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VEGF promotes osteogenic differentiation of ASCs on ordered fluorapatite surfaces

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Abstract

Vascular endothelial growth factor (VEGF) has been reported to mediate both osteogenesis and angiogenesis in bone regeneration. We previously found an up regulation of VEGF in adipose-derived stem cells (ASC) when obvious mineralization occurred on a novel fluorapatite (FA) coated surfaces. This study investigated the effect of FA and VEGF on the growth, differentiation and mineralization of (ASC) grown on ordered FA surfaces. Cells grown on FA and treated with VEGF demonstrated osteogenic differentiation as measured with ALP staining, and obvious mineralization as measured by Alizarin Red staining. A combined stimulating effect of FA and VEGF was seen using both indicators. VEGF signaling pathway perturbation using a specific VEGF receptor inhibitor showed the lowest levels of ALP and Alizarin Red staining, which was partially rescued when the cells were grown on FA and/or treated with the addition of VEGF. The osteogenic differentiation of ASCs stimulated by these FA surfaces as well as VEGF has been shown to be mediated through, but probably not only, the VEGF signaling pathway. The enhancement of osteogenic differentiation and mineralization supports the potential use of therapeutic VEGF and FA coatings in bone regeneration.

Keywords

Fluorapatite; vascular endothelial growth factor; differentiation; mineralization; signaling pathway

Introduction

Clinical demands have shifted the focus of medicine and dentistry to regenerative therapies. In this effort, osteo-progenitor cell lines such as mesenchymal stem cells have been used in bone regeneration and hard tissue engineering.^{1,2} More recent research has used adipose derived stem cells (ASCs), as they are capable of osteogenic differentiation under appropriate stimulus and are an abundant and accessible source with reduced donor

morbidity.^{2,3} Further, ASCs have been shown to be a source for both osteogenic and angiogenic precursor cells, both crucial for proper bone repair and regeneration.⁴⁻⁶

Hard tissue regeneration generally utilizes osteoinductive and/or osteoconductive materials. Optimization of these osteogenic processes has been the goal of new materials used in hard tissue engineering. Coatings of hydroxyapatite (HA) have been introduced to improve the osseointegration and fixation of implants and scaffolds.⁷ However the HA coating has been shown to have a potential risk of dissolution and debonding from the implant resulting in clinical failure.^{8,9} The limited stability of HA and the subsequent formation of heterogeneous calcium phosphates and its thermal decomposition products in a body environment has spurred interest in bioactive materials with increased resorption resistance.¹⁰ One such material is fluorapatite. Natural bone accumulates fluoride ions from the blood forming a fluoride containing HA. Fluoride ions within the lattice replace hydroxide ions creating a tighter lattice structure. This promotes increased stability and lowers the solubility of FA. Our lab has recently developed a method for synthesizing FA structures with the potential for biomedical applications.^{11,12} In our previous studies, a well-aligned, ordered FA structure has been created to show promising results in supporting the long term growth of dental pulp stem cell (DPSC), and stimulating the differentiation and mineralization of osteoblast-like MG-63 cells.^{13,14} In our previous studies, the disordered FA surfaces also stimulated the osteogenic differentiation and mineralization of cells compared to metal surfaces.^{14,15} However, compared to the disordered FA structure, the ordered FA surfaces show more pronounced bioactive effects on the adhesion, proliferation, differentiation and mineralization of several cell lines in vitro, and also stimulated a higher density of bone-like tissue formation in vivo, which shows important roles of the topography of FA in these biological processes.^{13,14,15} Therefore, ordered FA structure was used throughout this study.

Our recent work has reported on the promotion of differentiation of ASCs grown on the FA substrates. Pro-osteogenic transcripts were shown to be upregulated *in-vitro*.¹⁵ Interestingly, VEGF expression was found to be upregulated in these ASCs grown on FA surfaces when mineralization was evident.¹⁵

VEGF has been shown to have the capacity to promote both osteogenic and angiogenic differentiation.¹⁶ The use of VEGF for enhanced bone regeneration has been successful due to its promoting effects on angiogenesis, cell migration, and mineralization.^{17,18} Studies have combined therapeutic VEGF with osteoconductive scaffolds for the promotion of bone regeneration.¹⁹ However, the effect of VEGF on cells grown on FA substrates has not been tested. This study was carried out to investigate the effect of FA and VEGF on the growth, differentiation and mineralization of ASCs and to explore the potential involvement of VEGF pathway during these processes.

