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Characterization of the chemotactic and mitogenic response of SMCs to PDGF-BB and FGF-2 in fibrin hydrogels

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Abstract

The delivery of growth factors to cellularize biocompatible scaffolds like fibrin is a commonly used strategy in tissue engineering. We characterized SMC proliferation and chemotaxis in response to PDGF-BB and FGF-2, alone and in combination, in 2-D culture and in 3-D fibrin hydrogels. While both growth factors induced an equipotent mitogenic response in 2-D culture, only FGF-2 was significantly mitogenic for SMCs in 3-D culture. Only PDGF-BB was significantly chemotactic in a modified Boyden chamber assay. In a 3-D assay of matrix invasion, both growth factors induced an invasive response into the fibrin hydrogel in both proliferating and non-proliferating, mitomycin C (MMC) treated cells. The invasive response was less attenuated by the inhibition of proliferation in PDGF-BB stimulated cells compared with FGF-2 stimulated cells. We conclude that SMCs cultured in fibrin hydrogels have a more robust chemotactic response to PDGF-BB compared with FGF-2, and that the response to FGF-2 is more dependent on cell proliferation. Delivery of both growth factors together potentiates the chemotactic, but not mitogenic response to either growth factor alone.

Keywords

fibrin; PDGF-BB; FGF-2; smooth muscle cells; tissue engineering

Introduction

While growth factors have been utilized as attractive pharmacologic tools for the augmentation of tissue generation within biocompatible scaffolds, several limitations to their use remain.^{1–5} Among these is the ability of a single growth factor to activate broad responses among many different types of differentiated and undifferentiated cells, limiting the specificity and predictability of growth factor activity. This can have detrimental consequences, as many of the regenerative processes targeted by exogenous growth factors thought to be critical for tissue engineering often represent a continuum of host responses, which, if uncontrolled, can counterproductively limit the durability of tissue engineered organs.

The induction of mesenchymal cell recruitment and growth into hydrogels with exogenous growth factors is a critical strategy in tissue engineering, as they comprise the main cellular component of most organ stroma and are significant contributors to organ function. In the development of tissue engineered blood vessels (TEBVs), for example, the deposition of endogenous collagens and elastins, and the synthesis of metalloproteinases by *tunica media* smooth muscle cells (SMCs) is necessary for proper scaffold remodeling and thus TEBV biomechanical functionality and tissue incorporation after implantation.^{6–10} However, the promotion of SMC proliferation with growth factors in TEBVs can also lead to uncontrolled SMC growth and synthetic activity which can promote the myointimal hyperplastic response and lead to conduit stenosis. Thus, the characterization of the specific responses of SMCs within hydrogels to growth factors should provide for the future development of more targeted growth factor delivery strategies for tissue and organ engineering.

PDGF-BB and FGF-2 are known regulators of SMC behavior *in vivo*, impacting cell proliferation, migration, and phenotypic differentiation.^{11–12} This has made them attractive tools in tissue engineering for a variety of applications.^{13–16} While the mitogenic and chemotactic cellular responses to these growth factors have been studied in the context of pathologies such as atherosclerosis and intimal hyperplasia, they have not been extensively and studied in tissue engineering hydrogels.

In these studies, we utilize 3-D *in vitro* culture techniques to determine the relative chemotactic and mitogenic response of SMCs to PDGF-BB and FGF-2, alone and in combination, in fibrin hydrogels. We also investigate SMC proliferation and migration in response to these growth factors in 2-D culture to highlight potential differences in cellular behavior which may be attributable to the cell/matrix interactions present in 3-D culture.

