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Controlled release of vascular endothelial growth factor using poly-lactic-co-glycolic acid microspheres: In vitro characterization and application in polycaprolactone fumarate nerve conduits

Jing Rui [a,b], Mahrokh Dadsetan [c], M. Brett Runge [c], Robert J. Spinner [a], Michael J. Yaszemski [c], Anthony J. Windebank [d], Huan Wang [a,e,*]

[a] Department of Neurological Surgery, Mayo Clinic, Rochester, MN 55905, USA
[b] Department of Hand Surgery, Huashan Hospital, Fudan University, Shanghai 200040, People's Republic of China
[c] Departments of Orthopedic Surgery and Biomedical Engineering, Mayo Clinic, Rochester, MN 55905, USA
[d] Department of Neurology, Mayo Clinic, Rochester, MN 55905, USA
[e] Key Laboratory of Peripheral Nerve Microsurgery, Huashan Hospital, Fudan University, Shanghai 200040, People's Republic of China

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ABSTRACT

Vascular endothelial growth factor (VEGF) is a potent angiogenic stimulator. Controlled release of such stimulators may enhance and guide the vascularization process, and when applied in a nerve conduit may play a role in nerve regeneration. We report the fabrication and in vitro characterization of poly-lactic-co-glycolic acid (PLGA) microspheres encapsulating VEGF and the in vivo application of nerve conduits supplemented with VEGF-containing microspheres. PLGA microspheres containing VEGF were prepared by the double emulsion–solvent evaporation technique. This yielded 83.16% of microspheres with a diameter <53 μm. VEGF content measured by ELISA indicated 93.79 ± 10.64% encapsulation efficiency. Release kinetics were characterized by an initial burst release of 67.6 ± 8.25% within the first 24 h, followed by consistent release of approximately 0.34% per day for 4 weeks. Bioactivity of the released VEGF was tested by human umbilical vein endothelial cell (HUVEC) proliferation assay. VEGF released at all time points enhanced HUVEC proliferation, confirming that VEGF retained its bioactivity throughout the 4 week time period. When the microsphere delivery system was placed in a biosynthetic nerve scaffold robust nerve regeneration was observed. This study established a novel system for controlled release of growth factors and enables in vivo studies of nerve conduits conditioned with this system.

1. Introduction

Nerve regeneration requires cells, extracellular matrix, growth factors and a complex interplay between the three. When a nerve defect is repaired with a single lumen conduit these components are not present. Introduction of growth factors (GF) or cells into a conduit would encourage axon growth in tissue engineering implants [1]. Most studies have focused on application of GF with neurotrophic effects. Since in the process of nerve regeneration, especially that after nerve graft repair or conduit repair, revascularization precedes regeneration the current study aims at the delivery of pro-angiogenic GF to the regenerating nerve site. Pro-angiogenic GF promote angiogenesis, leading to increased transportation of oxygen and nutrients to the nerve tissue. One important pro-angiogenic GF is vascular endothelial growth factor (VEGF). VEGF is a homodimeric glycoprotein that increases microvascular permeability, stimulates the proliferation and migration of endothelial cells, and promotes angiogenesis [2–4]. VEGF also directly affects neurons and glial cells by inhibition of apoptosis, promotion of survival and stimulation of neurogenesis. The angiogenic effect of VEGF on vascular remodeling may play an important indirect role in nerve regeneration.

The mode and timing of GF delivery is as important as the GF itself. Two major strategies have been used to administer angiogenic growth factors: application of exogenous recombinant human protein and gene transfer to induce endogenous secretion of the target growth factor [2–5]. There has been some success in pre-clinical animal models and initial clinical trials of pro-angiogenic GF delivery [6–8]. However, double-blind clinical trials with large cohorts of patients failed to show the efficacy of intravenous infusions of recombinant human VEGF or VEGF gene transfer ther-

* Corresponding author at: Department of Neurological Surgery, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA. Tel.: +1 507 284 1188; fax: +1 507 284 3383.
E-mail address: wang.huan@mayo.edu (H. Wang).

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apy [9–12]. This lack of effect has been attributed to the requirement for a high level of biological activity (in the pico- to nanomolar range), pleiotrophic effects (acting on variety of targets) and short biological half-life [13]. To solve these problems many delivery systems that control the release of therapeutic agents have been developed. Examples include protein encapsulation or covalent linkage to an implant, protein encapsulation in microspheres, and subcutaneous implantation of osmotic minipumps [14]. Each delivery system has its advantages and disadvantages. Proteins encapsulated or covalently linked to implants are easily degraded and exhibit reduced bioactivity. Implanted osmotic pumps and injection devices require a second operation for device removal. Microsphere delivery systems are of increasing interest because the localized delivery systems release bioactive molecules directly to the desired site. This mitigates the risk of premature enzymatic degradation and allows sustained release of the therapeutic agent [15].

In this study we have fabricated PLGA microspheres containing VEGF121 by double emulsion–solvent evaporation. Degradation of PLGA microspheres and the release kinetics of VEGF were characterized. The bioactivity of released VEGF from PLGA microspheres was verified by the human umbilical vein endothelial cell (HUVEC) proliferation assay. These VEGF-containing microspheres were then implanted into the lumen of a polycaprolactone fumarate (PCLF) conduit to bridge a 1 cm gap in rat sciatic nerve.

