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Local delivery of a collagen binding FGF-1 chimera to smooth muscle cells in collagen scaffolds for vascular tissue engineering

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Abstract

We investigated the delivery of R136K-CBD (a collagen binding mutant chimera of fibroblast growth factor-1) with a type I collagen scaffold as the delivery vehicle to smooth muscle cells (SMCs) for vascular tissue engineering. The binding affinity of R136K-CBD to 3-D collagen scaffolds was investigated both in the presence and absence of cells and/or salts. 2-D and 3-D visualization of delivery of R136K-CBD into SMCs was accomplished by combined fluorescent and reflection confocal microscopy. The mitogenic effect of collagen-immobilized R136K-CBD on SMCs in 3-D collagen was studied by Cyquant assay at different time intervals. In the group devoid of salt and cells, no detectable release of R136K-CBD into overlying culture media was found, compared with burst-and-continuous release of R136K and FGF-1 over a 14-day period in all other groups. The release rate of R136K-CBD was 1.7 and 1.6 fold less than R-136K and FGF-1 when media was supplemented with 2M salt ($P < 0.0001$), and 2.6 and 2.5 fold less in cell-populated collagen hydrogels ($P < 0.0001$), respectively. R136K-CBD showed essentially uniform binding to collagen and its distribution was dependent on that of the collagen scaffold. Internalization of R136K-CBD into SMCs was documented by confocal microscopy. 3-D local delivery of collagen-immobilized R136K-CBD increased the proliferation of SMCs in the collagen matrix to significantly greater levels and for a significantly greater duration than R136K or FGF-1, with 2.0 and 2.1 fold more mitogenicity than R136K and FGF-1 respectively ($P < 0.0001$) at day 7. The results suggest that our collagen binding fusion protein is an effective strategy for growth factor delivery for vascular tissue engineering.

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Keywords

Controlled drug release; Growth factors; Cell proliferation; Collagen; Smooth muscle cell

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1. Introduction

Cardiovascular disease remains the leading cause of death in the United States, with an average of one death every 37 seconds[1]. Arterial bypasses are widely used in peripheral and coronary artery surgery for the treatment of vascular diseases. However, a large number of patients do not have suitable vessels for use as bypass conduits for various reasons[2]. Synthetic vessel alternatives commonly used for these patients continue to pose significant drawbacks [3,4]. For these reasons, efforts have been made in vascular tissue engineering to develop biologic conduits for bypass [5-7]. Typically, these constructions consist of two layers: an outer layer of a smooth muscle cell (SMC) or fibroblast populated gel equivalent of the blood vessel tunica media and an inner layer of an endothelial cell (EC) seeded equivalent of the blood vessel tunica intima. Promoting the transient proliferation of SMCs in the developing tunica media is critical to the development of a biologic blood vessel.

Various growth factors have been used to promote the proliferation of vascular SMCs in 2-D culture systems, such as platelet-derived growth factor[8], insulin-like growth factor-I[9] and fibroblast growth factor-1 (FGF-1)[10]. SMCs and other cell types, grown in 3-D scaffolds also show enhanced proliferation rates in response to soluble growth factors added into the culture medium [11-13]. thus relying on uncontrolled diffusion for growth factor delivery. In these systems, the even distribution of growth factors inside the scaffold typically takes hours to days and varies by scaffold composition, pore size and dimension and by the competitive presence of other proteins in the culture environment. In addition to the low delivery efficiency, another limitation of these systems is the significant waste of growth factor due to removal of non-diffused growth factors during media changes and the resultant inconstancy of the growth factor concentration.. This leads to the undesired compensatory requirement to deliver growth factors at concentrations which are greater than the likely cellular demand in order to maintain the minimal desired concentration within the scaffold.

