be removed from blood by plasmapheresis, double filtration

plasmapheresis and immune-absorbant plasmapheresis before gene transfer procedure to increase transduction rates of target tissues [168].

Plasmids alone or in combination with naked bacterial DNA can stimulate innate immune responses [152]. Plasmids, composed chiefly of bacterial DNA, contain far greater amounts of unmethylated CpG motifs than do the DNA in eukaryotic cells. DNA devoid of CpG motifs does not induce proinflammatory cytokine synthesis by macrophages *in vitro*. TLR 9 recognizes the unmethylated CpG motifs in immunostimulatory sequences of bacterial DNA which activate the cells responsible for innate immune responses (for example macrophages) after penetration of bacteria into the body [169]. Indeed, elimination or methylation of these sequences could be a method for suppressing the inflammatory response induced by unmethylated CpG sequences in plasmids [168].

5. Conclusion

An enormous amount of research has been done in the past few decades on the choice of the therapeutic gene, vectors and delivery approaches for effective vascular gene transfer. The low efficiency of gene transfer to vascular tissues still remains a major drawback.. Of the several approaches used so far, Ad-mediated gene transfer has been found to be the most efficient when compared to other methods. However, gene transfer using viral vectors has often caused ectopic expression and also an increased immunological response. The use of tropism modified vectors and plasmids with cell specific promoters are solutions for reducing the ectopic expression. Using "gutless" viral vectors devoid of the immunogenic regions of viral plasmid is an attractive option to reduce the immunologic response, but we have to wait for more *in vivo* data using these third-generation vectors to reach a conclusive result [160]. Non-viral methods have more barriers to overcome to successfully transfect the cell; however, with the advent of innovative technologies like nanobots [170], stimuli responsive polymers [171], novel erythrocyte based carriers [172], magnetically targeted delivery [173] and focused *in vivo* plasmid DNA delivery to the vascular wall via intravascular ultrasound destruction of microbubbles [174]; we expect enhanced transgene expression in vascular cells in future studies. This will also be a possible solution to tackle with the immune response associated with the viral vectors. Site specific biodegradable stent based gene delivery approach [175] and modified percutaneous gene delivery systems offer new opportunities for enhanced gene delivery to vascular cells.

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