Materials and methods

Materials

Chemicals, biological reagents, and experimental supplies were obtained as follows: Collagenase (Invitrogen); Human Thrombin (American Red Cross; Rockville, MD); Mitomycin C, PDGF-BB, FGF-2, L-ascorbic acid, Methylcellulose, Fibrinogen, Transferrin, Insulin, Anti- α -actin Antibody and Aprotinin (Sigma Chemical Co.; St. Louis, MO); Tritiated Thymidine (NEN Life Science Products; Boston, MA); Methanol, Trichloroacetic Acid, Acetic Acid, and Scintillation Fluid (Fisher Scientific; Fair Lawn, NJ); Bovine Lung Heparin (Upjohn; Kalamazoo, MI); Calcein AM (Molecular Probes, Eugene, Ore); 0.05% Trypsin/EDTA, HBSS, M199, DMEM, L-nonessential Amino Acids, Sodium Pyruvate, Penicillin, Streptomycin, DMEM-F12 (Gibco, Grand Island, NY); Fetal Bovine Serum (FBS) (Hyclone, Logan, UT); Woven Nylon Mesh Rings (ID = 7.5 mm, OD = 13 mm) (Sefar America Inc; Kansas City, MO); Parafilm M (American National Can, Greenwich, CT); 100-mm and 60mm Petri Dishes (Fisher Scientific; Pittsburgh, PA); 24 Wells Plates and Tissue Culture Flasks, Costar Transwell Polystyrene Plate (Corning Costar Corp; Cambridge, MA); Round Bottom 96 Well Plates (Greiner Bio-one; North Carolina); 96-Well Polystyrene Plastic Plates (Beckton Dickinson; Lincoln Park, NJ).

Animal care

All animal procedures complied with The Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, 1996) and The Principles of Laboratory Animal Care (National Institutes of Health publication no. 85–23, revised 1985).

Cell culture and treatments

Cell Isolation—SMCs migrating from explanted canine carotid arteries were harvested and cultured in complete media as previously described.¹⁷ Only cell populations with greater than 95% SMC α -actin expression were used for the assays. Carotid artery explants used as the source of cells for assays were passaged no more than two times.

Cell culture—Experiments were carried out in culture media containing 10% fetal bovine serum (FBS) as detailed below. There were two exceptions: 1) Media containing 1% FBS was used for 2-D migration assays in order to minimize basal levels of migration; and 2) 2-D proliferation assays were performed by stimulating serum-starved SMCs with growth factors diluted in serum free media as well growth factors diluted in media containing 10% FBS.

Mitomycin C (MMC) treatment—In experiments using MMC treatment to inhibit cell proliferation, 500,000 SMCs were plated onto 60mm Petri dishes and treated with 10 μ g/mL of MMC for one hour prior to use in experiments. SMCs which were plated but not treated with MMC served as controls.

Bioassays

2-D Proliferation Assay—Tritiated thymidine incorporation for evaluation of DNA synthesis was used to assay cell proliferation in 2-D culture as previously described.¹⁷ Briefly, SMCs were plated onto 96-well polystyrene plastic plates and cultured in complete media for 3 days until they reached 90% confluence. At that point, the media was changed to serum free quiescence media to synchronize cells in G₀ for a period of 48 hours. The cells were then stimulated for 24 hours by PDGF-BB, FGF-2, or both combined, diluted in invasion assay media consisting of M199, 10% FBS, 100 U/mL penicillin/streptomycin, 0.05 μ g/mL gentamicin, 100 KIU/mL aprotinin, 0.25 μ g/mL fungisone, and 5U/mL of heparan sulfate. A 24 hour incubation with tritiated thymidine quantified DNA synthesis. Preliminary studies were also carried out in which growth factors were added in the absence of serum. A standard concentration of 10 ng/mL, known to be maximally bioactive in SMC proliferation assays, was used for each growth factor delivered either alone or in combination in this and all subsequent assays. SMCs stimulated with invasion assay media without growth factors served as negative controls. There were four replicate wells per group, and the experiment was performed in triplicate with consistent results. 2-D proliferation assay data is presented as mean counts per minute (CPM) $\pm$ standard error of the mean (SEM). A representative experiment is presented in the results.

3-D Proliferation Assay—SMCs were mixed in a fibrinogen solution (2.5 μ g/mL fibrinogen and M199 at a pH of 8.0) at a concentration of 400,000 cells/mL prior to polymerization with the addition of thrombin (0.32 U/mL). Calcium content was standardized among hydrogels in all experiments at concentrations found in commercially available formulations of M199 (200 mg/L). Fibrin hydrogels were incubated in invasion assay media with PDGF-BB and/or FGF-2. SMC containing hydrogels incubated in invasion assay media without growth factor served as the negative control. After 72 hours of incubation, hydrogels were proteolytically digested for one hour with a combination of 0.05% Trypsin-EDTA and collagenase (2 mg/mL), and recovered cells were counted with a hemocytometer after trypan blue exclusion. Data from two independently performed experiments were normalized to negative controls and pooled results are presented as mean cells recovered per gel $\pm$ SEM (n=10–14).