2. Materials and methods

2.1. Materials

PLGA (5050 DLG 4A, Lakeshore Biomaterials, Birmingham, AL) with a 50:50 lactic acid to glycolic acid ratio, 0.35–0.45 dl g^{-1} inherent viscosity and 29 kDa molecular weight was used. Recombinant human VEGF (isoform 121) was purchased from R&D systems (Minneapolis, MN). A Quantikine VEGF ELISA kit was also obtained from R&D systems and used according to the manufacturer's instructions. Bovine serum albumin (BSA), poly(vinyl alcohol) (PVA) (87–89% hydrolyzed) and isopropyl alcohol were from Sigma–Aldrich (St. Louis, MO). Dulbecco's phosphate-buffered saline (PBS) was obtained from Invitrogen (Grand Island, NY). Methylene chloride was purchased from Fisher Scientific (Pittsburgh, PA). The HUVEC line and endothelial cell growth medium-2 (EGM-2) used in the bioactivity studies were both purchased from Clonetics (Walkersville, MD). A CellTiter 96 Q non-radioactive cell proliferation assay kit was obtained from Promega (Madison, WI) for the MTS assay and used according to the manufacturer's instructions. Syringe filters (Supor® Membrane, low protein binding, 0.2 μm) were purchased from Acrodisc (Port Washington, NY).

2.2. Preparation of VEGF-containing microspheres

A water in oil in water (w1–o–w2) double emulsion–solvent evaporation technique (L1) was applied to fabricate PLGA microspheres containing VEGF (0.1 μg mg microspheres^{-1}). Briefly, VEGF stock solution (250 μg ml^{-1}) was prepared under sterile conditions with PBS containing 0.25% (w/v) BSA as per the manufacturer's recommendation. 100 μl of VEGF stock solution was emulsified in a solution of 250 mg PLGA in 1 ml of methylene chloride for 30 s on a vortex device (Vortex Genie, Fisher, Pittsburgh, PA) at setting nine. The mixture was re-emulsified at the same vortex speed for another 30 s in 2 ml of 2% (w/v) PVA solution to create the double emulsion. This was then added to 100 ml of 0.3% (w/v) PVA solution and 100 ml of 2% (w/v) isopropyl alcohol solution and stirred for at least 1 h to evaporate the methylene chloride. The microspheres were collected and washed three times with distilled water by centrifugation at 2000 rpm for 3 min. A free flowing microsphere powder was prepared by freezing them at −80 °C for 1 h and vacuum drying overnight (Savant Speedvac Systems, NY). In order to obtain smaller and more even microspheres a 53 μm sieve (Humboldt) was used. Two batches of VEGF–PLGA microspheres were produced. Another three batches of PLGA microspheres without VEGF (plain microspheres) were produced for microsphere characterization. To investigate the effect of BSA on VEGF preservation and encapsulation microspheres containing VEGF and an excess of BSA (denoted VEGF–BSA(2000)-containing microspheres) were fabricated. The fabrication procedure was identical to the VEGF-containing microspheres with the exception that in addition to 100 μl of VEGF a 2000-fold excess of BSA was emulsified in a solution of 250 mg PLGA during the fabrication process.

2.3. Microsphere morphology

The morphology of the microspheres was visualized using scanning electron microscopy (SEM) (Hitachi S4700, Pleasanton, CA). A small quantity of plain microspheres was sprinkled on the SEM stubs and coated using a SEM coating system (Bio-Rad, Polaron Division) with gold palladium under an argon atmosphere using a gold palladium sputter module in a vacuum evaporator. Samples were then observed for their surface morphology by SEM and photographs were taken at 180× magnification. One stub was made for each batch and seven pictures were taken of each stub. ImageJ software (NIH) was used to measure the diameter of the microspheres from the pictures. All the microspheres (n = 1758) included in the micrographs were measured and the diameter of the microspheres was expressed as the mean ± SD.

2.4. Encapsulation efficiency

The encapsulation efficiency of sieved VEGF–PLGA microspheres was determined by extracting and quantifying the encapsulated VEGF. Ten milligram sieved VEGF–PLGA microspheres were dissolved in 2 ml of methylene chloride at 37 °C. VEGF was extracted from the organic layer with 2 ml of distilled water three times and combined. A total of 6 ml of supernatant was collected and the VEGF content measured by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's procedures. Briefly, 50 μl of assay diluent and 200 μl of VEGF standard solution or samples were added to the wells of a microplate coated with a monoclonal antibody against VEGF and incubated for 2 h at room temperature. The microplates were then washed three times with wash buffer. After complete removal of the wash buffer 200 μl of VEGF conjugate was added and incubated for another 2 h at room temperature. After washing, 200 μl of substrate solution was added and incubated for 20 min at room temperature in a light-proof container. Then 50 μl of stop solution was added and, following color development, the optical density (OD) was determined at 450 nm, corrected to 540 or 570 nm, within 30 min with a microplate reader (Spectramas Plus 384, Molecular Devices). The measured VEGF content was divided by the loaded VEGF content (0.1 μg mg^{-1} × 10 mg) to calculate the encapsulation efficiency. The encapsulation efficiency of VEGF in VEGF–BSA(2000)-containing microspheres was measured in the same way as that of VEGF-containing microspheres.

2.5. Degradation of PLGA microspheres

Both mass loss and molecular weight loss were determined in our experiment to delineate the degradation of PLGA microspheres. Twenty milligram of sieved plain microspheres were added to a 1.5 ml microcentrifuge tube and weighed on an electronic balance