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Effects of vascular endothelial growth factor (VEGF) on MC3T3-E1

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Structured Abstract

Osteogenesis and angiogenesis are closely correlated. Vascular endothelial growth factor (VEGF) is believed to play a critical role in skeletal development.

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Objective – To investigate whether VEGF has direct effects on bone cells activities and to better understand how VEGF promotes bone remodeling.

Materials and Methods – MC3T3-E1 cell line was cultured with and without VEGF *in vitro*. The cells in both control and test groups were collected at different culture time points of 24, 48 and 72 h. Real-time polymerase chain reaction (qPCR) was carried out to quantify the mRNA expression of VEGF receptor (VEGFR2), alkaline phosphatase (ALP) and osteocalcin (OCN), osteoprotegerin (OPG) and receptor activator of nuclear factor kappa β ligand (RANKL).

Results – The expression of VEGFR2 significantly increased by 53% at 24 h and remained increased by 8% at 72 h compared to control ($p < 0.05$). ALP showed an early increase by 73% at 24 h ($p < 0.001$), but dropped by 14 and 41% at 48 and 72 h, respectively ($p < 0.05$). OCN was down-regulated by 41% at 24 h but then up-regulated by 149% at 72 h ($p < 0.001$). The expression of OPG significantly decreased by 7% at 24 h ($p < 0.001$) while dramatically increased by 133% at 72 h ($p < 0.001$). RANKL remained unchanged at all three time points ($p > 0.05$).

Conclusion – VEGF promotes bone remodeling by direct effects on osteoblastic cells via regulating gene expression of ALP, OCN, and OPG through VEGFR2 signaling pathway.

Key words: bone remodeling; osteoblast; VEGF

Introduction

Osteogenesis and angiogenesis are two closely correlated processes during bone growth, development, remodeling, and repair (1). Vascular endothelial growth factor (VEGF), the best characterized angiogenic mediator, is believed to play a critical role in skeletal development by enhancement of angiogenesis. VEGF was first identified by Ferrara and Hanzel (2) in 1989, and it is expressed in highly vascularized tissues. In recent years, VEGF has been the subject of interest in basic science research because it is the pivotal link between angiogenesis and osteogenesis. On the one hand, VEGF promotes endothelial proliferation,

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1 survival, and migration from pre-existing vasculature.
2 On the other hand, it is found that VEGF acts as an
3 essential mediator during bone growth, development,
4 remodeling, and repair. It has been shown by Emad
5 et al.'s *in vivo* study (3) that VEGF enhanced bone
6 formation when used in combination with demineral-
7 ized bone matrix of intramembranous origin (DBM_{IM})
8 compared to DBM_{IM} alone. The addition of VEGF to
9 DBM_{IM} improves the quality and quantity of newly
10 formed bone in the grafted site. A direct cause and
11 effect is revealed by Rabie and coworkers (4) on
12 recombinant adeno-associated virus-mediated gene
13 delivery system that exogenous VEGF leads to signifi-
14 cant condylar growth. Besides, a recent laboratory
15 study on rabbits has shown that cell-based VEGF gene
16 therapy can enhance the healing of a segmental defect
17 in the long bone of rabbits (5). Hence, it is believed that
18 VEGF enhances ossification. The mechanism may
19 involve an increase in angiogenesis as well as a direct
20 effect on bone cells. Many researches focus on
21 revealing the role of VEGF in osteogenesis as an
22 angiogenic mediator. As for the direct effects on bone
23 cells, VEGF has been implicated to stimulate cell
24 migration and proliferation (6, 7), but the effects on cell
25 activities related to the function of bone formation and
26 bone resorption as well as the underlying mechanism
27 have remained unclear.

28 Bone remodeling requires a specific balance
29 between bone formation and bone resorption. In this
30 study, we chose VEGF receptor (VEGFR2) besides two
31 markers related to bone formation [alkaline phosphatase
32 (ALP) and osteocalcin (OCN)] and two factors
33 related to bone resorption [osteoprotegerin (OPG) and
34 the receptor activator of nuclear factor kappa β ligand
35 (RANKL)] to examine the effects of VEGF on MC3T3-E1
36 osteoblastic cell line, to find out whether VEGF has
37 direct effects to increase bone cells activities, in an
38 attempt to better understand how VEGF promotes
39 bone remodeling.