2-D Migration Assay—SMCs were seeded on the inner membrane of a Costar transwell polystyrene plate (8.0 μ m pore size in polycarbonate membrane). After allowing the cells to

attach for four hours, a test solution containing PDGF-BB, FGF-2, or the combination of the two diluted in invasion assay media with 1% FBS was placed in the lower chamber. Cells stimulated with invasion assay media with 1% FBS without growth factor served as the negative control. After two hours of migration, the media in the upper and lower chambers was gently suctioned off and the non-migratory cells were scraped from the inner membrane with a cotton-tipped applicator. The remaining cells were fluorescently labeled with Calcein AM. Four quadrants of the membrane were visualized under FITC at a magnification of 10x and fluorescent cells were counted. Each group was tested in replicates of three, and the experiment was duplicated, with similar results. Results from each experiment were normalized to negative controls and the pooled results are presented as mean migratory cells $\pm$ SEM (n=6).

3-D Matrix Invasion Assay—2,250 SMCs were suspended in an aggregating solution consisting of invasion assay media and a 20% methocel solution¹⁸ in round bottom 96-well plates and incubated at 37°C for 24 hours until cell aggregates formed. MMC treated or MMC untreated SMC aggregates were then embedded between two 150 μ L layers of fibrin that were polymerized on a nylon mesh ring such that the aggregates were completely surrounded by the hydrogel. Once completely polymerized, the disks were transferred into 24 wells plates and cultured in invasion assay media containing PDGF-BB, FGF-2, or both growth factors together at 37°C. Cells stimulated by invasion assay media without growth factor served as negative control. After a period of time, SMCs invade from the central aggregate into the surrounding ECM. Digital images were taken daily for up to five days at a magnification of 4x using a Zeiss Axiovert 200M microscope (Carl Zeiss, Inc, Oberkochen, Germany) and Axiovision software. The digital photographs were aligned with a grid evenly divided in 36 intervals using Adobe Photoshop for quantification (Fig. 3). For each assay, the distance of invasion (DOI) was determined by measuring the furthest point on each grid line with an invading chain of cells. The presence of cellular debris and discontinuity of previously continuous chains of invading cells is indicative of apoptotic cell death.¹⁸ For this reason, invasion was quantified by measuring only out to the final point of a continuous chain of cells. Experiments were carried out with replicates of four to five and duplicated with consistent results. Data from a representative experiment is presented in the results unless otherwise noted.

Statistical analysis

We used analysis of variance with Tukey posttests or Student's T-tests using SigmaStat (Systat Software Inc, San Jose, CA) at an α of 0.05 to determine statistical significance.

Results

SMC proliferation in 2-D culture

In the presence of serum, PDGF-BB and FGF-2 each demonstrate significantly greater mitogenicity compared with negative controls (Fig. 1a). Thymidine incorporation was 170.7 $\pm$ 7.5% and 183.6 $\pm$ 11.1% of negative controls in SMCs stimulated by PDGF-BB or FGF-2, respectively ($p < 0.001$ for each growth factor vs. negative control). Simultaneous delivery of PDGF-BB with FGF-2, however, did not induce any significant thymidine incorporation above negative controls (100.9 $\pm$ 8.0% of negative controls for PDGF-BB + FGF-2; $p = 0.93$). In the absence of serum, each growth factor, alone and in combination, induced significant thymidine incorporation compared with negative controls ($p < 0.001$ for each treatment group vs. negative control), with FGF-2 demonstrating significantly greater mitogenicity than PDGF-BB ($p < 0.001$) (Fig. 1b). In the absence of serum, thymidine incorporation in response to the combination of both growth factors added simultaneously

was not significantly different than PDGF-BB alone ($p = 0.09$), and was significantly less than FGF-2 alone ($p < 0.001$) (Fig. 1b).

