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Pigment Epithelium Derived Factor upregulates expression of Vascular Endothelial Growth Factor by human Mesenchymal Stem cells: Possible role in PEDF regulated matrix mineralization

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Abstract

Pigment epithelium-derived factor (PEDF) encoded by *serpinf1* is a potent anti-angiogenic factor found in a wide variety of fetal and adult tissues. Several reports have shown that lack of PEDF leads to osteogenesis imperfecta (OI) type VI whose hallmark is a defect in mineralization that leads to excessive osteoid build up that fails to mineralize. Because PEDF is antiangiogenic factor it would pose serious consequences on bone development and healing of fractures. To understand possible mechanisms by which PEDF plays a role in bone development and regulation of matrix mineralization, we determined the effects of exogenous PEDF on vascular endothelial growth factor (VEGF) expression by human mesenchymal stem cells (hMSCs) and mechanisms of its regulation by PEDF. Human MSCs incubated in normal medium supplemented with exogenous PEDF increased VEGF expression; this increase was also seen when PEDF was added to hMSCs undergoing osteogenic differentiation. MSCs maintained in osteogenic medium increased synthesis of both VEGF and PEDF but both factors were maintained relatively in balance during differentiation. To understand mechanisms by which exogenous PEDF regulated VEGF expression, hMSCs exposed to PEDF activated Erk signaling pathway in MSCs; inhibition of Erk signaling reduced VEGF mRNA expression as well as protein production suggesting that PEDF regulates VEGF expression in MSCs via Erk signaling pathway. In conclusion, PEDF increases

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Conflict of interest

All Authors do not have any conflict of interest with data reported in the manuscript.

VEGF expression by MSCs suggesting that regulation of VEGF by PEDF may be part of the mechanisms by which PEDF regulates osteoblastic mineralization.

Keywords

Pigment epithelium derived factor; Vascular endothelial growth factor; MSCs; mineralization; Erk signaling

Introduction

Pigment epithelium-derived factor (PEDF) is a potent anti-angiogenic factor found in a wide variety of fetal and adult tissues; it is believed to play a role in the regulation of angiogenesis during development [1,2,3]. PEDF was originally isolated from human fetal retinal pigment epithelial cells, it has now been shown to be synthesized by a variety of other cell types including osteoblasts [4,5,6]. Several reports have shown that patients with a recessive form of osteogenesis imperfecta (OI) type VI lack expression of PEDF leading to severe bone defects and frequent fracturing [7,8,9,10]. Synthesis, posttranslational modification and secretion of type I collagen were reported to be normal. The major bone defect in these patients is presence of excessive osteoid build up that fails to mineralize [7–10]. We and others have shown that PEDF promotes mesenchymal stem cell differentiation and increases osteoblast mineralization [11,12]. In addition, we reported that PEDF reduced expression of sclerostin by osteocytes [13]. Mechanisms by which PEDF regulates osteogenesis and osteoblast mineralization however, remain unclear.

Most studies on PEDF have focused on its role in antiangiogenic activities and as a potential therapeutic factor for inhibiting cancer cell migration as well as survival, and potential application in some diabetic related pathologies like retinopathy [14,15,16]. PEDF has been reported to downregulate expression of VEGF in retinal cells but PEDF also inhibited VEGF binding to the type 2 VEGF receptor [17,18]. In bone, VEGF plays important role in bone development; there is also evidence that VEGF can directly promote osteoblast differentiation and or matrix mineralization [19,20]. Several reports indicate that a balanced VEGF/PEDF ratio a key factor in maintaining the function of ocular neovascularization and osteoblastogenesis [21,22]. Because VEGF plays important role in bone, we set out to determine if PEDF regulates VEGF expression in hMSCs. In this report, we examined effect of exogenous PEDF on VEGF expression by MSCs in normal medium as well as during mineralization. We also determined mechanisms by which PEDF regulates VEGF expression in hMSCs.

Materials and Methods

Human Mesenchymal stem cells (MSCs) isolation

Human MSCs were harvested from the reaming of patients bones undergoing hip surgery with approved IRB protocol by Penn State college of Medicine (ages 50–70). The cells were isolated using the methods described previously [23]. Briefly, bone reaming were washed extensively in sterile PBS by repeated centrifugation at 1000 rpm for 5 min. Pellets were

suspended in DMEM supplemented with 10% FBS and plated in Petri dishes. The cells were maintained in culture in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin for 7 days. Bone chips and non-adherent cells were removed; adherent cells were maintained in culture with medium changes and passaged when they reached confluence. The cells were used at passages 2 or 3. Prior to use, cells were assessed for stem characteristics as we described previously [11,24]. hMSCs were treated with PEDF protein at 250ng/ml either in osteogenic or maintenance medium. PEDF used for the experiments was donated by Dr. Tombran-Tink who isolated and characterized it as described previously [25].