40 Materials and methods

41 Cell culture: MC3T3-E1 cell line

42 MC3T3-E1 cells were cultured in Dulbecco's modified
43 Eagle's medium (DMEM) containing 10% fetal bovine
44 serum (FBS) and antibiotics (100 IU/ml of penicillin G
45 and 100 μ g/ml of streptomycin) and incubated at 37°C

in a 5% CO₂ humidified atmosphere. Cells were cul-
tured with final concentration of 10 ng/ml mouse
VEGF (R&D System, Minneapolis, MN, USA) in the test
group and cultured without any intervention in the
control group. The cells in both control and test groups
were collected at different culture time points of 24, 48,
and 72 h, respectively.

46 Total RNA extraction and cDNA synthesis

Total RNA was isolated from cells cultured in a 6-well
plate using TRIzol reagent according to the manufac-
turer's instructions (Invitrogen, Carlsbad, CA, USA).
Total RNA (1 μ g) was used to generate cDNA in each
sample using SuperScript II reverse transcriptase
(Invitrogen) following the manufacturer's instructions.
The reaction mixture consisted of 1 μ g of total RNA,
1 μ l of Oligo(dT)₁₂₋₁₈ (500 μ g/ml) (Invitrogen), 1 μ l of
dNTP Mix (10 mM each) (Invitrogen), 4 μ l of 5X First-
Strand Buffer (Invitrogen), 2 μ l of 0.1 M DTT (Invitro-
gen), and 1 μ l of SuperScript™ II RT (Invitrogen).

47 Real-time polymerase chain reaction

Real-time polymerase chain reaction (qPCR) was car-
ried out to quantify the expression of VEGFR2, ALP,
OCN, OPG, and RANKL at mRNA level with glyceral-
dehyde-3-phosphate dehydrogenase (GAPDH) as a
house-keeping gene. PCR amplification was performed
on a StepOnePlus® Real-time PCR System (96-well
plate) using SYBR® Green PCR Master Mix (ABI, Foster
City, CA, USA) as PCR mixture. Amplification of cDNA
included an initial denaturation at 95°C for 10 min,
followed by 40 cycles (95°C denaturation for 15 s, 60°C
annealing for 1 min) and final extension at 95°C for
15 min. Primers are shown in Table 1. Experiments
were repeated three times.

48 Statistical analysis

Real-time quantitative data analysis was performed
with InStat (version 2.04a, 1994, Graphpad, San Diego,
CA, USA). The arithmetic mean of cDNA copy number
and SD were calculated according to Δ Ct and standard
curves for both control and test group. *T*-test was used
to compare the mean difference between the two
groups. The probability level of $p < 0.05$ was regarded
as statistically significant.

Table 1. Primers of VEGFR2, ALP, OCN, OPG, and RANKL for real-time PCR

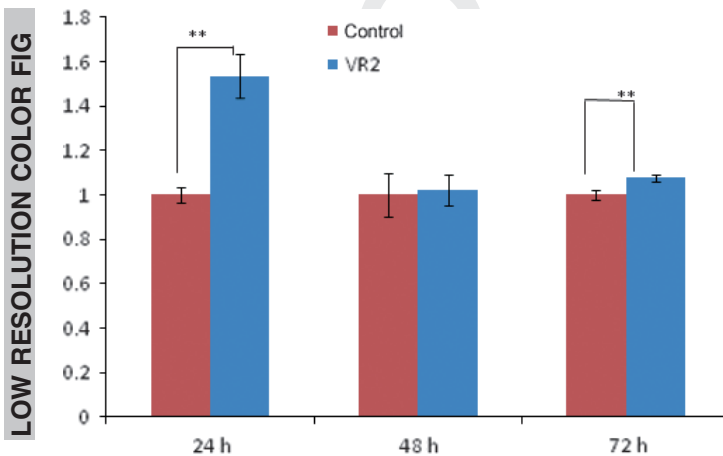
Gene	Sequence of primers		Product size (bp)
VEGFR2	Forward	5'AGATGCCCGACTCCCTTTG3'	100
	Reverse	5'CCAGAGCAACACACCCGAAAGA3'	
ALP	Forward	5'-TGGCTCTGCCTTTATTCCCTAGT-3'	100
	Reverse	5'-AAATAAGGTGCTTTGGGAATCTGT-3'	
OCN	Forward	5'-GCATAAGCTCACCGTCCACAA-3'	117
	Reverse	5'-GCCACATCCCCCAAAT-3'	
OPG	Forward	5'-CACCTGAAGGGCCTGATGT-3'	59
	Reverse	5'-TTTTGGGAAAGTGGGATGTTTT-3'	
RANKL	Forward	5'-GCTTCTCAGGAGCTCCAGCTAT-3'	58
	Reverse	5'-CCTCGCTGGCCACATC-3'	

