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Magnetically enhanced cell delivery for accelerating recovery of the endothelium in injured arteries

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ABSTRACT

Arterial injury and disruption of the endothelial layer are an inevitable consequence of interventional procedures used for treating obstructive vascular disease. The slow and often incomplete endothelium regrowth after injury is the primary cause of serious short- and long-term complications, including thrombosis, restenosis and neoatherosclerosis. Rapid endothelium restoration has the potential to prevent these sequelae, providing a rationale for developing strategies aimed at accelerating the reendothelialization process. The present studies focused on magnetically guided delivery of endothelial cells (EC) functionalized with biodegradable magnetic nanoparticles (MNP) as an experimental approach for achieving rapid and stable cell homing and expansion in stented arteries. EC laden with polylactide-based MNP exhibited strong magnetic responsiveness, capacity for cryopreservation and rapid expansion, and the ability to disintegrate internalized MNP in both proliferating and contact-inhibited states. Intracellular decomposition of $BODIPY_{558/568}$-labeled MNP monitored non-invasively based on assembly state-dependent changes in the emission spectrum demonstrated cell proliferation rate-dependent kinetics (average disassembly rates: 6.6 ± 0.8% and 3.6 ± 0.4% per day in dividing and contact-inhibited EC, respectively). With magnetic guidance using a transient exposure to a uniform 1-kOe field, stable localization and subsequent propagation of MNP-functionalized EC, markedly enhanced in comparison to non-magnetic delivery conditions, were observed in stented rat carotid arteries. In conclusion, magnetically guided delivery is a promising experimental strategy for accelerating endothelial cell repopulation of stented blood vessels after angioplasty.

1. Introduction

The introduction of intravascular stenting and the more recent redesigning of arterial stents into combination devices providing controlled release of therapeutic agents (namely, drug eluting stents) have dramatically improved therapeutic outcomes of interventional procedures clinically used to relieve obstruction of coronary arteries in vascular disease patients [1]. However, the compression of the endoluminal surface of the blood vessel and extensive trauma inevitably associated with stent implantation together cause extensive endothelial denudation [2,3], and the subsequent recovery of functional endothelium is further markedly delayed by potent antiproliferative drugs released by DES [4]. In absence of an intact endothelial cell layer forming a barrier that modulates local hemostasis and fibrinolysis [5,6], the vulnerability period for late stent thrombosis, a rare but severe complication associated with the use of DES, is markedly prolonged [7,8]. Delayed arterial healing with incomplete endothelialization also contributes to in-stent neoatherosclerosis, the primary cause of late stent failure [9,10].

Rapid restoration of a continuous and functional endothelial layer is essential for mitigating these untoward effects [6,11]. The recognized therapeutic potential of approaches aimed at accelerating arterial re-endothelialization [11] and accessible sources of autologous endothelial cells (EC) [12–14] have prompted exploring endoluminal delivery or direct seeding of EC on vascular stents in experimental settings [15–17]. However, the results of early studies focusing on evaluating these experimental approaches pointed to rapid elimination and low rates of cell engraftment at the injury site [11,14,18] among factors limiting the clinical utility of endothelial cell delivery [19]. The performance of more recently introduced CD34 antibody-coated stents designed to capture endothelial progenitor cells from the blood stream *in situ* has also been shown to be suboptimal, likely due to their insufficient specificity causing recruitment of non-endothelial cells [20–22].