SMC chemotaxis in 2-D culture

PDGF-BB, but not FGF-2, induced a significant chemotactic response in SMCs compared to negative controls (353.7 $\pm$ 47.9% of negative controls for PDGF-BB, $p < .001$; and 170.8 $\pm$ 57.2% of negative controls for FGF-2, $p = 0.28$) (Fig. 2). Simultaneous stimulation with PDGF-BB and FGF-2 induced a synergistic migratory response in comparison to either growth factor alone (659.2 $\pm$ 86.9% of negative controls; $p \leq 0.01$ vs. either PDGF-BB or FGF-2 alone).

SMC matrix invasion in 3-D culture

Both PDGF-BB and FGF-2 induced significant invasion into fibrin hydrogels compared to negative controls (Fig. 3). At 48 hours, the average distance of invasion in SMCs stimulated by PDGF-BB or FGF-2 alone was 200.1 $\pm$ 22.2 μ m, and 231.0 $\pm$ 14.6 μ m, respectively, vs. 64.1 $\pm$ 5.9 μ m in negative controls ($p < 0.05$ for each growth factor vs. negative controls). Simultaneous stimulation with PDGF-BB and FGF-2 induced an additive invasive response (367.3 $\pm$ 28.8 μ m; $p < 0.05$ vs. either growth factor delivered alone) (Fig. 3). Similar results were seen at 72 hours, which was the last time point accurately quantifiable due to the invasion of cells beyond the limits of the camera lens field at a magnification of 4x. No significant differences were observed between the groups treated with PDGF-BB or FGF-2 alone at any time point.

Proliferation and chemotaxis in 3-D matrix invasion

To investigate the relative role for proliferation vs. chemotaxis in the invasive response to PDGF-BB and FGF-2 in 3-D culture, we first quantified their mitogenicity on SMCs homogeneously distributed in fibrin hydrogels (Fig. 4). All 3-D culture experiments were carried out in the presence of serum in order to maximize nutrient delivery to cells embedded within ECM, and to most accurately model the culture conditions likely required for *ex vivo* or *in vivo* tissue development. After 72 hours of culture, there were 231.3 $\pm$ 19.7% more cells in the PDGF-BB stimulated group compared to negative controls ($p = 0.07$), 509.5 $\pm$ 69.3% more cells in the FGF-2 stimulated group compared to negative controls ($p < 0.001$), and 392.6 $\pm$ 32.0% more cells in the PDGF-BB plus FGF-2 stimulated group compared to negative controls ($p < 0.001$). FGF-2 delivered alone was significantly more mitogenic in 3-D than PDGF-BB delivered alone ($p < 0.001$). The combination of PDGF-BB and FGF-2 delivered simultaneously was significantly more mitogenic than PDGF-BB delivered alone ($p = 0.01$), and non-significantly less mitogenic than FGF-2 delivered alone ($p = 0.10$).

To quantify the non-proliferation dependent component of SMC invasion into fibrin hydrogels in response to these growth factors, cells were pretreated with MMC and compared to MMC untreated SMCs. Significant inhibition of tritiated thymidine incorporation within 24 hours of MMC treatment confirmed the inhibition of cell proliferation (468 $\pm$ 36 CPM vs. 2927 $\pm$ 116 CPM in MMC treated cells vs. MMC untreated cells, respectively at 24 hours, $p < 0.001$; and 37 $\pm$ 21 CPM vs. 4624 $\pm$ 109 CPM in MMC treated cells vs. MMC untreated cells, respectively at 48 hours, $p < 0.001$) (Fig. 5a). Cell viability studies using trypan blue exclusion along with the persistence of viable phenotypic characteristics of cells in 2-D and 3-D culture confirmed that MMC treatment was not cytotoxic (data not shown). In addition, no differences in cell morphology under light microscopic examination in MMC treated cells were noted in comparison to MMC untreated cells. In the absence of any growth factors, MMC treatment attenuated the distance of SMC invasion compared to MMC untreated controls (Fig. 5b and Table 1). MMC treatment decreased the average distance of SMC invasion to a maximal reduction of

40.0 $\pm$ 2.2% of MMC untreated controls ($p < 0.001$) at Day 5, with significant reductions in the distance of invasion during the entirety of the experiment (Table 1).