In order to address these problems, several alternative delivery strategies have been developed. One of these is the local delivery of growth factors by incorporating them into the construct scaffold[12]. Petrigliano et al [14] incorporated basic fibroblast growth factor (bFGF, or FGF-2) into the scaffold, which allowed for direct contact with the cells and directly increased delivery efficiency. However, a limitation of this approach was the early burst release of 25% of incorporated bFGF over the first 10 hours and 40% over the first 24 hours. This was partly due to the large pore size of the scaffold material compared with the relatively small size of most growth factors. For example, the pore size of type I collagen scaffolds commonly varies from 66.8 μm (at 1.67mg/ml concentration) to 37.5 μm (at 2.5mg/ml concentration)[15], while the size of most growth factors are at the nanometer level or lower. Thus strategies which promote the retention of growth factor within the tissue engineering scaffold, such as improving binding of the growth factor to the scaffold, would be potentially beneficial provided retention of cellular recognition and signaling.

We have constructed a fusion protein consisting of R136K, which is a relatively thrombin-resistant mutant derivative of FGF-1, and a collagen binding domain (CBD) [16]. Previous research in our lab demonstrated that R136K-CBD had greater binding affinity to 2-D collagen surfaces compared with FGF-1, and had similar chemotactic and mitogenic effects on ECs in

a 2-D culture system compared with FGF-1. We also found that R136K-CBD demonstrated greater retention in fibrin hydrogels versus R136K and FGF-1[16]. Because R136K-CBD should have stronger affinity to collagen hydrogels compared to fibrin, we predicted that R136K-CBD would demonstrate even greater retention in collagen scaffold compared to R136K and FGF-1. This would thereby prolong the bioavailable of this growth factor subsequently promoting both SMC proliferation and endothelialization.

In this paper, we studied the affinity and distribution of R136K-CBD to 3-D collagen scaffolds. We used fluorescent and reflection confocal microscopy to visualize the internalization of R136K-CBD into SMCs within the scaffold. In addition, the mitogenic effect of collagen-immobilized R136K-CBD on SMCs in the scaffold was studied.

2. Material and Methods

2.1 Construction of R136K and R136K-CBD—R136K and R136K-CBD was constructed as described in our previous published protocols [16] [17]. In brief, R136K was generated using MORPH Site-Specific Plasmid DNA Mutagenesis Kit (5 Prime–3 Prime, Inc., Boulder, Co). R136K-CBD was generated by amplification of R136K by PCR and ligated to the TOPO vector. Both R136K and CBD were digested and transformed into H101 Cells. Miniprep DNA was purified and R136K-CBD Pet3c construction was transformed into BL21 cells for expansion. Cells were then collected and R136K-CBD was purified by heparin Sepharose affinity chromatography and reverse phase HPLC.

2.2 Free release assay in 3-D collagen scaffold—Bovine type I collagen scaffold was prepared according to the instructions from the manufacture (MP Biomedicals, Solon, OH). In brief, collagen stock was neutralized and mixed with 10X PBS (Sigma-Aldrich, St. Louis, MO) to form a collagen solution. Equimolar amounts of R136K-CBD, R136K or FGF-1 were mixed with the collagen solution for a final concentration of 0.03 nmol/well of growth factor, and 2mg/ml of collagen. 500µl of each mixture was transferred into wells of a 24-well plate in triplicate and incubated at 37°C. After one hour of incubation, 1ml of “release” media consisting of phenol red-free Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA), 50 µg/mL gentamicin (Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen) was added on top of the polymerized collagen scaffold. Media was changed at 4 hours, 8 hours and then daily for two weeks and the collected media was stored at 80°C until tested by ELISA assay. The wells of 96-well plates (Nalge Nunc International, Rochester, NY) were coated with 50ul of release media in triplicate at 4°C overnight. Wells were rinsed with PBS+0.05% Tween and blocked with 1% bovine serum albumin (BSA) at 37°C for 1h. After subsequently washing twice with PBS-Tween, the wells were coated with 50ul anti-human FGF-1 antibody (Peprotech, Rocky Hill, NJ) for 2 h at room temperature. The plates were washed again and then incubated with HRP-conjugated goat anti-rabbit secondary Ab (Bio-Rad, Hercules, CA). After further washing with PBS-Tween, 100µl of TMB (Sigma-Aldrich, St. Louis, MO) was added into the wells and the reaction was stopped by adding 50ul 2N HCL after 15 minutes. The plates were read at a wavelength of 450nm with a plate reader (PerkinElmer, Waltham, MA).