Endowing EC with capacity for physical guidance via functionalization with magnetic nanoparticles (MNP) can potentially be used as part of a targeted delivery strategy effectively confining cells to the stented region and dramatically increasing the rate of endothelial cell repopulation after arterial injury [23,24]. Using biodegradable MNP formulated with strong magnetic responsiveness, such functionalization

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can be achieved quickly and dose-efficiently through magnetically enhanced endocytosis [25]. In our recent studies, we developed polylactide-based superparamagnetic MNP providing strongly magnetizable EC without compromising cell viability, identified experimental variables controlling the kinetics of magnetically driven cellular uptake, and examined disassembly patterns of the biodegradable MNP using a Förster resonance energy transfer-based approach [25,26]. In the present study, we applied cell functionalization with MNP to investigate feasibility of achieving stable homing and site-specific expansion of syngeneic EC in stented arteries using a two-source magnetic guidance scheme. Unlike single magnetic field sources that fail to provide a sufficiently strong and focused translational force for targeting non-superficial sites in the human body, this targeted delivery approach uses uniform magnetic fields readily achievable in the clinical setting for magnetizing strongly responsive MNP while concomitantly concentrating the magnetic force at the site of stent implantation. The combination of a far-reaching uniform field and strong field gradients induced in the vicinity of the magnetizable implant (secondary source) at the target site makes the two-source strategy potentially scalable for magnetic guidance in human subjects as predicted theoretically [24,27] and more recently confirmed by experimental results in human-sized blood vessels [28,29]. In the context of targeted vascular therapy, this approach has previously been shown effective by our group at localizing small-molecule drugs, gene delivery vectors and xenogeneic cells in injured arteries [24,30,31]. Herein, we evaluated the efficiency of this magnetic guidance strategy and subsequent fate of stent-targeted EC in a rat carotid stenting model by two complementary methods: direct tissue analysis of cell-associated MNP and quantitative bioluminescent imaging of syngeneic EC stably expressing firefly luciferase as a reporter. Due to its short half-life of ~3 h in mammalian cells [32], stably expressed firefly luciferase serves both as an indicator of the number of viable cells capably of continuously expressing the transgene, and as a marker of their spatial distribution in the region of interest. However, the applicability of the luciferase-based bioluminescent assay for organ distribution analysis is limited by inhomogeneous tissue uptake and highly variable availability of the substrate (luciferin) [33,34]. To address this limitation, an additional approach based on direct fluorimetric analysis of EC functionalized with MNP stably labeled with a boron dipyrromethene fluorophore, $BODIPY_{558/568}$, was applied in this investigation.

2. Materials and methods

2.1. MNP formulation and characterization

$BODIPY_{558/568}$-labeled particle-forming polymer containing 5.7 μmol per g of the covalently bound fluorophore was synthesized as previously described [26] from poly(D,L-lactide) with M_n of 50 kDa (Lakeshore Biomaterials, Birmingham, AL).

Uniformly sized polylactide (PLA)-based magnetic nanoparticles were formulated using a modification of the emulsification-solvent evaporation method [25]. In brief, ethanolic solution of ferric chloride hexahydrate and ferrous chloride tetrahydrate (170 and 62.5 mg, respectively, in 2.5 ml) was added to an equivalent amount of sodium hydroxide dissolved in deionized water (5 ml). The precipitate was maturated for 1 min at 90 °C, cooled on ice and separated on a magnet. The obtained magnetite was stirred with a solution of oleic acid in ethanol (200 mg in 2 ml) at 90 °C for 5 min. Unbound oleic acid was phase-separated with deionized water (4 ml) and removed by decantation. Oleic acid-coated magnetite was washed with ethanol and dispersed in 4 ml of chloroform. $BODIPY_{558/568}$-labeled and plain PLA (80 mg and 120 mg, respectively) were dissolved in 4 ml of chloroform and combined with the chloroformic dispersion of magnetite to form an organic phase. The organic phase was emulsified by sonication on ice in an aqueous solution of bovine serum albumin (2% w/v, 10 ml), and the solvent was removed under reduced pressure using a rotary evaporator. MNP were washed twice by magnetic decantation, resuspended in 6 ml of aqueous trehalose solution (10% w/v), passed through a sterile 5.0 μm polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA) and lyophilized. Lyophilized MNP were kept at −80 °C and resuspended in deionized water before use at 24 mg/ml.