ALP, alkaline phosphatase; OCN, osteocalcin; OPG, osteoprotegerin; RANKL, nuclear factor kappa β ligand.

Results

The expression of VEGFR2 significantly increased by 53% at 24 h and remained increased by 8% at 72 h compared to control ($p < 0.05$) (Fig. 1). ALP showed an early increase by 73% at 24 h ($p < 0.001$), but dropped by 14 and 41% at 48 and 72 h, respectively ($p < 0.05$) (Fig. 2). OCN was down-regulated by 41% at 24 h but then up-regulated by 149% at 72 h ($p < 0.001$) (Fig. 3).

The expression of OPG significantly decreased by 7% at 24 h ($p < 0.001$) while dramatically increased by 133% at 72 h ($p < 0.001$) (Fig. 4). RANKL remained unchanged at all three time points ($p > 0.05$) (Fig. 5). Thus, OPG/RANKL ratio was down-regulated first, and then up-regulated. Experiments were repeated three times with similar results.



4, 10, 12 Fig. 1. Effect of vascular endothelial growth factor on expression of VEGFR2 in MC3T3-E1 *in vitro*.

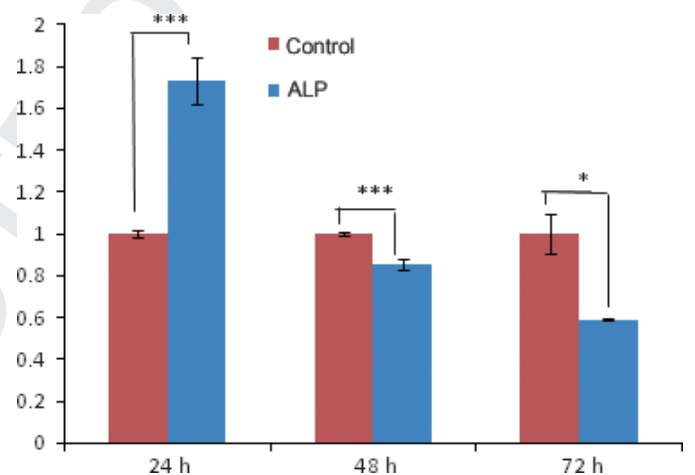


Fig. 2. Effect of vascular endothelial growth factor on expression of alkaline phosphatase in MC3T3-E1 *in vitro*.

Discussion

Biological effects of VEGF are mediated by specific tyrosine kinase receptors (VEGFRs), i.e. VEGFR-1/Flt-1 and VEGFR-2/KDR. In this study, expression of VEGFR2 significantly increased by 53% at 24 h and remained increased by 8% at 72 h compared to control (Fig. 1), which indicates that VEGF up-regulates expression of VEGFR2 in MC3T3-E1 osteoblastic cells. VEGF was found to increase bone formation indicated by increase in ALP activity and OCN expression (Figs 2 and 3). Furthermore, it was found to affect bone resorption through regulation on OPG/RANKL ratio (Figs 4 and 5). These findings indicate that VEGF has a direct effect on osteoblasts and subsequently on bone remodeling. Bone remodeling is a lifelong process by