In MMC treated SMCs, all growth factor groups induced significantly greater matrix invasion compared to MMC treated, growth factor unstimulated negative controls ($p < 0.001$ in all groups vs. negative controls) (Fig. 6). By 48 hours, the simultaneous stimulation of MMC treated SMCs with PDGF-BB and FGF-2 induced a significantly greater invasive response compared to either growth factor alone (317.7 $\pm$ 24.0 μ m vs. 143.0 $\pm$ 8.5 μ m for PDGF-BB + FGF-2 vs. FGF-2, $p < 0.001$; and 317.7 $\pm$ 24.0 μ m vs. 188.1 $\pm$ 11.5 μ m for PDGF-BB + FGF-2 vs. PDGF-BB, $p < 0.005$) (Fig. 6). Similar statistically significant results were seen at the 72 hour time point.

After 48 hours of 3-D culture, MMC treatment significantly diminished the SMC invasive response to FGF-2, but not to PDGF-BB or PDGF-BB plus FGF-2 (Fig. 7, a and c). By 72 hours, MMC treatment significantly decreased the SMC invasive response to all three growth factor stimulated groups to 76.0 $\pm$ 3.0%, 56.1 $\pm$ 2.1%, and 81.9 $\pm$ 4.5% of respective MMC untreated controls when stimulated by PDGF-BB, FGF-2, and PDGF-BB plus FGF-2, respectively (Fig. 7, b and c). At 72 hours, the overall attenuation of SMC matrix invasion by MMC treatment was significantly greater in the FGF-2 stimulated groups in comparison to the PDGF-BB or PDGF-BB plus FGF-2 stimulated groups ($p < 0.05$) (Fig. 7c).

Discussion

In cardiovascular tissue engineering, the use of acellular biomaterials for valves, stents, and TEBVs is dependent on the eventual population of the scaffold with mesenchymally derived stromal cells. These cells provide vasomotor and tonal functions, deposit endogenous extracellular matrix (ECM), and remodel the biomaterial scaffold.¹⁹ While the strategic delivery of growth factors may be beneficial in the promotion of scaffold cellularization, the uncontrolled growth of SMCs and other mesenchymal cells can result in the early proliferative process key to the pathogenesis of stenotic neointimal and atherosclerotic lesions. For this reason, the identification of growth factors which promote the chemotactic recruitment of surrounding stromal cells into the biomaterial scaffold without promoting an excessive proliferative capacity of these cells can minimize unwanted effects of growth factor therapy, and potentially provide for synergistic benefits with the development of designer growth factors.²⁰⁻²¹

Differential mitogenic and chemotactic responses by SMCs to PDGF-BB and FGF-2 have been suggested in studies *in vivo*.²²⁻²⁵ This observation provides potential utility for the targeting of specific SMC activities for tissue engineering applications. However, the relative chemotactic and mitogenic response of SMCs to these growth factors in fibrin hydrogels, a commonly used scaffold and growth factor delivery biomaterial, has not been investigated. In these studies, we demonstrate that both PDGF-BB and FGF-2 are potent inducers of SMC invasion into fibrin hydrogels *in vitro*. The mechanism of this effect, however, differs between the two growth factors, with PDGF-BB functioning primarily as a chemotactic agent demonstrating limited mitogenicity in 3-D culture, and FGF-2 functioning primarily as a mitogenic agent, with less pronounced chemotactic effects. Thus, while matrix invasion into fibrin in the presence and absence of each of these growth factors is a function of both migration and proliferation, we show that the invasive response to PDGF-BB is less dependent on proliferation than FGF-2. These results indicate that PDGF-BB would be an attractive growth factor for the induction of SMC invasion into tissue engineered constructs in situations where the minimization of cell proliferation is desirable. This might be especially useful for growth factor delivery strategies in devices or constructs

which utilize anti-proliferative drugs for the inhibition of myointimal hyperplasia as used in drug eluting stents.²⁶ The relative dependence of SMC invasion on cell proliferation in response to FGF-2 suggests that it would be a less effective choice in those circumstances.