2.3 Binding assay in 3-D collagen against salt elution—R136K-CBD, R136K and FGF-1 were each labeled with Alexa Fluor 488 (Molecular probe, Invitrogen, Carlsbad, CA) according to the instruction of the manufacture. In brief, R136K-CBD was mixed with Alexa Fluor 488 dye in PBS supplemented with 100 mM sodium bicarbonate and reacted at room temperature for 1h. Unbound dye was eliminated by passing through a layer of Bio-Gel (Bio-Rad Laboratories, Hercules, CA). The concentrations of the labeled growth factors were quantified using a plate reader according to the manufacturer's instruction.

The labeled growth factors were mixed with collagen and incubated as described above. After 4 hours, NaCl solution was added into the release media at a final concentration of 2.0M. Release media without NaCl served as a control. The release media was then transferred to 96-well plates and read with a plate reader. The concentration of protein is linear to the intensity of fluorophore and the results were expressed as the percentage of total growth factor which was released into the media.

2.4 Binding assay in 3-D collagen populated by SMCs

2.4.1 Canine SMC harvest and culture: Animal care complied with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996) and the Principles of Laboratory Animal Care (National Institutes of Health publication no. 85-23, revised 1985).

SMCs were obtained from canine carotid arteries and identified and expanded by our previously published explant cell culture tech Pniques [18]. In brief, the carotid arteries were opened longitudinally, and the intima and adventitia were removed by scraping and dissecting with a scalpel. The medial layer was minced and placed into SMCs growth media consisting of Dulbecco modified Eagle medium (DMEM) (Invitrogen), 10% FBS (Hyclone, Logan, UT), 10 mmol/L L-nonessential amino acids (Invitrogen), 100 mmol/L sodium pyruvate (Invitrogen), 50 µg/mL gentamicin (Invitrogen), 100 U/mL penicillin, and 100 µg/mL streptomycin. Primary SMCs migrating from the explants were used in all experiments and only cells exhibiting 95% or greater positive staining for SMC α -actin were used in the assays.

2.4.2 Determination of the amount of growth factor maximally increasing the

proliferation of SMCs: In order to determine the growth factor concentration associated with maximal SMC proliferation, 500 µl of a mixture which consisted of serial concentrations of growth factors (0.003nmol to 0.06nmol per hydrogel based on our previous results from 2-D SMC proliferation studies [16]), 1mg collagen and 5×10^5 SMCs, was transferred to the wells, in triplicates, of 24-well plates and incubated for 1 h at 37°C with 5% CO₂. One ml of culture media was then added to each well, and incubated for 16 hours. Collagen hydrogels were digested by collagenase(Worthington, Lakewood, NJ) and cell proliferation was quantified by the CyQUANT Cell Proliferation Assay Kit (Invitrogen) according to the instructions from the manufacture. In brief, collected cells were frozen at -80°C and stored until tested. Cells were thawed and stained with CyQUANT GR dye and read with a plate reader and analyzed with Workout software (Wallac, Turku, Finland). The dye exhibits strong fluorescence enhancement when bound to cellular nucleic acid, and the rate of proliferation is calculated based on the intensity of fluorescence.

2.4.2 Binding assay: R136K-CBD, R136K or FGF-1 was mixed in equimolar concentrations with SMC-populated collagen solutions prior to polymerization. 500 µl of the mixture, which consisted of 0.09 nmol of growth factors (based on the results from studies of Section 2.4.2 above, which is three times the maximal effective concentration) and 1mg of collagen and 5×10^5 SMCs, was transferred to the wells of 24-well plates and incubated for 1 h at 37°C with 5% CO₂. After polymerization, one ml of culture media was added to each well and then incubated for 16 hours. The culture media was then collected and analyzed by ELISA with results expressed as above.

