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Improvement of vascular function by magnetic nanoparticle-assisted circumferential gene transfer into the native endothelium

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ABSTRACT

Gene therapy is a promising approach for chronic disorders that require continuous treatment such as cardiovascular disease. Overexpression of vasoprotective genes has generated encouraging results in animal models, but not in clinical trials. One major problem in humans is the delivery of sufficient amounts of genetic vectors to the endothelium which is impeded by blood flow, whereas prolonged stop-flow conditions impose the risk of ischemia.

In the current study we have therefore developed a strategy for the efficient circumferential lentiviral gene transfer in the native endothelium under constant flow conditions. For that purpose we perfused vessels that were exposed to specially designed magnetic fields with complexes of lentivirus and magnetic nanoparticles thereby enabling overexpression of therapeutic genes such as endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF). This treatment enhanced NO and VEGF production in the transduced endothelium and resulted in a reduction of vascular tone and increased angiogenesis. Thus, the combination of MNPs with magnetic fields is an innovative strategy for site-specific and efficient vascular gene therapy.

1. Introduction

Cardiovascular diseases are a pandemic problem, the underlying pathophysiology is most commonly determined by atherosclerosis. At early stages of the disease endothelial dysfunction occurs, which is characterized by a reduced bioavailability of vasorelaxants such as nitric oxide (NO) and impaired vasodilation. Because endothelial dysfunction is a reversible disorder, the direct therapeutic enhancement of NO production in the endothelium appears to be a promising treatment strategy to restore cell biological properties and vascular function. At later stages of the disease process atherosclerotic plaques are formed that lead to vascular stenosis limiting blood flow and oxygen supply to organs. Consequently, the exogenous application of vasoprotective and angiogenic growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) should promote the development of functional collaterals thereby enhancing tissue perfusion and preventing end organ damage.

To enable a sustained and long-lasting release of NO or growth factors in the vessel a gene therapeutic approach is advantageous. Vascular gene delivery of endothelial nitric oxide synthase (eNOS) has been demonstrated to augment NO-dependent vasorelaxation in animal models. This was found in healthy rabbits [1], [2] but also in spontaneous hypertensive rats [3] and diabetic [4] or hyperlipidemic rabbits [5]. Moreover, eNOS overexpression reduced VSMC proliferation and intima hyperplasia in vascular injury models [6–8] and prevented the progression of atherosclerosis [9,10]. VEGF gene therapy successfully induced neovascularization and collateral flow in pre-clinical models of cardiovascular disease. These effects were observed after intravascular [11] and intramuscular [12] application in hindlimb ischemia of the rabbit or after intramyocardial [13], [14] application in pig. However, the transfer of such strategies to humans has generated rather disappointing results in clinical trials [15]. A so far unsolved problem is the lack of site-specificity when genetic vectors are applied. To affect endothelial function or the growth of collaterals an intravascular administration route would be optimal. However, this requires vascular occlusion to enhance contact time and transfection efficacy of the vector, which also increases the risk of ischemic events. To avoid the interruption of blood flow magnetic nanoparticles (MNPs) and magnetic fields appear to be ideally suited for the retention of genetic vectors at the vascular wall [16]. In a previous study we tested the feasibility of local reporter

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gene overexpression using lentivirus (LV)/MNP complexes in combination with external magnets under flow conditions in vessels [17]. Based on these findings in the current study we established a new approach by which an efficient circumferential transduction of the native endothelium results in the functional improvement of endothelial cell biological properties. Therefore, we applied an optimized magnet configuration providing a radially symmetric magnetic field and directly overexpressed the therapeutic genes eNOS or VEGF in the native vascular endothelium of perfused vessels using LV/MNP complexes. Our experiments demonstrate that this strategy improves vascular endothelial function shown by the efficient reduction of vascular tone and the enhancement of angiogenesis.

2. Methods

2.1. Animals

CD1 wildtype mice, C57BL/6 wildtype mice (both Charles River, Sulzfeld, Germany) or eNOS deficient mice ($eNOS^{-/-}$, kindly provided by Dr. A. Goedecke, University of Duesseldorf) were used and held under conditions approved by the local ethics committee. Experiments were carried out according to the guidelines of the German law of protection of animal life with approval by the local government authorities (LANUV, NRW, Germany).

2.2. Lentiviral vectors

For transduction experiments self-inactivating (SIN), VSV.G pseudotyped lentiviral vectors (LVs) of the 3rd generation were used. They were purified by ultracentrifugation of cell culture supernatant from transfected HEK293T producer cells as described before [17]. For perfusion of polyethylene tubing an eGFP-labeled replication-defective HIV-1 derivative (pCHIV) was applied [18]. As expression vectors cytomegalovirus (CMV) promoter-driven constructs for enhanced green fluorescent protein (eGFP), human endothelial NO synthase (eNOS) and human vascular endothelial growth factor (VEGF) were used. As control a LV without transgene expression cassette LV(rrl) was chosen. Physical virus titers (virus particles (VP)/ml) and infectious virus titers (infectious particle (IP)/ml) were determined by calculation of active viral reverse transcriptase using an enzyme-linked immunosorbent assay (ELISA) [19] or by transduction of HEK293T cells and flow cytometry analysis, respectively.