Several caveats to the interpretation of these results should be considered. First, we should note that these experiments were carried out at a single concentration of 10 ng/mL, a concentration known to have mitogenic and chemotactic potency, and to mediate interactions between both growth factors.^{27,28} The results in these studies should be interpreted with the understanding that the responses demonstrated here could be dependent on the specific concentrations used.²⁹ Additionally, we observed that the mitogenic response to these growth factors was partially dependent on the presence of serum. Observations from other groups which demonstrated altered PDGF receptor expression in cells cultured in the absence of serum factors may offer an explanation for these results.³⁰ Second, the replicability of these results in other cell types, as well as to other species should be a consideration prior to application in other animal or human studies. Finally, while fibrin is a commonly used hydrogel in tissue engineering for many applications, the possibility that these responses are specific to fibrin compared to other extracellular matrices should be considered.^{8,9,31–36} Recent evidence has demonstrated that fibrinogen alone can augment the proliferation rates of specific carcinoma cells and endothelial cells in response to FGF-2, and that this appears to be attributable to fibrinogen-FGF-2 binding.^{37,38} Similarly, thrombin promotes endogenous FGF-1 expression in endothelial cells³⁹ and FGF-2 in SMCs,⁴⁰ upregulates VEGF expression in SMCs via endogenous PDGF and FGF-2 activity,⁴¹ can itself be a significant SMC mitogen and chemoattractant *in vitro* at concentrations of 0.5 U/mL in a process likely involving FGF-2 release and FGFR-1 transactivation,⁴² and can significantly impact fibrin gel structural and mechanical properties which could affect cellular behavior.⁴³ While thrombin can affect SMC proliferation rates in 2-D culture systems, the role of thrombin in modulating SMC proliferation in 3-D culture systems is less established. Previous studies by Rowe et. al., for example, have suggested that thrombin may have no significant effect on the proliferation, phenotype, or actin and cellular alignment of SMCs cultured within fibrin hydrogels.⁴³ Due to the low concentrations of thrombin (0.32 U/mL) in our model, it is unlikely that a significant amount of free thrombin is present after gelation and likely plays a minimal role in modulating cellular responses. However, observations that individual components of fibrin can significantly impact cellular responses within the hydrogel as well as their response to growth factors do imply that the responses seen in these experiments may not extrapolate directly to other extracellular matrix configurations.

In our studies, the simultaneous delivery of PDGF-BB with FGF-2 completely eliminated the mitogenic response to either growth factor alone in 2-D culture, and appeared to attenuate the mitogenic response to FGF-2 in 3-D culture. In contrast, the combination of both growth factors induced a synergistic chemotactic response in 2-D culture, and an additive invasive response in 3-D culture, both in the presence and absence of proliferation. Inhibiting proliferation had little to no effect on SMC matrix invasion induced by the co-delivery of both growth factors. These results were similar to those that were seen in SMCs stimulated by PDGF-BB alone. Thus, our results suggest that the co-delivery of PDGF-BB with FGF-2 in fibrin hydrogels potentiates the invasive SMC response by a preferentially greater chemotactic signal, rather than increased mitogenesis.

The mechanism of this effect is yet unclear. There is known interaction between PDGF-BB and FGF-2 and growing evidence that PDGF-BB and FGF-2 are intimately involved in the regulation of one another's activity.⁴⁴ PDGF in the arterial wall is thought to interact with FGF-2 to promote SMC proliferation in a rat carotid artery filament injury model.²⁵ It has been demonstrated that PDGF-BB-induced SMC proliferation and late activation of ERK in

