Characterization of a Vascular Endothelial Growth Factor–loaded Bioresorbable Delivery System for Pulp Regeneration

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Abstract

Introduction: Vascular endothelial growth factor (VEGF) is a signal protein that stimulates angiogenesis and vasculogenesis and has been used in tissue regeneration and pulp regeneration experimental models. The purpose of this study was to develop a delivery system composed of a biodegradable fiber and controlled release of VEGF to promote cell viability and secure an adequate blood supply for the survival of human stem cells of the apical papilla (SCAP) favoring endodontic regenerative procedures. **Methods:** We developed a polydioxanone fiber, 50 μm in diameter, loaded with VEGF at a linear concentration of 12.2 ng/cm. Cytotoxic effects of the VEGF-loaded fiber (VF) on SCAP and mouse fibroblasts were assessed by using a multiparametric assay kit (XTT-NR-CVDE [Xenometrix, Allschwil, Switzerland]). We evaluated VF-induced mRNA expression of downstream growth factors by using a human growth factor Taqman array in real-time polymerase chain reaction. We also assessed the *in vivo* subcutaneous reaction of C57BL/6 mice to implants of VF alone and human root fragments (10 mm in length) filled with VF after 10, 20, and 45 days. Statistical analyses were performed by using analysis of variance and Student *t* tests or non-parametric alternatives. **Results:** Enzyme-linked immunosorbent assay verified detectable concentrations of released VEGF in solution for 25 days. No cytotoxicity was observed on SCAP and mouse fibroblasts treated with VEGF. In addition, VEGF treatment also induced the expression of additional growth factors with roles in tissue and blood vessel formation and neuroprotective function. Implantation of VF and root fragments filled with VF showed biocompatibility *in vivo*, promoting new blood vessels and connective tissue formation into the root canal space with negligible inflammation. **Conclusions:** Our results show that the VF used in this study is biocompatible and may be a promising scaffold for additional optimization and use in endodontic regenerative procedures. *(J Endod 2017;43:77–83)*

Keywords

Biomaterials, growth factors, pulp regeneration, VEGF

Significance

VEGF is able to induce stem cell differentiation into endothelial cells and modulate tooth development and dentin formation. This study supports that VEGF-loaded fiber may be considered a viable option to stimulate angiogenesis and new tissue formation during endodontic regeneration procedures.

Treatment of immature necrotic teeth remains a challenge in endodontics. Arrested root development after pulp necrosis can lead to weak root structure with thin dentinal walls, which makes the tooth susceptible to fracture and reduces its survival rate (1). Tissue engineering has become a viable option and an attractive strategy for dental pulp tissue regeneration through the combined use of stem cells, bioactive molecules such as growth factors, and a biomaterial support system or scaffold (2). Current protocols for pulp regeneration show promising results in terms of healing of periapical lesions and survival rate of treated teeth compared with traditional methods (3). However, the outcomes are still unpredictable (4).

Stem cells play essential roles in organ development and tissue repair (4). Pluripotent embryonic stem cells can give rise to multipotent stem cells, including epithelial, mesenchymal, endodermal, and other tissue-specific stem cells, to generate all the tissues in an organism (4, 5). Lovelace et al (6) showed the accumulation of stem cell markers such as CD73, CD105, and STRO-1 in the blood collected from the root canal system after bleeding was evoked during a clinical regenerative endodontic protocol, suggesting that the evoked-bleeding step in regenerative procedures triggers the significant accumulation of undifferentiated stem cells into the canal space, and these cells might contribute to the regeneration of pulpal tissues. The stem cells of the apical papilla (SCAP) have been

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described as an important population of stem cells with potent osteogenic and dentinogenic capacity similar to dental pulp stem cells and are likely involved in endodontic regenerative procedures (7, 8).

Although SCAP are crucial for pulp regeneration, a suitable scaffold of extracellular matrix and appropriate growth factors are also important factors in regenerative therapies. Hyaluronic acid, platelet-rich plasma, fibrin, and a variety of hydrogels have been studied as potential scaffolds for pulp regeneration (9–11). Growth factors released from dentin are critical for the generation of a favorable pulp tissue regenerative microenvironment. More specifically, the controlled release of growth factors such as transforming growth factor beta 1 and fibroblast growth factor 2 sustained their biological activities after 21 days and significantly increased dental pulp cell proliferation *in vitro* (12).

It has been suggested that the use of angiogenic-inducing factors is needed to ensure or enhance pulp angiogenesis (13). In fact, angiogenesis is a key step for regeneration after pulpal injury because adequate blood supply is essential to accomplish tissue regeneration, and without it, necrotic or scar tissues are formed (1). Vascular endothelial growth factor (VEGF) is the most potent angiogenic and vasculogenic factor, promoting endothelial cell proliferation, migration, and survival (1, 14). Studies have shown that VEGF is able to induce stem cell differentiation into endothelial cells and to modulate tooth development and dentin formation (15, 16). On the basis of these observations, we hypothesize that the biological activity of VEGF released from a biodegradable fiber would serve as a growth-factor delivery system able to induce angiogenesis as well as cell migration, proliferation, and differentiation when placed in the root canal system for pulp regeneration. In this study, we investigated the usefulness of a VEGF-loaded polymer fiber as a growth factor–loaded scaffold for pulp tissue regeneration.