LOW RESOLUTION COLOR FIG

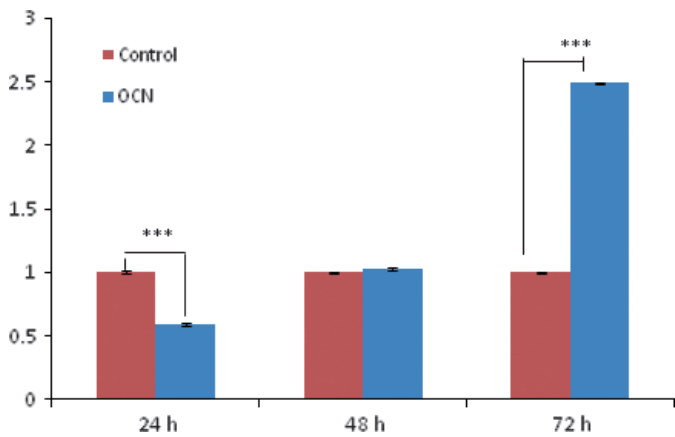
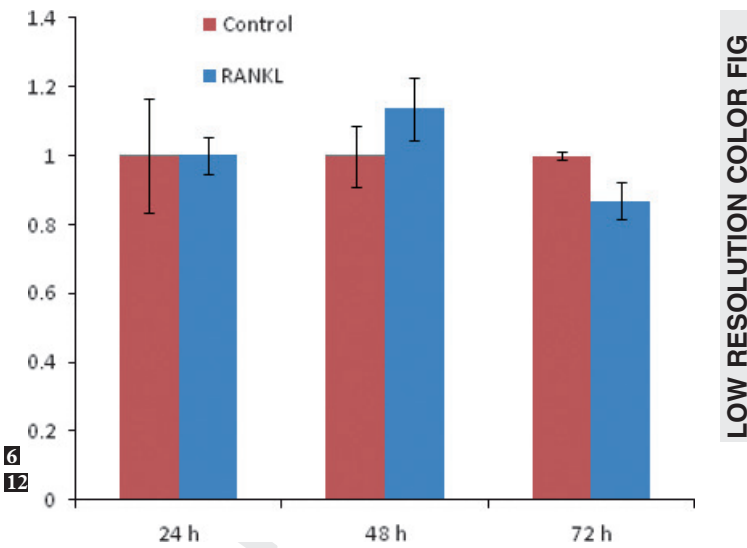


Fig. 3. Effect of vascular endothelial growth factor on expression of osteocalcin in MC3T3-E1 *in vitro*.



LOW RESOLUTION COLOR FIG

Fig. 5. Effect of vascular endothelial growth factor on expression of nuclear factor kappa β ligand in MC3T3-E1 *in vitro*.

