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Nanoparticular delivery system for a secretoneurin derivative induces angiogenesis in a hind limb ischemia model

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ABSTRACT

Common therapeutic strategies for peripheral arterial disease often fail to re-establish sufficient blood flow within legs and feet of patients for avoiding critical limb ischemia, what is characterized by a substantial risk for amputation. The neuropeptide secretoneurin induces angiogenesis in models of limb and myocardial ischemia and might be a promising tool in the treatment of patients without the option of revascularization therapy for severe ischemia. Within this manuscript, the biologically active part of secretoneurin was identified, modified by induction of a cysteine residue to gain higher stability against enzymatic degradation and further packed into S-protected thiolated chitosan nanoparticles, which enable intra-muscular application of secretoneurin. Secretoneurin nanoparticles restored blood flow in a mouse hind limb ischemia model within one week, whereas control particles did not. In vitro testing also revealed the angiogenic, antiapoptotic and proliferative effects of the new secretoneurin derivate, as tested in primary human umbilical vein endothelial cells. With the work from this study we provide a new promising tool for treatment of peripheral arterial disease.

1. Introduction

Peripheral arterial disease is the most common disease concerning arteries, mostly of the lower limb and might lead to critical limb ischemia (CLI). It is a gradual process in which the artery becomes blocked, narrowed, or weakened. Despite therapeutic strategies like surgical or interventional revascularization, a substantial number of patients are not eligible for those treatments and show significantly reduced quality of life, as in some cases, amputation is the only possibility left [1]. Therefore therapeutic angiogenesis, which aims for revascularization of the ischemic tissue by means of re-growth of capillaries and arteries out of existing vessels, has been extensively studied [2]. Amongst many promising growth factors, secretoneurin (SN) has shown its capacity to act as angiogenic factor in a mouse cornea neovascularization assay [3,4]. Furthermore, a SN plasmid therapy vector was generated and showed induction of angiogenesis, arteriogenesis, and vasculogenesis in the mouse hind limb ischemia model [5,6]. Based on this plasmid vector, we could recently provide evidence on improvement of heart function in a rat model of myocardial infarction [7].

Nevertheless, there are obvious limitations to gene therapy as a large phase III clinical trial with plasmid treatment failed to reach the primary endpoint of amputation-free survival in CLI patients [8]. Probably the plasmid gene therapy, which is characterized by low transfection efficacy, led to a limited concentration of the active compound in the targeted tissue. On account of this, alternative strategies like application of small angiogenic proteins embedded in nano-particulate formulations might lead to more promising therapies.

Therefore it was the aim of this study to develop a small, biologically active and stable SN derivative, which can easily be incorporated into a nano-particular formulation. We chose chitosan as matrix for those particles, as chitosan has been previously reported to be suitable for intramuscular application [9]. S-protected chitosan reveals a further development in polymeric drug delivery, as its potential to provide sustained release over longer time periods on the one hand and protection from enzymatic digestion on the other hand, make this highly engineered polymer the candidate of choice for our studies [10]. Furthermore, an even more sustained release can be achieved by covalent attachment of thiol bearing drugs via disulfide bond formation to it [11].

In this manuscript we identified the biologically active amino acid sequence of SN and attached a cysteine residue to this angiogenic

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peptide in order to enable stable incorporation in the S-protected chitosan nanoparticles. Potential SN derivatives were further tested for stability, in vitro angiogenesis, proliferative and anti-apoptotic features. The most promising candidate identified from in vitro investigations was embedded in S-protected thiolated chitosan nanoparticles (CS-TGA-MNA) and tested for effects on in vivo blood perfusion in a mouse hind limb ischemia model. The results of this work might lead to a novel therapeutic approach in critical limb ischemia.

2. Materials and methods

2.1. Secretoneurin and derivatives

Secretoneurin (SN) and derivatives were purchased from piCHEM (Austria). Peptide information and amino acid (AA) sequences are listed in Table 1.

2.2. Cell culture experiments

Human umbilical vein endothelial cells (HUVECs) between passage 2 and 5 were used. Cells were purchased from PromoCell and cultured in EBM-2 medium containing 4.76% (v/v) fetal calf serum (FCS) and EGM-2 Single Quots (Clonetics, Lonza). For all in vitro assays using HUVECs, cells were incubated with medium without supplements containing 0.5% bovine serum albumin (BSA, Sigma-Aldrich). Stability studies were performed on C2–C12 mouse muscle cells, which were grown on 75cm^2 plastic culture flasks in DMEM containing L-Glutamine, penicillin/streptomycin (1000 U penicillin, 0.1 mg/l streptomycin) and 20% (v/v) FCS. Cells were seeded at a density corresponding to 5×10^5 cells per flask and cultured at 10% CO_2, 37 °C, and 95% air humidity. Cell passages between 3 and 10 were used for all stability studies. Non-adherent cells were removed by complete change of medium every day for C2–C12.