humans depends at least in part on FGF-2 release and FGFR-1 activation,²⁷ and that PDGF-BB induces upregulation of the high molecular weight form of FGF-2 in a mechanism dependent on ERK-1/2 kinase activity.⁴⁵ Given the role of ERK as a cell cycle progression factor, it is notable that the co-delivery of both growth factors in our studies did not result in any additive mitogenic effects. The effects on SMC chemotaxis by the co-delivery of both growth factors can be equally confounding based on contrary finding in the literature. Consistent with our results, Pickering et. al. had demonstrated in 2-D migration assays that pretreatment of human SMCs with 48 hours of exposure to FGF-2 potentiates their chemotactic response to PDGF-BB on type I collagen coated surfaces in a mechanism dependent on the upregulation of the integrin receptor $\alpha 2\beta 1$.⁴⁶ In contrast, other studies have suggested that FGF-2 inhibits PDGF-BB induced migration in rat SMCs via a PDGF- α receptor dependent mechanism.²⁸ We speculate that alterations in growth factor or integrin receptor expression, differential expression of competence factors, alterations in cellular phenotype and growth factor responsiveness, or a combination of all may account for the synergistic chemotactic effects observed after growth factor co-delivery in our studies. Specifically, investigating ERK expression and activation, integrin receptor expression, and PDGF receptor expression and activation in fibrin based 3-D culture models are warranted in understanding the mechanism of PDGF-BB and FGF-2 interactions seen in our studies.

Finally, differences in the mitogenic response of SMCs to PDGF-BB, with or without FGF-2, in 2-D vs. 3-D culture are notable. First, PDGF-BB and FGF-2 demonstrated near equipotent mitogenicity in 2-D culture, while the mitogenicity of PDGF-BB in 3-D culture was insignificant. Second, while the co-delivery of both growth factors eliminated the mitogenic response to either growth factor alone in 2-D culture, this was not observed in 3-D culture. While these discrepancies may be attributable to incomplete penetration of growth factors into the ECM altering effective growth factor concentrations, it is more likely due to cell/matrix/growth factor interactions which may modulate the growth factor response, either by altering cell signaling or cellular FGF or PDGF receptor expression and occupancy.^{47–50} Alterations in cellular phenotype by the 3-D ECM conformation may also account for these changes, leading to differential expression of competence factors and cellular organelles, growth factors and growth factor and integrin receptors, and altered synthetic capacity in response to exogenous growth factor delivery.⁵¹ Stegemann had demonstrated that SMCs in 3-D collagen matrices demonstrate less expression of α -SMA, a marker of the “contractile” SMC phenotype compared to cells cultured on a 2-D collagen lattice. They further demonstrated that exogenous PDGF-BB decreased α -SMA expression in SMCs cultured in 3-D collagen gels, but not in 2-D culture.⁵² In our studies, SMCs appeared to maintain a spindled, elongated shape under light microscopy indicating a “contractile” phenotype in 3-D culture, but we did not further characterize markers indicative of a “contractile” vs. “synthetic” phenotype as this was not the primary purpose of these studies.⁵³ Future studies investigating the phenotypic characteristics of SMCs in 3-D vs. 2-D culture and its impact on SMC chemotaxis and proliferation in response to PDGF-BB and FGF-2 are required to provide further insight.

Conclusions

In summary, we demonstrate that both PDGF-BB and FGF-2 are potent growth factors for the induction of SMC invasion into fibrin hydrogels *in vitro*. PDGF-BB functions primarily as a chemotactic agent demonstrating limited mitogenicity in 3-D culture, and FGF-2 functions primarily as a mitogenic agent with less pronounced chemotactic effects. Thus, PDGF-BB appears to be a superior choice over FGF-2 for growth factor delivery systems which aim to promote SMC invasion into fibrin hydrogels with limited cell proliferation. The delivery of both growth factors added in combination with one another can promote an invasive response superior to either growth factor alone, and this appears to be primarily a

function of augmented chemotaxis rather than mitogenesis. The observation that the simultaneous delivery of both growth factors at these concentrations can increase the mitogenic response above that of PDGF-BB alone, however, should be considered when choosing this strategy. Finally, the presence of serum and 3-D extracellular matrix conformations can alter SMC responsiveness to PDGF-BB and FGF-2 alone, or in combination, confirming previous studies demonstrating the importance of considering the extracellular environment as a regulator of cellular behavior. Further studies are required to elucidate the mechanism of these differences, as well as the applicability of these studies to other cell types within other biomaterials used in tissue engineering.