LOW RESOLUTION COLOR FIG

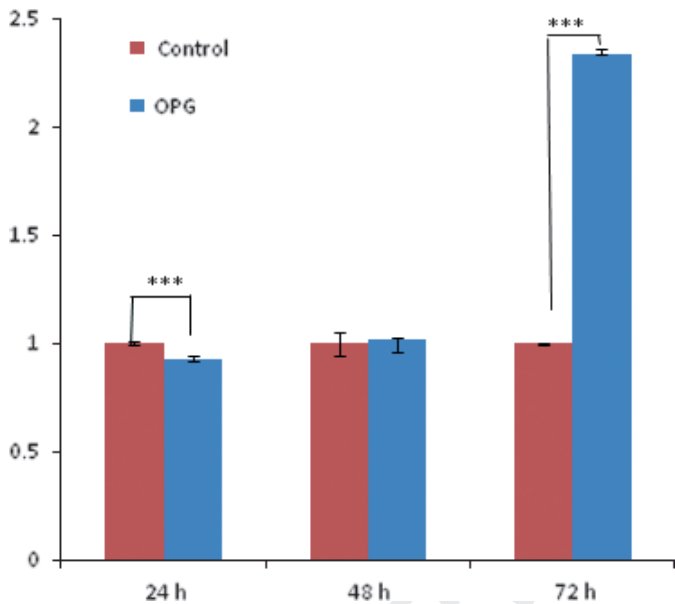
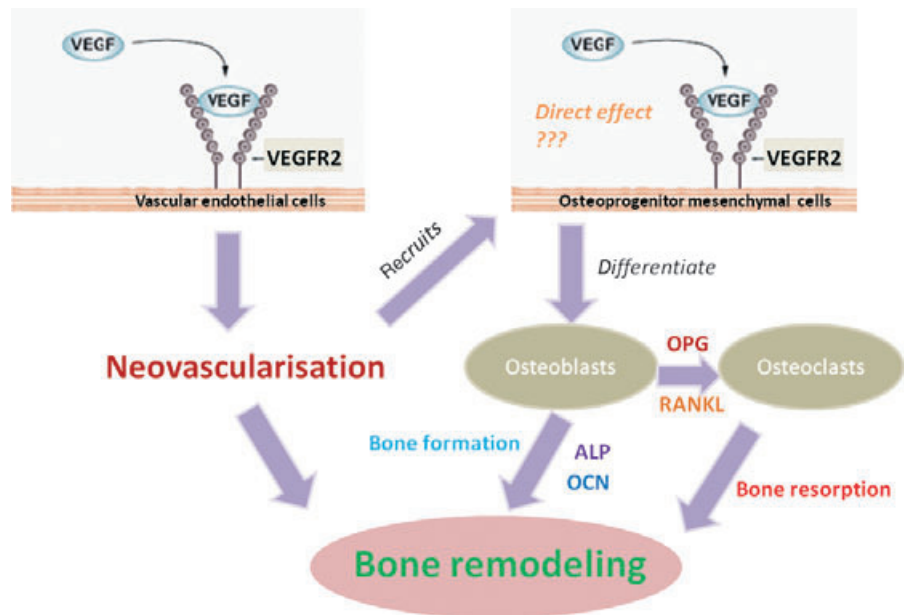


Fig. 4. Effect of vascular endothelial growth factor on expression of osteoprotegerin in MC3T3-E1 *in vitro*.

along the osteoblast pathway in the presence of ascorbic acid and is able to express mineralization markers. It has been used as a model system for studying the effect of Quercetin on pre-osteoblasts *in vitro* (10). In this study, this model was used similarly to study the effect of VEGF on osteoblasts *in vitro*.

The first step of bone remodeling is removal of 'old' bone followed by new bone formation. Removal of 'old' bone is based on bone resorption, which is accomplished by several important steps: recruitment of osteoclasts into the resorption site, osteoclasts differentiation, dissolution of bone minerals, and degradation of organic matrix (11). Yasuda et al. (12), Katagiri and Takahashi (13) identified two important key factors for osteoclastogenesis, RANKL, and OPG. These two factors are expressed by osteoblasts and are directly related to the regulation of activation and function of osteoclasts. The discovery of RANKL-RANK interaction confirms the hypothesis that there is a direct contact between osteoblasts and osteoclasts and that osteoblasts play an essential role in osteoclast differentiation. Osteoblasts express RANKL as a membrane-associated factor. Osteoclast precursors that express RANK, a receptor for RANKL, recognize RANKL through the cell-cell interaction and differentiate into osteoclasts. OPG is the decoy receptor of RANKL, which inhibits osteoclast differentiation by binding to RANKL, thereby preventing interaction with RANK. Thus, OPG inhibits osteoclast formation, whereas RANKL has the reverse action. Our result showed significantly

which bone grows and turns over. It involves a precise regulation between bone formation and bone resorption during development and maintenance of the skeleton. Osteoclasts, the multinucleated giant cells that resorb bone, develop from hematopoietic cells of the monocyte/macrophage lineage (8). Osteoblast is a mononucleate cell, arising from osteoprogenitor cells located in the periosteum and the bone marrow that is responsible for bone formation (8). Interestingly, besides bone formation, osteoblast is also known to regulate osteoclasts' formation and activity, i.e. osteoclastogenesis (9). Therefore, osteoblast is the key to investigate the effects on bone remodeling. The mouse pre-osteoblastic cell line, MC3T3-E1, can differentiate



3.9 Fig. 6. Xxxx.

decreased OPG expression with unchanged RANKL at the beginning (at 24 h). As RANKL remains, OPG/RANKL ratio decreased. The result suggests increased bone resorption to remove the 'old' bone that is essential for the trigger of bone remodeling.