2.5 Visualization and quantification of distribution of R136K-CBD in 3-D collagen

2.5.1 Image acquisition: R136K-CBD was labeled and mixed with collagen solution as above. The mixture was transferred to 20 mm diameter glass bottom Petri dishes (MatTek, Ashland, MA) and incubated one hour at 37°C. After polymerization of the hydrogel, 2 ml of PBS was added, and samples were incubated overnight. Hydrogels were then rinsed with PBS three

times and studied using a ZeissLSM510 laser scanning confocal microscope (Carl Zeiss, Inc. Thornwood, NY). Fluorescent mode was used to visualize labeled R136K-CBD using excitation with the 488 nm Argon laser and detection of emitted light through 585nm long-pass filter. Collagen fibers were visualized in reflection mode according to our previous protocol [19,20]. Zeiss LSM software was used for image acquisition. Z-stacks were also acquired with 1µm incensement and five random different visual fields were chosen in each of four gels to take z-stack images for analysis.

2.5.2 Image processing and quantification: ImageJ software and our self-written software were used for Image processing[20]. In brief, 3-D image reconstructions were made and images in fluorescent and reflection channels were segmented into binary images with a threshold. The area occupied by the pixels of R136K-CBD represents the distribution of the growth factor. Quantification of the distribution was made according to the pixel distribution in the binary image. In each focal plane, the density of R136K-CBD was normalized to that of collagen in order to eliminate the variations of collagen density using the following equation:

$$100 \times \frac{\text{Total pixels in the fluorescent channel}}{\text{Total pixels in the reflection channel}}$$

2.6 Visualization of R136K-CBD delivery to SMCs in 3-D collagen

2.6.1 Sample preparation: R136K-CBD was labeled as above and SMCs were labeled with PKH26 (Sigma) per our previous protocol[21]. The labeled R136K-CBD (0.015 nmol) and SMCs (2.5×10^5) were mixed with collagen solution (0.5mg), and 250 µl of the mixture was transferred to 20 mm diameter glass bottom Petri dishes (MatTek, Ashland, MA). After one hour of incubation, the collagen hydrogel was fully polymerized and 2ml of culture media supplemented with 5U/ml heparin was added on top of the collagen for incubation overnight. Then the hydrogels were used for the assays described below. Another group was similarly assayed immediately after the one-hour polymerization-incubation in order to assess potential R136K-CBD internalization during polymerization when R136K-CBD is soluble or semi-soluble.

2.6.2 2-D visualization: Collagen hydrogel mixtures were transferred to 15ml centrifuge tubes and incubated with 1.5ml of collagenase (600U/ml) (Worthington Biochemical Corp., Lakewood, NJ) for 0.5 to 1 h in an incubator shaker at 37°C. Bovine serum, 300 µl (Hyclone), was then added to stop the collagenase digestion. The digested solution was centrifuged, the supernatant aspirated, and the SMCs were resuspended in culture media and seeded on top of a cover slip in a 60mm Petri dish. After 6h of incubation, the cells were fixed with 4% formaldehyde for 20 minutes, mounted on a slide, and observed using a Zeiss LSM510 confocal microscope. SMCs were visualized with differential interference contrast (DIC) microscopy and internalization of labeled R136K-CBD was visualized with fluorescent microscopy.

Polymerized collagen hydrogels containing R136K-CBD but no SMCs were also created and digested by collagenase as described. The digested media was analyzed by ELISA to confirm it has been digested completely by collagenase in order to eliminate the possibility of internalization during collagen digestion when R136K-CBD could be in soluble or semi-soluble condition if not digested completely.

2.6.3 3-D visualization: 2.6.3.1 Confocal Microscopy: The collagen hydrogels were rinsed three times with PBS and fixed with warm formaldehyde and were studied under confocal microscopy. Fluorescent microscopy was used to visualize Alexa Fluor 488 labeled R136K-CBD and PKH26 labeled SMCs. Collagen fibers were visualized using reflection microscopy.