Western Blotting

Total protein from differentiating hMSCs either in presence or absence of PEDF at different time points was extracted with RIPA lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 50 mM sodium fluoride, 2 mM sodium vanadate, 0.1% BSA, and complete protease inhibitors) (Pierce, Rockford, IL). The cell lysates were centrifuged and the supernatant was collected. The protein concentration of cell lysates was determined using a Protein Assay kit (Bio-Rad, USA). Total cell extracts containing equal amounts of protein were separated by 12% SDS-PAGE and electroblotted onto PVDF membranes (Bio-Rad Laboratories). Only proteins associated with cells were collected, proteins secreted in the medium were not assessed. The membranes were probed with anti-human antibodies to proteins of interest for analysis; PEDF (1:2500, SC-25594, Santa Cruz, CA), VEGF (1:1000, SC-7269, Santa Cruz, CA), total ERK1/2 (1:1000, # 9102, Cell Signaling Technology, CA) and phosphorylated ERK1/2 (1:1000, #9106, Cell Signaling Technology, CA). Mouse anti-human GAPDH (1:2500, SC-32233, Santa Cruz, CA) antibodies were also used for normalization. For visualization, horseradish peroxidase-conjugated secondary antibodies were used and the bands were developed by SuperSignal West Pico substrate (Thermo Scientific). Specific bands were quantified using the Bio-Rad Quantity One 1-D Analysis Software (Bio-Rad, USA).

Signaling pathways analysis

Human MSCs were pretreated with 10 mM of Erk docking domain inhibitor (294675-79-9, Sigma Aldrich, CA) or control solution (DMSO). This was followed by incubation of MSCs in a medium supplemented with 250 ng/ml of PEDF for 5, 15, 30 min, 1h, 2h, 4h, 8h, and 24 h. Cells were extracted with RIPA buffer and the cell lysates were collected for analysis.

Quantitative Real-time PCR

Total RNA was extracted from cells using TRIzol (Invitrogen). Synthesis of cDNA was performed with 1 µg of total RNA at 37 °C for 50 min using oligo (dT) primers and reverse transcriptase (M-MLV; Invitrogen). cDNA was analyzed in a StepOnePlus™ Real-Time PCR system (Applied Biosystems, CA). Amplification was carried out in a total volume of 20 µl and PerfeCTa SYBR Green FastMix® ROX reagents (Quanta Bioscience, MD) with the following cycling conditions initial denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 3s, 60 °C for 30 s, and Melt Curve: 95°C, 15s (100%); 65°C, 1min, 95°C 15s (100%), +0.3°C/cycle. The data were calculated with 2^{-Ct}. Primer sequences are as VEGF: forward, 5'-AGGAGGAGGGCAGAATCATC and reward 5'-ATGTCCACCAGGGTCTCGAT-3', beta-actin, Forward 5'-CGTCTTCCCCTCCATCG-3',

and Reward5' - CTCGTTAATGTCACGCAC-3'. All determinations were measured in triplicates. The cycle threshold (*Ct*) values corresponded to the PCR cycle number at which fluorescence emission in real time reached a threshold above the base-line emission and were analyzed. The *Ct* value of the PCR product of interest and a control mRNA (β -actin) was then used to calculate relative quantities of mRNA between samples.

Statistical Analysis

For statistical evaluations of the calculated relative expression variations, data were analyzed for significant differences by ANOVA using approximate post-hoc tests. A level of $p < 0.05$ was considered statistically significant.

Results

Exogenous PEDF increases VEGF expression by hMSCs in maintenance medium

We first examined whether exogenous PEDF had an effect on VEGF expression by hMSCs cultured in maintenance medium (DMEM, 10% FBS, 1% P/S v/v), at different days of culture. The results showed that MSCs exposed to PEDF exhibited increased VEGF expression compared to cells not incubated in presence of PEDF (Fig. 1). These data indicate that PEDF increases expression of PEDF by hMSCs under static conditions.

Exogenous PEDF increases VEGF expression in differentiating MSCs

Next, we determined whether hMSC cultured in osteogenic medium supplemented with exogenous PEDF will increase VEG expression. The data showed that hMSCs incubated in osteogenic medium and supplemented with exogenous PEDF increased VEGF expression. The major increase was at day 7 of osteogenic differentiation (Fig. 2). These data indicate that PEDF increases expression of VEGF during osteoblastic differentiation. Because both PEDF and VEGF are synthesized by MSCs and they increase during differentiation (see below), the results imply that PEDF does not inhibit VEGF expression by differentiating MSCs but rather it promotes or regulates its expression.

hMSCs increase expression of PEDF and VEGF during osteoblastic differentiation

Because exogenous PEDF expression by differentiating MSCs cultured in osteogenic medium, we assessed we examined relative expressions of PEDF and VEGF during MSCs-osteoblastic differentiation. The results showed that both PEDF and VEGF increased in expression during MSCs-osteoblastic differentiation showing maximal expression at day 14 (Fig. 3). The data indicate that increase in PEDF synthesis did not affect VEGF synthesis during MSCs differentiation. Thus a balance between PEDF and VEGF is maintained in MSCs during osteoblastic differentiation suggesting that the two factors play a role in enhancing osteoblast differentiation and matrix mineralization.