Some researchers suggest that an interaction between VEGF and matrix metalloproteinase (MMP) occurs during skeletal development (14, 15). MMP, also called matrixin, is one of the two major classes of proteolytic enzymes besides cysteine proteinase, which is thought to play an essential role in degradation of the organic matrix in bone (16, 17). MMP-9 is able to trigger angiogenesis by the release of extracellular matrix (ECM)-bound VEGF (14). In a word, both MMP-9 and VEGF are related to angiogenesis as well as degradation of bone matrix. Lu and Rabie (18) have demonstrated that expression of MMP-9 is essential for graft bone resorption as it has influence on the removal of the cartilage matrix to allow for invasion of new blood vessels. In our study, increased osteoclastogenesis implicated by decreased OPG/RANKL ratio at the beginning of VEGF induction is coincident with the effect of MMP, which may benefit invasion of new blood vessels together with undifferentiated mesenchymal cells and osteoprogenitor cells that then engage in osteogenesis (19). This implies that more bone resorption at the beginning of bone remodeling may be one essential step for osteogenesis. The interaction between MMPs and VEGF was not analyzed in this

study, and further research on this field may provide evidence to the available data.

At 72 h, OPG expression increased dramatically with unchanged RANKL. Thus, OPG/RANKL ratio increased. This indicates that less bone resorption occurs at the later stage for the balance of bone remodeling. Several researchers reported enhanced expression of RANKL with VEGF (20–22). Moreover, Yao et al. (21) found that VEGF alone can neither promote osteoclastogenesis nor stimulate proliferation of the osteoclast precursors *in vitro* and a low dose of colony-stimulating factor-1 (CSF-1) in combination of VEGF is needed. However, this is not shown in our results as we found that RANKL remained unchanged at all three time points. This could probably be explained by the varied concentrations of VEGF used by different researchers. For instance, Nakagawa et al. (22) used 100 ng/ml of VEGF, and the concentration used in this study was 10 ng/ml.

New bone is formed following the removal of 'old' bone. Osteoblasts arise from osteoprogenitor cells that are responsible for bone formation (8). The process of osteogenesis consists of cell proliferation, cell differentiation, and mineralization. Cell differentiation is at the completion of proliferation when genes associated with matrix development and maturation are up-regulated, and mineralization occurs at the onset of ECM calcifies. A series of signaling mechanisms are illustrated, whereby the proliferation period supports the synthesis of a type I collagen–fibronectin ECM, which

continues to mature and mineralize. ALP and OCN are two common bone formation markers (23–25). ALP is known as an early marker responsible for osteoblastic differentiation, whereas OCN is considered a late marker as it is closely related to calcification. In this study, we found that ALP expression was up-regulated at the early stage (at 24 h), while OCN increased at the later stage (at 72 h). These results coincide with the time-serial function of ALP and OCN.

The formation of ECM down-regulates proliferation, and matrix mineralization down-regulates the expression of genes associated with the ECM maturation period (26). It explains our results that ALP was up-regulated at the early stage but OCN was down-regulated at the same time, while OCN increased at the late stage but ALP decreased at the same time. The postproliferative low-level basal expression of OCN increases transcriptionally during the period of active ECM mineralization reflecting maturation of the bone cell phenotype, and synthesis of OCN peptide parallels mRNA levels (26).

Taken together, it has been shown that VEGF has direct effects on osteoblastic cells *in vitro*. Combined with the results of *in vivo* studies (4–6) that VEGF enhances bone formation, the role of VEGF to enhance

osteogenesis is established, and this is mediated by either inducing neovascularization or by direct effects on bone cells. Figure 6 summarizes the effect of VEGF on bone remodeling.

Conclusion

VEGF promotes bone remodeling by direct effects on osteoblastic cells via regulating gene expression of ALP, OCN, and OPG through VEGFR2 signaling pathway.

Clinical relevance

This article provides evidence on how VEGF promotes bone remodeling. By doing so, VEGF could be applied in clinical settings, for example, to promote mandibular condylar growth in skeletal class II patients.

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