2.6.3.2 Image Processing: 3-D image reconstructions were made using ImageJ 3-D Viewer and our self-written software as above.

2.7 3-D proliferation assay—Equimolar concentrations of R136K-CBD, R136K or FGF-1 were mixed with SMCs and the collagen. 500 μ l of the mixture, which consisted of 0.15 nmol growth factors, 1mg collagen and 5×10^5 SMCs, was transferred to the wells in 24-well plates and incubated for 1 h at 37°C with 5% CO₂. The mixture scaffold was then transferred to a 60mm Petri dish and cultured in 5ml of complete culture media supplemented with heparin. One group cultured in the absence of growth factor served as the control.

Collagen scaffold constructs were digested by collagenase at Day 1, 3, 5 and 7, and SMCs were collected as above. Proliferation of collected cells was analyzed by Cyquant assay. The results were normalized to that of the control.

2.8 Statistics—One-way ANOVA was used to evaluate the differences of among groups with SPSS software (SPSS Inc, Chicago, IL) with the level of significance at $P < 0.05$. Tukey post-hoc analysis was also performed to determine differences between every two groups. The results are expressed as mean $\pm$ SD.

3. Results

3.1 R136K-CBD binding to 3-D collagen—R136K-CBD showed significantly greater binding affinity to collagen compared with R136K and FGF-1. R136K-CBD was undetectable in release media for up to 2 weeks, compared with R136K and FGF-1, which demonstrated $(9.27 \pm 0.59)\%$ and $(8.47 \pm 1.43)\%$ burst release in the first two days and continuous release for the remaining 12 days (Fig. 1). There were no significant differences in release between R136K and FGF-1 groups. After salt elution with 2M NaCl, (Fig. 2), the percentage release of R136K-CBD ($36.18 \pm 4.16\%$) was 1.7-fold less than that of R136K ($61.01 \pm 6.41\%$), and 1.6-fold less than FGF-1 ($59.14 \pm 4.61\%$) ($P < 0.0001$ for R136K-CBD vs. either R136K or FGF-1). A 2M salt concentration was selected as the optimum concentration for generating the highest elution effect based on our previous results in 2-D assays. More significantly, R136K-CBD showed even stronger binding to SMC-populated collagen (Fig. 3). After 17 hours of incubation when most cell generated collagen hydrogel compaction had occurred, burst release of R136K-CBD ($12.37 \pm 2.87\%$) from collagen into media was 2.6 fold and 2.5 fold less than that of R136K ($32.04 \pm 5.05\%$) and FGF-1 ($30.81 \pm 7.11\%$) respectively ($P < 0.0001$ for R136K-CBD vs. either FGF-1 or R136K). No burst was found in the condition without cells or salt.

3.2 Visualization of R136K-CBD in 3-D collagen—As shown in Fig. 4, we used fluorescent confocal imaging to visualize the labeled R136K-CBD and reflection confocal microscopy to visualize collagen fibers without any fluorescent label. By overlaying the images from two channels, we are able to visualize the distribution of R136K-CBD in collagen fibers, and demonstrated that R136K-CBD co-localized to collagen fibers and formed very small aggregated particles.

3.3 Quantification of R136K-CBD distribution—In each channel of R136K-CBD or collagen, we thresholded and segmented the images into binary images. After normalizing the pixel density to that of the collagen, we did not find significant differences in the pixel intensities, which represent the density of R136K-CBD, between any two randomly selected z-stacks. The cone column chart (Fig 5) graphically represents the distribution of R136K-CBD throughout the collagen hydrogels in all z-stacks analyzed. We found that R136K-CBD bound to about 50% of the area of the collagen matrix. Because the intensity of fluorescence is normalized to that of the collagen, variability in collagen density is eliminated, and thus the results demonstrate that the distribution of R136K-CBD is totally dependent on the density of

the collagen fibers. This suggests that R136K-CBD is bound to collagen rather than suspended in the media within collagen fibers.