Particle size measurements were performed by dynamic light scattering. Magnetite content was determined spectrophotometrically against a suitable calibration curve ($\lambda = 335$ nm) in MNP samples digested for 30 min with sodium hydroxide (1 N) at 37 °C to produce precipitate, which was subsequently dissolved in hydrochloric acid (1 N) by heating to 90 °C for 5 min. Magnetic hysteresis measurements were made using an alternating gradient magnetometer (Princeton Measurements Corporation, Princeton, NJ, USA).

2.2. EC functionalization with MNP and in situ measurements of intracellular MNP disassembly

Rat aortic endothelial cells (EC) from male Lewis rats were purchased from Cell Biologics (Chicago, IL, USA), stably transduced with firefly luciferase under the CMV promoter using Firefly Luciferase Lentifect™ (GeneCopoeia Inc., Rockville, MD, USA) and expanded to passage 4. For MNP functionalization, EC were seeded at 60% of confluence on 96-well plates. On the next day, MNP (3.6 mg) were reconstituted in 0.75 ml rat serum (Bioreclamation, Inc., Westbury, NY, USA) and incubated at 37 °C for 15 min. MNP suspension was diluted 1:50 with serum-free Cell Biologics medium, incubated at 37 °C for an additional 15 min, and added to plated cells at 100 μl per well. The plates were positioned for 24 h on 96-well magnetic separators with an average field gradient of 32.5 T/m (LifeSep 96F, Dexter Magnetic Technologies, Elk Grove Village, IL) to achieve quantitative uptake [25]. The medium was replaced with fresh Cell Biologics medium containing 2% rat serum for 2 h. MNP-laden EC were washed with serum-free culture medium and the amount of internalized MNP was measured fluorimetrically ($\lambda_{em}/\lambda_{ex} = 540/575$ nm). The iron oxide loading per cell was calculated based on the magnetite content in MNP determined as above. Cells were trypsinized. Reconstituted at 2.4×10^5 cells per ml in a freezing medium composed of Pluronic F-68 and dimethyl sulfoxide (1% w/v and 10% v/v, respectively) in rat serum, aliquoted and frozen in Nalgene System 100™ cryogenic tubes (Thermo Fisher Scientific, Rochester, New York, USA) by gradually reducing the temperature to −80 °C at a rate of 1.5 °C/min. For in vitro experiments and animal studies, frozen cells were thawed over 4 min and washed twice with normal saline.

Growth of MNP-functionalized EC seeded on 96-well plates at a density of 2×10^3 cells per well was longitudinally monitored in comparison to non-functionalized EC by luminometry using D-luciferin potassium salt (PerkinElmer, Bridgeville, PA, USA) as a substrate (50 μg/ml), and the obtained results were verified by cell counting. Intracellular disassembly of endocytosed MNP was monitored in EC initially seeded at 2×10^3 and 5×10^4 cells per well based on the ratio of fluorescence intensities at 612 nm and 575 nm (F_{612}/F_{575}) after establishing their correlation in accelerated degradation experiments as previously described [35]. In brief, proteinase K (Sigma-Aldrich, MO, USA) was added at 150 μg/ml to MNP diluted 1:1000 in PBS. Samples were incubated at 37 °C, their emission at $\lambda = 612$ and 575 nm were measured at each time point using $\lambda_{ex} = 540$ nm, and a part of each sample was passed through an aluminum oxide membrane with a 0.02 μm pore size (Anotop, Whatman Inc., NJ, USA) impermeable to intact particles. Aliquots taken before and after filtration were digested with acetonitrile and analyzed by fluorimetry ($\lambda_{ex}/\lambda_{em} = 540$ nm/575 nm) to determine the fluorophore fraction enzymatically cleaved off from MNP. The F_{612}/F_{575} ratio of triplicate MNP samples was plotted as a function of the fluorophore fraction dissociated from the particles, and the obtained inverse correlation showing linearity up to 70% of MNP disassembly (Fig. 3C) was applied for determining disintegration of endocytosed MNP by dividing and contact-inhibited EC seeded on 96-well plates.