Materials and Methods

Synthesis of ordered FA surfaces on etched stainless steel (SSE) discs

The hydrothermal synthesis and coating of the ordered crystal film on SSE discs was carried out as previously described.^{12,13} Briefly, before the FA synthesis, the metal discs were

treated overnight with an etching solution containing 50% H₂SO₄ and 50% H₂O₂. For a typical synthesis of FA crystals, 9.36 g ethylenediaminetetraacetic acid calcium disodium salt (EDTA-Ca-Na₂) and 2.07 g NaH₂PO₄·H₂O were mixed with about 90 mL distilled water. The suspension was stirred continuously until the powder dissolved. The pH was adjusted to 6.0 using NaOH. Then 0.21 g NaF was dissolved in 10 mL water (pH 7.0) and stirred continuously. The FA crystal growth on the substrates (15mm 316 stainless steel discs, Ted Pella, CA) was achieved by adding the plates to 100 mL of newly prepared EDT-Ca-Na₂/NaH₂PO₄/NaF mixture and then autoclaving at 121°C at pressure of 2.4×10^5 Pa for 10 h. The ordered crystal structure, which was produced on the undersurfaces of the stainless steel discs, is composed of single layer of crystals. The thickness of the coated FA layer was equivalent to the length of the c-axis of single crystals, which were approximately 15–20 µm. For the coating of dental implants, the implants were suspended in the solutions and subjected to the same hydrothermal conditions to ensure the well-aligned, oriented single layer crystal structure.

Scanning Electron Microscopy

Characterization of FA coated surfaces was performed to observe apatite structure and ordered crystal arrangement using scanning electron microscopy (SEM). SEM analysis was conducted on a Phillips XL30FEG Scanning Electron Microscope (FEI company, OR, United States) operated at 10kV (Resolution: 2.0 nm at 30 kV, 5.0 nm at 1kV). Samples were coated with Au/Pd film to prevent specimen charging.

Cell culture and cell attachment assay

The StemPro[®] Human Adipose-Derived Stem Cell (ASC) Kit (Invitrogen, NY, USA) was purchased, which contains cryopreserved normal human ASCs and MesenPRO RS[™] Medium. The ASCs were then subcultured in reduced-serum (2%) MesenPRO RS[™] medium under standard culture conditions at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air. Before cell seeding, the discs were incubated with the complete growth media for 2 h. The ASCs were seeded onto the etched stainless steel (SSE) and FA coated discs at a density of 2×10^4 cells/mL, with or without the addition of VEGF at a working concentration of 100ng/µl and cultured for 3 and 7 days. The medium was changed every two days. At the end of each culture period cells were detached with trypsin/EDTA, stained with trypan blue and counted using a hemocytometer. An average of 3–5 specimens was used for the experiment.

VEGF Signaling Perturbation

ASCs were seeded on FA and SSE surfaces in a 12-well plate and subjected to the same treatment groups as above. Then the ASCs were further treated with VEGFR Tyrosine Kinase Inhibitor IV, a specific VEGF receptor inhibitor used to perturb the VEGF signaling pathway. The inhibitor was prepared in medium to a working concentration of 30nM. ASCs grown on SSE and FA surfaces without the addition of VEGF and VEGFR inhibitor served as controls.

Alkaline Phosphatase (ALP) Staining

After day 7, the specimens were washed with PBS, fixed in 4% paraformaldehyde for 30 min, and then incubated with 0.5 mg/mL naphthol AS-MX phosphate (Sigma-Aldrich, St. Louis, MO) and 1 mg/mL Fast red TR (Sigma-Aldrich) pre-dissolved with 1 % N, N dimethylformamide (Sigma-Aldrich) in 0.1 M Tris buffer (pH 9.2) at 37°C for 40 minutes. The specimens were counterstained in DAPI (300mM, Molecular Probes) for 10 minutes, and washed in PBS before fluorescence imaging. Image J (NIH) was used to compare the intensity of fluorescence staining between the different groups, and triplicate samples from each group were used for statistical analysis.

Alizarin Red staining and quantitative analysis

At day 28, the specimens were washed with PBS, fixed with 70% ethanol and stained with Alizarin red (Osteogenesis Assay Kit, Millipore, Temecula, CA) according to manufacturer's protocols. After staining, quantitative analysis was carried out through extraction of Alizarin red from stained samples using acetic acid. The OD405 was measured for each sample and compared to a standard generated starting with a 10× ARS Dilution buffer. Alizarin red concentration was calculated as micromoles per liter with three to five samples from each group being used for quantification.

Statistical analysis

The cell counting, enzyme-linked immunosorbent assay (ELISA), quantitative osteogenesis assay, and the ImageJ analysis results were analyzed statistically using GraphPad Prism 5 for one-way ANOVA (and post hoc pairwise comparisons) of an average of three to five replicates and significance was considered at $p < 0.05$. Data are expressed as mean $\pm$ standard deviation.

Results

FA synthesis and surface characterization

SEM images at low (Fig. 1a) and high (Fig. 1b) magnification demonstrated ordered crystal arrangement on the SSE surfaces, with crystals aligning parallel along their c-axis and presenting to be characteristic apatitic hexagonal shape. Figure 2 showed that the synthesized FA crystals were evenly distributed and coated on a dental implant.