3.4 Visualization of internalization of R136K-CBD into SMCs—In 2-D analysis, as shown in Fig 6, R136K-CBD localized to the cytoplasm and perinuclear area of the SMCs isolated from collagen-bound R136K-CBD. No detectable R136K-CBD was found in SMCs in control hydrogels which were digested immediately after polymerization, eliminating the possibility of internalization during digestion.

In 3-D analysis, we then analyzed the R136K-CBD (Fig. 7 A) and SMC channels in a focal plane inside the SMC within the collagen hydrogel in the Z axis (Fig. 7 B). Because PKH-26 is a general cell membrane label, unlabelled areas in the X-Y axis within the red outline of the cell represent intracellular areas. Green fluorescent R136K-CBD localized to the intracellular portions of the images in the X-Y-Z dimensions (Fig. 7 B). The 3-D image (Fig. 7 C) shows that SMCs located within the collagen fibers to which the R136K-CBD was bound, and that there was R136K-CBD located in the cytoplasm and perinuclear area of the cell.

3.5 Mitogenic effect of R136K-CBD on SMCs in 3-D collagen—At Day 3, all of the collagen hydrogels containing growth factors demonstrated a significantly higher mitogenic effect than control groups without growth factor ($p < 0.001$). R136K-CBD induced a significantly greater mitogenic effect than either R136K or FGF-1. There were no significant differences between R136K and FGF-1. After a media change at Day 3, R136K-CBD-containing hydrogels showed a significantly greater mitogenic effect compared to R136K and FGF-1 at both Day 5 and Day 7. Our previous work showed that the pore size of collagen fibers decreased significantly after SMC-induced remodeling [20], making media and nutrient transport into the center of the hydrogel more difficult, thus attenuating cell proliferation in response to added soluble growth factor. In the current study, a decrease in mitogenicity of R136K and FGF-1, but not R136K-CBD, was found at Day 7. Proliferation in response to R136K decreased from 3.3 fold of controls at Day 3 to 2.6 fold over the controls at Day 7. Similarly, in 3-D assays, proliferation in response to FGF-1 decreased from 3.1 fold of controls at Day 3 to 2.5 of control at Day 7. In contrast, proliferation in response to R136K-CBD increased from the 4.9 fold of controls at Day 3 to 5.3 fold of controls at Day 7. Proliferation in R136K-CBD stimulated groups at Day 7 was 2 fold greater than R136K and 2.1 fold greater than FGF-1.

4. Discussion

Optimizing growth factor delivery to engineered tissues is a critical strategy in tissue engineering [22-24]. Standard methods, such as adding growth factors into culture media, rely on diffusion within the media for growth factor interaction with cells to induce a biologic response. In these systems, strategies to modify the biologic response are limited to alterations in growth factor concentration, and do not address the uncontrolled, random delivery modality to the cells [25,26].

In order to more precisely control kinetics of growth factor delivery, the development of new delivery strategies is critical. Recently, the strategy of specifically binding growth factors to the scaffold substrate has been used to promote cell-demanded growth factor delivery [27,28]. With these methods, patterned local control of growth factor concentrations within the scaffold allows for more precise biologic responses.

As collagen is a widely used cell culture substrate, growth factors fused to collagen binding domains attracted research interest [29,30]. Collagen-binding growth factors bound to collagen coated culture dishes have in some studies [16,29,31,32] demonstrated altered and often greatly improved biologic effects compared to their soluble counterparts. In tissue engineering, these

techniques must be translated into 3-D scaffold based culture systems, which are fundamentally different from 2-D systems [33]. Recently, a semi-3-D culture system was successfully used for the local delivery of collagen binding hepatocyte growth factor (CBD-HGF) [30]. In this study, a single layer of endothelial cells were seeded on top of a 2-D collagen surface bound to CBD-HGF and overlaid with a layer of hydrogel. Bound CBD-HGF produced an enhanced angiogenic effect compared to soluble HGF.

In our study, we have further translated this approach to a growth factor delivery system in a true 3-D culture system while preserving all advantages of the collagen binding domain.