2.3. Magnetic nanoparticles (MNPs)

In this study PEI-Mag2 and SO-Mag5 MNPs of the core-shell-type were used. The magnetic core of both MNPs is composed of magnetite (Fe(II)Fe(III)2O4). The core of PEI-Mag2 has a diameter of 9 nm and is coated with fluorosurfactant lithium 3-[2-(perfluoroalkyl)ethylthio] propionate (ZONYL® FSA) and branched polyethyleneimine with a size of 25 kDa [20]. The core of SO-Mag5 has a diameter of 6.8 nm and is surrounded by a silica coating with surface phosphonate groups [19]. The ζ potential of the MNPs was determined to -9.7 ± 0.4 mV (SO-Mag5) and 11.1 ± 0.4 mV (PEI-Mag2) in $HBSS^{++}$, $n = 3$.

2.4. Assembly of magnetic complexes

Assembly of magnetic complexes was obtained as described previously [21]. Briefly, LVs and MNPs were incubated in 800 μl Hank's Balanced Salt Solution with calcium and magnesium ($HBSS^{++}$, Life Technologies, Darmstadt, Germany) for 20 min at room temperature. For perfusion of aortas a total amount of 35 μg of iron and $8.7 * 10^8$ VPs (heNOS, hVEGF) or $1.2 * 10^8$ IPs (eGFP) were used. This corresponds to ratios of 40 fg iron/VP and 300 fg iron/IP, respectively. The size and electrokinetic potential of the complexes was determined by dynamic light scattering using a ZetaSizer Nano (Malvern Instruments Ltd., U.K.). The average magnetic moment of the complexes was determined by analyzing the clarification of the complex suspension in a well-defined magnetic field. This was done by registration of the time course of the light absorption at 420 nm (Specord 210, Analytik Jena, Jena, Germany).

The diameters of magnetic complexes showed a log-normal distribution with a mean value of $\bar{d}_H = 1031$ nm for SO-Mag5 and 612 nm for PEI-Mag2. Average magnetic moments were determined as 14.44 ± 11.03 fAm^2, $n = 3$ for LV(eGFP)/SO-Mag5 and 3.18 ± 3.08 fAm^2, $n = 3$ for LV(eGFP)/PEI-Mag2, $n = 3$ as described in [21].

2.5. Numerical simulations

The calculations of the magnetic fields yielded by the two different magnet configurations used for aortic perfusion experiments were based on a cylindrical model of the mouse aorta with an inner diameter of 0.75 mm and a length of 30 mm.

The magnetic fields were generated by 12 cuboidal rare-earth permanent magnets (see specifications below) that were arranged in four triplets perpendicular to each other. For configuration A the magnets within the triplets have the same orientation, while the different triplets have an opposite alignment. For configuration B the magnets within the triplets have an alternating orientation, while the opposing triplets have an equal alignment.

Calculation of magnetic flux densities and the gradient of magnetic flux densities was performed by means of finite element calculations with the AC/DC module of the package Comsol Multiphysics 4.1 (Comsol Multiphysics GmbH, Goettingen, Germany) as described before [21]. The resulting field values were exported into MATLAB (MathWorks, Natick, MA, USA) on a previously defined equally spaced grid. To provide smooth and continuous data sets for all variables 3-dimensional smoothing B-splines were used.

To estimate the amount of retained LV/MNP complexes for the perfusion experiments, the trajectories of 1000 complexes were calculated. The sizes of the complexes were randomly generated according to the measured characteristics and generated with the Mersenne Twister method [22]. The starting positions of the complexes were also randomly generated, while the positions are normally distributed in radial direction and uniformly distributed in flow direction. The equation of motion was subsequently solved in MATLAB (MathWorks, Natick, MA, USA) with an implicit Runge-Kutta algorithm (TR-BDF2) until the complex left the domain of the aorta. For calculations with multiple passages of the fluid, complexes, which were not trapped in the previous passage, started at newly generated positions. The error of the trapping rate was estimated by the bootstrapping method to estimate the level of accuracy delivered by the algorithm for the given boundary conditions [23]. The number of samples for the bootstrapping method was chosen to be equal to the number of complexes.

2.6. Quantification of iron content

To quantify the iron content of LV/MNP complexes in polyethylene tubes and in aortas the colorimetric 1,10-phenantroline method for detection of non-heme-iron was used [24]. In order to ionize the iron the iron content of the tubes or the whole aortas were incubated over night at 65 °C in an acid mixture (3 M hydrochloric acid, 0.6 M trichloric acid). The colorimetric detection of non-heme-iron was performed on the following day. Using pre-defined amounts of ammoniumferrum(II)sulfate monohydrate a calibration curve was generated.

2.7. Quantitative RT-PCR

Native aortas incubated in DMEM + 20% fetal calf serum (FCS) for 24 h, aortas treated with 10 μg/ml poly (I:C) (Sigma-Aldrich, Germany) in DMEM + 20% FCS for 24 h or aortas after perfusion (see below) were