Cell culture and cell attachment assay

After day 3 and 7, the numbers of ASCs grown on FA surfaces were significantly lower than those on the SSE surfaces (Fig. 3). VEGF treatment significantly inhibited the proliferation of ASCs grown on FA surfaces at day 3 and 7 and the cells grown on SSE surfaces at day 7.

ALP staining

After culture of ASCs for 7 days, increased ALP staining was observed for all cells grown on FA surfaces compared to SSE surfaces. For the ASCs grown on FA surfaces, VEGF treatment resulted in the most intense ALP staining. The weakest level of ALP staining was observed with treatment of the VEGF receptor inhibitor. Inhibition of ALP staining by

VEGF receptor inhibitor appears to be partially rescued by the addition of VEGF (Fig. 4). Similarly, the staining intensities of the cells grown on SSE surfaces with VEGF and/or VEGFR inhibitor supplement were much weaker compared to those of the cells grown on FA surfaces (Fig. 4).

Alizarin Red staining and quantitative analysis

At day 28, ASCs grown on FA surfaces showed stronger Alizarin red staining compared to cells grown on SSE surfaces. For cells grown on FA surfaces, treatment with VEGF resulted in the strongest staining. The VEGF receptor inhibitor resulted in the weakest staining of the cells, which was partially rescued by the addition of VEGF. A similar staining pattern, but to a markedly lesser degree, was observed for the experimental groups with ASCs grown on SSE surfaces (Fig. 5a). Subsequent quantitative assay further confirmed these results (Fig. 5b).

Discussion

This study was conducted to show the potential for using FA and therapeutic VEGF in hard tissue engineering. The results showed a greater number of cells on SSE surfaces than that on FA surfaces, which was consistent with our previous results.¹⁴ The VEGF appeared to inhibit the proliferation of ASCs grown on both SSE and FA surfaces. This effect was probably a result of a greater percentage of differentiating ASCs over the percentage of cells undergoing proliferation as both proliferation and differentiation could occur simultaneously within the given population of ASCs under the same micro environment.²⁰ As we have discussed before, the most favorable surfaces for initial cellular proliferation might not necessarily result in the favorable or desired cell fate selection or differentiation pathway.¹⁴ In this study, the FA surfaces were shown to promote osteogenic differentiation and mineralization, even though the initial cellular proliferation on the FA surfaces was not as great as those seen on SSE surfaces, as reported in our previous results.¹⁵ The enhanced osteogenic response was further improved through the addition of VEGF to cell culture. This was confirmed by the strongest ALP staining at day 7 when the cells grown on FA surfaces were further treated with VEGF. ALP is known to be a reliable marker for early osteoblast differentiation.²¹ Consistently, mineral formation, as shown by the Alizarin red staining and subsequent quantitative analysis, demonstrated the same trend as ALP staining. This suggests a combined stimulating effect of VEGF and FA surface on the enhanced ASC differentiation and mineralization.

The need for angiogenesis in bone healing and formation is well known and is supported by studies demonstrating angiogenesis preceding bone formation, BMP2 secretion by vascular tissues, and disruption of angiogenesis resulting in a disturbance of bone formation.^{22–24} Considering the close coupling of angiogenesis and osteogenesis, it is not surprising to observe VEGF expression at the time of osteoblast differentiation.²⁵ Previously, we also showed an elevated expression of VEGF transcripts when evident mineralization occurred in ASCs compared to their early non-mineralization stage.¹⁵ Osteoblastic production of VEGF in promotion of angiogenesis to support bone growth is evident; however, a more direct or autocrine role of VEGF in osteogenesis is less supported. Mayer et al. has demonstrated

recombinant human mesenchymal stem cells expressing VEGF during mineralization formation.²⁶ It was also shown that the addition of a VEGF antagonist reduced mineralization suggesting an autocrine role of VEGF for osteoblast differentiation.²⁶ By similarly demonstrating an interruption in osteoblastic differentiation and mineralization through VEGF signaling perturbation, this study further supports direct involvement of VEGF in osteogenesis. In this study when ASC cultures were treated with VEGFR Tyrosine Kinase Inhibitor IV, there was a marked decrease in early ALP activity and late stage mineralization of the ASCs grown on both SSE and FA surfaces. VEGFR inhibitors generally interrupt VEGF signaling by binding to ATP-binding catalytic domain site of the receptor, inhibiting the receptor's kinase activity and thus inhibiting intracellular signaling.²⁷ VEGFR tyrosine kinase inhibitors have reliably been shown to interrupt VEGF signaling when used to interrupt angiogenesis in tumor progression via treatment with various VEGFR tyrosine kinase inhibitor agents.^{28–30}