First we tested the binding affinity of R136K-CBD to a 3-D collagen scaffold. R136K-CBD showed strong affinity to the collagen scaffold in both the free release assay and the salt elution assay, consistent with the result of our previous work and other collagen binding growth factor chimeras [16,29,31,34]. We recognize that high concentrations of salt do not represent physiological levels of salt, and, therefore, the significance of utilizing CBD to improve the retention of R136K requires further exploration. Encouraged by these results as proof of concept, we further tested binding affinity of R136K-CBD in the presence of SMCs to study the benefits of growth factor retention on cellular responses which would be required for tissue engineering. It is likely that via extracellular matrix proteolysis, SMCs increase the rate of growth factor release from the matrix, as would be seen *in vivo*. Encouragingly, R136K-CBD showed significantly greater retention by collagen than either FGF-1 or R136K when SMCs were present within the scaffold. In previous studies, *in vivo* assays have shown the strong binding characteristics of CBD fusion growth factors, as, for example, by direct injection of growth factor into the subcutaneous tissue of an animal [29].

After characterizing the retention of R136K-CBD in 3-D collagen hydrogels, we studied the distribution of R136K-CBD within scaffolds using our previously published imaging techniques[20]. Our results showed that the distribution of R136K-CBD was almost completely dependent on that of collagen fibers. This suggests that incorporated R136K-CBD would likely have essentially uniform biologic effect on SMCs throughout the collagen, an important point for engineering a blood vessel or other tissue constructs.

We then turned our attention to studying the efficiency of delivery of R136K-CBD to the SMCs, as our ultimate goal is to optimize cellular internalization and biologic response. In order to directly visualize R136K-CBD internalization, we fluorescently labeled both R136K-CBD and the SMCs. After a period of incubation, we isolated the SMCs from the collagen and visualized internalized R136K-CBD with confocal microscopy. Z stack scanning into the middle focal plane of the cell confirmed the cytoplasmic and perinuclear localization of R136K-CBD. Our results are consistent with previous studies of the localization of internalized soluble FGF-1[35,36]. Because R136K-CBD exists in both a soluble and semi-soluble state during collagen polymerization and digestion, there is the possibility that R136K-CBD transferred into the SMCs in these two conditions. However, results suggesting that internalization of collagen-immobilized growth factor occurs is supported by two points: 1) We didn't find the internalized R136K-CBD immediately after complete polymerization of collagen; and 2) As we used high concentration collagenase-digestion (600U/ml) to isolate, any R136K-CBD remaining extracellularly would be susceptible to non-specific proteolytic digestion by the collagenase. Therefore, we concluded that the internalization of R136K-CBD occurred in a process which involves cell-demanded release and internalization.

We further studied the delivery in 3-D by “on-site” visualization of the internalization. Reflection confocal imaging showed collagen fibers and fluorescent confocal imaging showed the R136K-CBD bound to collagen fibers. We visualized the internalized R136K-CBD within

labeled SMCs. By 3-D structure reconstruction, their spatial relationships were clearly visualized.

After confirmation of longer retention, efficient delivery, and cellular internalization, we assayed the biologic activity of R136K-CBD by studying its mitogenic effect in the 3-D collagen scaffold. From our results, all three growth factors delivered within the scaffold showed similar significant mitogenicity above negative controls during the first two days of culture. However, after the first media change at Day 3, proliferation of R136K-CBD groups significantly exceeded proliferation of all other groups. This effect persisted throughout the remainder of the experiment. We believe this is likely due to the high affinity of R136K-CBD to collagen compared to FGF-1 and R136K. At Day 5, R136K-CBD demonstrated 1.5 fold greater mitogenic potential than R136K and 1.6 greater than FGF-1. At Day 7 R136K-CBD demonstrated 2.0 and 2.1 fold greater mitogenic effect than R136K and FGF-1, respectively. The persistent mitogenicity of R136K and FGF-1 at Day 5 is likely due to delayed cell proliferation in response to growth factor activity prior to removal by media change. This is supported by results at Day 7 which demonstrated a significant drop in cell proliferation in R136K and FGF-1 stimulated groups compared to the increasing proliferation in R136K-CBD stimulated groups.