PEDF increases VEGF expression in hMSCs via ERK signaling pathway

We reported previously that PEDF promotes expression of osteoblast associated genes via Erk signaling pathway [11]. In this report, we asked whether PEDF increases and regulates VEGF expression via similar mechanisms. Exposure of MSCs to PEDF activated Erk

signaling pathway as indicated by Erk phosphorylation, (p-Erk1/2) (Fig. 4A). Treatment of MSCs with PEDF in presence of Erk inhibitor (iErk) suppressed Erk activation as indicated by reduction in Erk phosphorylation (Figure 4A). We then examined if inhibition of Erk activation reduced VEGF expression. Figure 4B shows that PEDF increases VEGF expression; treatment of MSCs with PEDF in presence of Erk inhibitor reduced VEGF mRNA expression (Fig. 4B). Following mRNA analysis, we determined if inhibiting Erk had an effect on VEGF protein synthesis by hMSCs. The data showed that within 3 days following incubation of MSCs with PEDF in presence of Erk inhibitor, VEGF synthesis was reduced suggesting that increased VEGF expression and regulation by PEDF is mediated via Erk signaling pathway (Fig. 4C).

Discussion

Null mutations in *serpinf1* gene that encodes PEDF lead to the development of OI type VI whose hallmark is presence of extensive osteoid build up that fails to mineralize [7,8,9,10]. Mechanisms by which PEDF regulates bone mineralization remain undefined. We and others showed that PEDF increased MSCs-osteoblastic mineralization; in addition, we showed that PEDF enhanced expression of genes that encode proteins which promote matrix mineralization [11,12]. In this report, we examined whether VEGF which plays important role in bone development is also regulated by PEDF. Because PEDF is known as a potent antiangiogenic factor its expression in bone would have undesirable consequences. Previous reports showed that VEGF can have a direct effect on osteoblast differentiation and bone mineralization [19,20,26]. VEGF plays important role in fracture healing through coupled angiogenesis and osteogenesis. The present report has established that VEGF expression was increased by differentiating murine mesenchymal stem cells incubated in osteogenic medium suggesting that it plays a role during osteoblastic differentiation. Interestingly, VEGF expression we examined was associated with cells not the medium. A report by Olsen and colleagues showed that intracellular VEGF was crucial for MSCs differentiation and matrix mineralization [27]. Our present data imply that PEDF may increase expression of intracellular VEGF by MSCs which then leads to the enhancement of MSCs differentiation and matrix mineralization. These assumptions remain to be further investigated.

The present findings may be unique to MSCs, because PEDF has been reported to reduce expression of VEGF in retinal capillary endothelial cells and muller cells [18]. Because increased synthesis of PEDF during differentiation by MSCs did not appear to have an effect on VEGF synthesis, the findings suggest that PEDF and VEGF are coordinately regulated in MSCs to maintain a balance between the two factors. Our findings also suggest that PEDF regulates VEGF in hMSCs differently in MSCs than in other cell types and that this may be part of the mechanisms by which PEDF regulates osteoblastic mineralization.

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Abbreviations

PEDF	Pigment epithelium derived factor
VEGF	Vascular endothelial growth factor

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Figure 1. Exogenous PEDF increases synthesis of VEGF by MSCs in maintenance medium at different days

A) hMSCs incubated in maintenance medium supplemented with exogenous PEDF increased expression of VEGF mRNA . B) Western blot showing VEGF synthesis by MSCs in maintenance medium at different days. Medium supplemented with PEDF showed increased synthesis of VEGF. NM=maintenance medium; NM+PEDF=maintenance medium supplemented with PEDF.

Figure 2. Exogenous PEDF enhanced expression of VEGF by hMSCs in osteogenic medium
 A) A western blot showing increased VEGF synthesis by hMSCs incubated in osteogenic medium supplemented with exogenous PEDF. B) Quantitation of VEGF synthesis by hMSCs maintained in osteogenic medium shows cultures supplemented with exogenous PEDF increased expression of VEGF by day 7 of culture in osteogenic medium.

Figure 3. hMSCs incubated in osteogenic medium increase synthesis of PEDF and VEGF during differentiation

A) Western blot of VEGF and PEDF proteins synthesized by hMSCs incubated in osteogenic medium. B) Quantitative analysis of VEGF and PEDF synthesis during MSCs differentiation in osteogenic medium. The data indicate that that PEDF and VEGF are coordinately regulated during MSCs-osteoblastic differentiation, both VEGF and PEDF showed maximal expression at day 14.

Figure 4. Analysis of PEDF-mediated activation of ERK signaling pathway in hMSCs to regulate VEGF expression

A) Incubation of hMSCs in presence of exogenous PEDF activated Erk signaling pathway (pERK1/2). Erk docking domain inhibitor (iERK) blocked activation of Erk following exposure of hMSCs to PEDF. B) VEGF gene expression was reduced by blocking Erk activation. C) Western blot indicated that increased expression of VEGF by exogenous PEDF was reduced by inhibition of Erk activation. These data indicate that PEDF regulates VEGF expression through Erk signaling pathway in hMSCs.