By blocking the VEGF receptor, osteogenic differentiation and mineralization had been significantly inhibited, and this inhibiting effect was shown to be partially rescued by the addition of VEGF. This suggested a promoting effect of VEGF on osteogenesis and the involvement of VEGF pathway during this process. Zhang et al. has implied a crosstalk between VEGF and BMP pathways.³¹ In their study, ASCs treated with BMP-6 and VEGF *in vitro* demonstrated enhanced ALP expression and mineralization and significant increases in pro-osteogenic genes (COL1A1, osterix, and Dlx5).³¹ The gene expression was significantly greater than when ASCs were treated by BMP-6 or VEGF alone.³¹ Similar synergistic effects were demonstrated through the addition of VEGF and BMP-2 in culture, with the sequence of growth factor delivery imparting additional enhancements in osteoblastic differentiation.³² Conversely, other co-culture studies have shown an inhibitory effect of VEGF on BMP-2 release.³³ Thus, the temporal regulation of VEGF and BMP-2 secretion appear to be significant.

Interestingly, we performed an ELISA assay for BMP-2 from the supernatant of ASCs grown on FA surfaces with or without the VEGF supplement and found no detectable BMP-2 present (data not shown). The absence of BMP-2, a potent promoter of osteogenesis, in our study suggest VEGF may work directly or indirectly through the promotion of other pro-osteogenic factors and transcription factors, e.g. BMP-6 and Runx2, resulting in enhanced osteogenesis. Runx2 has been shown to stimulate the expression of VEGF at both gene and protein levels in mesenchymal cells; whereas, treatment of preosteoblast cells with Runx2 siRNA reduced the VEGF expression under both normoxia and hypoxia conditions.³⁴ Previously, involvement of the FGF pathway has demonstrated synergistic effects in both angiogenesis and osteogenesis. VEGF and FGF-2 have shown synergistic promotion of angiogenesis (*in vivo* and *in vitro*).³⁵ Most recently, we have revealed that coculture with microvascular endothelial cells significantly promoted the osteogenic differentiation and mineralization of ASCs, which has been mediated, in part, by the FGF pathway.³⁶ Similarly, FGF-2 combined with BMP-2 has also shown a synergistic promotion of osteogenesis over BMP-2 alone *in vitro*.³⁷ This again supports a sensitivity in the temporal and spatial secretion of growth factors during the osteogenesis process. Together

with our studies, it is suggested that the angiogenesis and bone formation are intimately related processes.

Growth factor delivery via integration of growth factors onto an osteogenic supporting scaffold or surface has become a common strategy in tissue engineering. One method for integrating growth factors to a scaffold surface uses the deposition of a mineral layer on the surface to allow for binding of the proteins to the crystalline surface.³⁸ Studies have shown strong interactions of biological proteins to calcium phosphate (CaP) and hydroxyapatite surfaces.^{39,40} Recent work has shown VEGF successfully loaded onto a CaP mineralized scaffold surface with resulting sustained protein release *in-vitro*.³⁸ This supports further investigation into loading VEGF onto a similar mineralized surfaces such as FA as we have already demonstrated in this study the combined effect of FA and VEGF for enhanced *in-vitro* osteogenesis promotion.

Conclusion

The osteogenic differentiation of ASCs stimulated by these FA surfaces as well as VEGF has been shown to be mediated through, but probably not only, the VEGF signaling pathway. The enhancement of ASC osteogenic differentiation and mineralization supports the potential use of therapeutic VEGF and FA coatings in hard tissue engineering. In future studies, investigation on other upstream signal molecules and the downstream effectors of VEGF pathways and identification of other pathways that are associated or activated by VEGF/VEGFR signaling during these processes needs to be further explored.

Acknowledgement

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Figure 1.
Scanning electron microscopy images of the ordered fluorapatite crystal surface at low magnification (a) and high magnification (b).

Figure 2.
Low magnification scanning electron microscopy image of the surface of a fluorapatite coated dental implant.

Figure 3.

ASCs grown on SSE and FA surfaces with and without the treatment of 100ng/ μ l VEGF at day 3 and day 7. Triplicate samples were used for one-way ANOVA test and significance was considered at $p < 0.05$.

Figure 4.

Fluorescence microscopy images of Alkaline phosphatase (ALP) staining of ASCs grown on FA and SSE surfaces with the treatment of VEGF and/or VEGF receptor inhibitor at day 7. a: DAPI stained nuclei. b: ALP staining (Fast red TR). c: merged pictures of a and b.

Figure 5. Alizarin Red staining of ASCs grown on FA and SSE surfaces with the treatment of VEGF and/or VEGF receptor inhibitor at day 28 (a). Quantitative analysis of Alizarin Red staining of ASCs grown on FA surfaces with the treatment of VEGF and/or VEGF receptor inhibitor at day 28 (b).