2.3. Preparation and characterization of SN4C nanoparticles

The synthesis of S-protected thiolated chitosan (CS-TGA-MNA) was accomplished as described previously by Dünnhaupt et al. [10]. The first modification involved the covalent attachment of thioglycolic acid (TGA) to chitosan (CS, 150 kDa) due to the formation of amide bonds between the primary amino groups of the polymer and carboxylic acid groups of TGA by using of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC). Therefore, 1 g of chitosan (Low viscous chitosan with an average molecular weight of 150 kDa and a degree of deacetylation >85% obtained from Sigma Aldrich, Austria) was dissolved in 0.1 M hydrochloric acid. In order to activate the carboxylic acid moieties, 250 mg of TGA were chemically treated with EDAC in a final concentration of 100 mM corresponding to a molar ratio of 1:4:0.5 free amino groups on chitosan to EDAC to ligand. The second modification step was then achieved by disulfide bond formation between free thiol groups of the thiolated chitosan and the aromatic ligand mercaptonicotinic acid (MNA). As the dimeric ligand 2,2′-dithiodinicotinic acid results in comparatively higher coupling rates demonstrated by Dünnhaupt et al. [10] the dimer was previously synthesized out of the monomer mercaptonicotinic acid using hydrogen peroxide as oxidizing agent. In the following, free thiol groups on the polymer matrix were S-protected by reacting 1 g of CS-TGA with 400 mg of aromatic dimeric ligand at a pH of 6.0. The content of thiol groups and disulfide bonds was quantified by Ellman's reagent [12]. Nanoparticles were prepared by in situ gelation of the resulting CS-TGA-MNA S-protected polymer with the corresponding counter-ion tri-polyphosphate (TPP) under aseptic conditions. Therefore, all appropriate solutions for particle production were sterile filtered before use and particles were prepared in a laminar flow. First, a 0.2% (m/v) solution of CS-TGA-MNA was prepared in saline. Afterwards, 1.7 mg of the promising SN candidate was dissolved in 300 μl saline and added to the polymer solution. Due to the high reactivity of S-protected thiol groups of chitosan SN4C is thereby likely quantitatively bound to the polymer [11]. Then, pH was adjusted to 4.8 with 0.2 M hydrochloric acid and a 0.2% (m/v) TPP solution was added to generate nanoparticles [13]. Finally, particle suspension was lyophilized with 3% (w/v) trehalose (lyoprotectant) and stored in the fridge at 4 °C for further use. Size distribution and zeta potential of particles were determined by dynamic light scattering using a Zeta Potential/Particle Sizer (Malvern Zetasizer Nano ZSP, Malvern Instruments Ltd., Worcestershire, UK). For size measuring particles were suspended in demineralised water at room temperature and the intensity distribution was fit to a Gaussian size distribution curve.

In case of SN4C release studies, loaded nanoparticles were distributed in 1 ml of DMEM without phenol red, aliquoted in 24-well plates and subsequently incubated for 71 h in an incubation chamber at 37 °C. At predetermined time points samples were taken, centrifuged for 15 min at 12500 rpm (9800 rcf) and the supernatant was used for detection via HPLC as described above.

For quantification of peptide release in presence of a reducing agent, particles were incubated with reduced glutathione (GSH). Therefore, particles were suspended in 500 μl demineralised water and mixed with 500 μl of reduced glutathione (50 μM). After 15 min of incubation, particles were separated by centrifugation at 12500 rpm (9800 rcf) for 15 min and the supernatant was analyzed via HPLC as described above.

Table 1
lists SN and SN derivatives with information about abbreviations, amino acid (AA) sequences and biological activity due to MAPK activation.

Peptide	AA sequence	Biological activity
Secretoneurin (SN)	TNEIVEEQYTPQSLATLESVFQELGKLTGPNNQ	Yes
15 c-terminal AA (SN1)	SVFQELGKLTGPNNQ	Yes
10 c-terminal AA (SN2)	LGKLTGPNNQ	No
15 c-terminal AA + cysteine (SN4C)	SVFQELGKLTGPNNQC-NH2	Yes

2.4. In vitro stability assay

For stability studies C2–C12 mouse muscle cells were cultured on 24-well plates at a density of 1×10^5 cells per ml in 500 μl DMEM for 5 days. Afterwards, cells were incubated with different SN derivatives in an appropriate concentration ratio to the cell culture medium. After predetermined time points within 4 h of incubation the supernatant was removed, lyophilized and the residue was dissolved in 1 ml of mobile phase and 20 μl of the aliquot was injected into the HPLC system for analysis. HPLC analysis was achieved using a Nucleosil 100-5 C18 HPLC column, 125 × 4.6 mm i.d., 5 μm particle size, 100 Å pore size (Macherey-Nagel, Germany) as stationary phase and eluents for mobile phase were (A) 0.1% trifluoroacetic acid in water and (B) in acetonitrile. A gradient elution from 10% to 90% of (B) was performed with a flow rate of 1 ml/min, an oven temperature of 40 °C and a running time of 20 min. Products were detected at a wavelength of 215 nm with a diode array absorbance detector. A calibration curve was established by linear regression from peak areas versus nominal concentrations. In case of SN4C release studies, loaded nanoparticles were distributed in 1 ml DMEM without phenol red, aliquoted in 24-well plates and subsequently incubated for 71 h in an incubation chamber at 37 °C. At predetermined time points samples were taken, centrifuged and the supernatant was used for detection via HPLC.

2.5. Western blotting

HUVECs were maintained as described above, plated on 60 mm tissue culture dishes and starved with EGM-2 medium without supplements for 16 h. Cells were stimulated with SN or SN derivatives at a concentration of 10^{-8} M for different time periods. Afterwards, cells