Materials and Methods

VEGF-loaded Fiber

A biodegradable drug-loaded fiber was developed by using a polydioxanone fiber of 50 μm in diameter and loaded with VEGF at a linear concentration of 12.2 ng/cm. Fiber fabrication was contracted through Tissuegen Inc (http://www.tissuegen.com; Dallas, TX). Quality control tests such as *in vitro* protein release and degradation assays were provided by Tissuegen. Enzyme-linked immunosorbent assays confirmed the kinetic release of detectable concentrations of released VEGF in solution during a period of 25 days. A peak initial release of VEGF was observed during the first 5 days, and then a controlled slow release was observed until day 25. Results of *in vitro* degradation test in phosphate-buffered saline solution in pH 7.3 at 37°C indicated that a significant reduction of molar mass and reduction of mechanical properties occur in approximately 180 days.

Cell Culture

Human SCAP were provided by Dr Anibal Diogenes (University of Texas Health Science Center at San Antonio School of Dentistry) and cultured following published protocol (7). NIH-3T3 mouse fibroblasts were purchased from American Type Culture Collection (Manassas, VA) and cultured according to the supplier's protocol. Cells between passages 2 and 5 only were used in this study for the *in vitro* assays.

Multiparametric Cytotoxicity Assay

All sample preparations were performed inside a laminar flow hood as previously described (17). In brief, 10 mm VEGF-loaded fiber was inserted into 1000-μL pipette tips that were cut at 2.2 cm from their end to standardize the tip diameter at 0.8 mm. The tip containing VEGF fiber was then attached to the lid of a microcentrifuge tube by using an O ring (5 mm in diameter and 2 mm in thickness) and inserted into the tube containing 0.5 mL culture medium so that at least 1 mm of the tip containing the experimental material was completely immersed in the culture medium. Samples were further incubated at 37°C, 95% humidity, and 5% CO_2 for 12 hours, 24 hours, 48 hours, 72 hours, 7 days, and 14 days. The extracted elutes from the experimental material were clear in appearance and were used unfiltered.

We assessed the cytotoxic effects of the VEGF-loaded fiber in SCAP and mouse fibroblasts by using a multiparametric assay kit (In Cytotox; Xenometrix, Allschwil, Switzerland) as previously described (17). The multiparametric assay combines the use of 3 different parameters of cell survival and integrity on the same sample: 2,3-bis[2-methoxy-4-nitro-5-sulfopheny]-2H-tetrazolium-5-carboxyanilide inner salt (XTT), neutral red (NR), and crystal violet dye elution (CVDE) assays. The XTT assay measures mitochondrial dehydrogenase activity, in other words, the ability of mitochondrial enzymes from metabolically active cells to reduce XTT molecules to a soluble salt of formazan. The NR assay assesses cell membrane integrity as the vital NR dye is incorporated through endocytosis and accumulates preferentially on the lysosomes of membrane-intact viable cells. CVDE stains the DNA of the cells and estimates the density of cells.

SCAP and mouse fibroblast cells were seeded at a concentration of 2×10^4 cells/well in 2 separate 96-well plates and incubated for 24 hours. Cells were then exposed to fresh culture medium (negative control), 0.1% sodium dodecylsulfate (positive control), or elutes of each test material (incubated at each time point of 12 hours, 24 hours, 48 hours, 72 hours, 7 days, and 14 days) and incubated for 24 hours. All experiments were performed in triplicates in 3 independent reactions. Results were analyzed by using an enzyme-linked immunosorbent assay plate reader (Thermo Scientific, Asheville, NC) by using light absorbance at 450 and 530 nm, as recommended by the assay manufacturer.

Growth Factors mRNA Expression

We investigated the mRNA expression of growth factors in SCAP with and without VEGF-loaded fiber treatment. SCAP were cultured as previously described, treated with the VEGF-loaded fiber for 24 and 72 hours, and evaluated for growth factor mRNA expression by using a human growth factor real-time polymerase chain reaction Taqman array (Life Technologies, Grand Island, NY) (Supplemental Table 1).

A total of 100,000 SCAP/well were plated in a 12-well plate and incubated for 24 hours. The culture medium was then replaced with 1 mL of the 24-hour and 72-hour VEGF fiber elutes. Untreated cells served as negative controls. Total RNA extraction was performed, followed by cDNA synthesis by using the Taqman Cells-to-Ct kit (Life Technologies) according to the manufacturer's instructions. Total RNA concentration was measured by using a Nanodrop spectrophotometer (ND-1000 V3.30; Thermo Scientific).

Real-time quantitative polymerase chain reaction was performed in a ViiA7 Sequence Detection System (Life Technologies). Reaction conditions were as follows: 95°C (10 minutes), 40 cycles at 95°C (15 seconds), 60°C (1 minute), 95°C (15 seconds), then 60°C (1 minute), and a final stage at 95°C (15 seconds). Endogenous genes contained in the array plate were used for normalization. Reactions were performed in a final reaction volume of 20 μL in triplicates. Results were depicted as threshold cycle (Ct) values. Expression levels were calculated by using the comparative $2^{-\Delta\Delta}$Ct method (18).

In Vivo Subcutaneous Implantation

This study was approved by the University of Texas Health Science Center at Houston Committee for Protection of Human Subjects (HSC-DB-14-0303) and Animal Welfare Committee (AWC-13-150).