Previous research has shown that the proliferative activity of SMCs in 3-D collagen is often negligible [13] and supplementing growth factor in the culture media showed relatively little or no increase in proliferation. We believe one important explanation is the large extent of collagen volume compaction impeding soluble growth diffusion into the scaffold. We confirmed this by adding growth factor into the media at day 5 after collagen compaction hoping to enhance the proliferation in a group in the absence of growth factor initially (data not shown). The cells' proliferative response was minimal.

5. Conclusion

In this paper, we demonstrated a novel growth factor delivery strategy employing a collagen binding fusion mutant protein-R136K-CBD in a 3-D collagen scaffold as a delivery vehicle and documented its 3-D binding characteristics, 3-D visualization, cellular internalization and mitogenic activity on SMCs. In the context of previous work from our lab which demonstrated positive effects of R136K-CBD on EC behavior, we believe the delivery system discussed in this paper should be a very promising tool for use in engineering clinically applicable blood vessels and other tissue constructs.

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Fig. 1.

The cumulative release curve of R136K-CBD, R136K and FGF-1. R136K-CBD showed undetectable release for up to 14 days and is significantly less than release of R136K and FGF-1 ($P < 0.0001$). Both R136K and FGF-1 demonstrated burst release during the first two days and continuous release in the following days.

Fig. 2. R136K-CBD showed significantly lower percentage release from collagen hydrogel when supplemented with 2M salt in the media compared with R136K and FGF-1. Asterisk (*) indicates statistical difference at $P < 0.0001$.

Fig. 3.

After 16-hour culture, R136K-CBD showed significantly lower percentage release in collagen hydrogels populated with SMCs compared with R136K and FGF-1. R136K-CBD showed 2.6 and 2.5 fold less release than R136K and FGF-1, respectively. Asterisk (*) indicates statistical difference at $P < 0.0001$.

Fig. 4.
2-D and 3-D view of Alexa Fluor 488 labeled R136K-CBD inside the collagen scaffold. R136K-CBD and collagen fibers are visualized by florescent and reflection confocal microscopy, respectively.

Fig. 5. The column chart shows the distribution of Alexa Fluor 488 labeled R136K-CBD within the collagen scaffold. The height of the column represents the mean percentage fluorescence intensity of each stack image. Five stack images were randomly selected from each scaffold and four collagen scaffolds were analyzed. There is no significant difference between any two stack images ($n=20$, $P>0.05$).

Fig. 6. R136K-CBD localized to the cytoplasm and perinuclear area of the SMC. A) An orthogonal view of Alexa fluor 488 labeled R136K-CBD. Middle square box is a focal plane within the SMC in Z axis and the two rectangular boxes on the top and right are the side views of the whole stack image. The existence of R136K-CBD in the middle layer in Z axis proved the internalization. B is the DIC image and C is the overlay.

Fig.7.

A is the channel of Alexa fluor 488 (green) labeled R136K-CBD and B is the overlay of R136K-CBD and PKH26 (red) labeled SMC, both visualized with fluorescent confocal microscopy. A and B are the focal planes within the cell in Z axis. C is the 3-D view of R136K-CBD internalizing into the SMC in collagen. Collagen fibers were visualized with reflection confocal microscopy (blue). The green dots inside SMCs in B and the triple-color overlaid white dots inside the SMC demonstrate the internalization of R136K-CBD.

Fig. 8.

Proliferation in response to R136K-CBD continued for the full 7 day period of times compared to the decrease in proliferation of R136K and FGF-1 stimulated groups at day 7. By day 7, proliferation in response to R136K-CBD was 5.5, 2.0 and 2.1 fold greater than R136K, FGF-1 and control groups respectively. Asterisk (*) indicates statistical differences at $P < 0.0001$.