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Figure 1. SMC proliferation in 2-D culture in response to PDGF-BB and FGF-2. Serum starved SMCs seeded on 96 well plates were incubated with indicated growth factors diluted in a) invasion assay media (10% serum) or b) serum free media. SMC proliferation was quantified by determining tritiated thymidine incorporation. Results represent the mean CPM $\pm$ SEM from a representative experiment (n=4). * $p < 0.001$

Figure 2.

SMC chemotaxis in response to PDGF-BB and FGF-2. SMCs were seeded on the inner surface of an 8 μ m porous membrane and allowed to migrate in response to respective growth factor gradients. Each column represents the mean number of migratory cells $\pm$ SEM of pooled results from two independently performed experiments (n=6). * $p < .001$ vs. negative control; # $p < 0.01$ vs. PDGF-BB; $\pm$ $p < 0.001$ vs. FGF-2.

Figure 3. SMC invasion into fibrin hydrogels in 3-D culture in response to PDGF-BB and FGF-2. (Top panel) SMC invasion after 48 hours of 3-D culture (Magnification - 4x). (Bottom panel) Quantification of SMC invasion into fibrin hydrogels. Results represent the mean distance of invasion $\pm$ SEM from a representative experiment (n=4-5). # $p < 0.05$ vs. PDGF-BB; $\pm p < 0.05$ vs. FGF-2.

Figure 4.

SMC proliferation in 3-D fibrin culture in response to PDGF-BB and FGF-2. SMCs were distributed homogenously in fibrinogen solution prior to gel polymerization with the addition of thrombin. 3-D cell cultures were incubated in media $\pm$ respective growth factors. After 72 hours, the gels were digested and trypan blue excluding cells were counted with a hemocytometer. Each column represents the mean number of cells/gel $\pm$ SEM of pooled results from two independently performed experiments (n=10–14). * $p < 0.001$ vs. negative control; # $p \leq 0.01$ vs. PDGF-BB.

Figure 5.

The effects of MMC treatment on SMC proliferation and matrix invasion in the absence of growth factors. (a) SMC proliferation. SMCs either treated or untreated with MMC were seeded onto 96 well plates and cultured in indicated media. SMC proliferation was determined by quantifying tritiated thymidine incorporation. Results are mean CPM $\pm$ SEM from a representative experiment (n=4). (b) SMCs either treated or untreated with MMC were embedded within fibrin hydrogels and matrix invasion was quantified. Results represent the mean distance of invasion $\pm$ SEM from a representative experiment (n=4-5). * $p < 0.05$ vs. MMC untreated control.

Figure 6.

Invasion of MMC treated SMCs into fibrin hydrogels in 3-D culture in response to PDGF-BB and FGF-2. SMCs were treated with MMC, embedded in fibrin hydrogels, and then incubated in invasion assay media with or without indicated growth factors. Results represent the mean distance of invasion $\pm$ SEM from a representative experiment (n=4-5). # $p < 0.005$ vs. PDGF-BB; $\pm$ $p < 0.001$ vs. FGF-2.

Figure 7. Invasion of MMC treated and untreated SMCs into fibrin hydrogels in response to PDGF-BB and FGF-2. (a) Invasion quantified after 48 hours of culture. (b) Invasion quantified after 72 hours of culture. (c) Matrix invasion of MMC treated SMCs as % of MMC untreated controls in the presence of indicated growth factors. Results represent the mean distance of invasion $\pm$ SEM from a representative experiment ($n=4-5$). * $p < 0.05$ compared to respective MMC untreated controls; $\pm p < 0.05$ vs. FGF-2.

Table 1

Matrix invasion of MMC treated SMCs as % of MMC untreated controls.

	Day 1	Day 2	Day 3	Day 4	Day 5
Distance of Invasion (μ m)	68.7 $\pm$ 14.2	50.1 $\pm$ 4.2 *	51.6 $\pm$ 3.6 *	42.1 $\pm$ 3.5 *	40.0 $\pm$ 2.2 *

Data are means $\pm$ SEM of pooled results from two independently performed experiments (n=8-10).

* p<0.001 vs. MMC untreated controls.