

Mechanical stress up-regulates RANKL expression via the VEGF autocrine pathway in osteoblastic MC3T3-E1 cells

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Received October 10, 2008; Accepted December 23, 2008

DOI: 10.3892/mmr_00000088

Abstract. Although it has been reported that vascular endothelial growth factor (VEGF) promotes not only angiogenesis but also osteoclast and osteoblast differentiation, few reports exist regarding VEGF/VEGF receptor (VEGFR) signaling in osteoblasts, which regulate osteoclast differentiation and generate VEGF. This study examined the expression of the bone remodeling factor VEGF-A and its receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), in murine osteoblastic MC3T3-E1 cells with the application of mechanical stress. The protein concentration of VEGF-A in the mechanical stress group increased markedly compared with the control group, while that of macrophage colony-stimulating factor in the mechanical stress group was lower than in the control group. VEGFR-2 mRNA expression was not detected in osteoblastic MC3T3-E1 cells. Mechanical stress up-regulated VEGF-A, VEGFR-1 and the receptor activator of nuclear factor- κ B ligand (RANKL) mRNA expression. In particular, VEGF-A and RANKL mRNA expression increased immediately after mechanical stress. We examined the VEGF/VEGFR system on anti-mouse VEGF neutralizing antibody in osteoblasts with mechanical stress. Neutralizing antibody to VEGF partially inhibited the increase of VEGF-A and RANKL mRNA expression compared with the non-mechanical stress group. VEGFR-1 mRNA expression was completely suppressed to control levels by the neutralizing antibody to VEGF. These findings suggest that mechanical stress up-regulates RANKL expression via the VEGF/VEGFR-1 autocrine pathway in osteoblastic MC3T3-E1 cells, indicating the possibility that, in response to mechanical stress, osteoblasts increase bone resorption by an autocrine up-regulation of VEGF/VEGFR-1 and RANKL expression.

Introduction

Bone remodeling requires bone resorption and formation and is controlled by osteoclasts and osteoblasts. It was reported that macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL) are necessary and sufficient for osteoclast differentiation (1,2). However, M-CSF (also known as CSF-1) was instead found to stimulate osteoclast survival (3), and the binding of RANKL to its receptor RANK proved essential for osteoclast differentiation (4). Osteoprotegerin (OPG) is the decoy receptor for RANKL and inhibits RANKL-RANK signaling (5). Osteoblasts express RANKL and OPG, and coordinate osteoclast differentiation and bone resorption (6,7).

Vascular endothelial growth factor (VEGF) is the most important mediator of angiogenesis (8). VEGF-A (a member of the VEGF family), as a substitute for M-CSF, can enhance osteoclastic bone resorption through its VEGF-A receptors (VEGFR) (9,10). VEGF-A is a significant regulator of angiogenesis and hematogenesis (11,12). The tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) receive VEGF-A (13,14) and are expressed in mature osteoclasts and osteoblasts (15). *In vivo*, osteoclasts appear concurrently with blood vessel invasion, and capillary angiogenesis and bone matrix resorption are essential for bone remodeling (16). Therefore, bone vascularization by VEGF-A may lead to the recruitment of osteoblasts and osteoclasts for bone matrix remodeling. Previous studies have reported that the differentiation and migration of osteoblasts are supported by VEGFR-1 signaling (17-19).

Mechanical stress is a key regulator of osteoblast and osteoclast activity in bone. Mechanical stress (mechanical stimulus and loading) regulates the activation and differentiation of osteoblasts (20,21), induces RANKL availability (22) and increases VEGF and M-CSF expression (23). It has been indicated that collagenase-3 (matrix metalloproteinase-13; MMP-13) may act as a 'coupling factor', allowing osteoblasts to exert control over osteoclast resorptive activity (24). Mechanical strain up-regulates MMP-13 expression in MC3T3-E1 osteoblastic cells (25).

In the process of bone remodeling caused by orthodontic force, osteoclasts are induced by osteoblasts. Subsequently, the osteoblasts should form new bone and remodel the resorbed area. Consequently, the function of osteoclasts is enhanced by

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Key words: mechanical stress, osteoblast, vascular endothelial growth factor, flexercell tension system

mechanical stress in the orthodontic tooth movement. Since osteoblasts express VEGF-A, VEGFR-1 and VEGFR-2, autocrine action by the VEGF/VEGFR system may up-regulate the differentiation and function of osteoclasts. However, few reports exist on how the VEGF/VEGFR system functions and affects the bone remodeling process under mechanical stress. This study aimed to investigate the VEGF/VEGFR system of osteoblasts with mechanical stress using the flexercell tension system.

Materials and methods

Cell culture. Murine osteoblastic MC3T3-E1 cells (Riken Cell Bank no. RCB1126, Japan) were used. The cells were cultured in α MEM (Invitrogen, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum, 66.7 μ g/ml kanamycin-sulfate and 284 μ M L-ascorbic acid 2-phosphate at 37°C in a humidified atmosphere of 95% air and 5% CO₂ (26). Subsequently, the cells were cultured to subconfluence in 100 mm standard dishes (Falcon™, Becton-Dickinson Labware, Franklin Lakes, NJ, USA) and transferred to Bioflex collagen I-coated 6-well plates (Flexcell Corp., Hillsborough, NC, USA) after treatment with 0.25% trypsin/EDTA (Invitrogen).

Flexcell tension system and cell culture. Mechanical stress was applied to the MC3T3-E1 cells with a Flexercell Strain-Unit (Flexcell Corp.) (27,28). After being transferred to Bioflex collagen I-coated 6-well plates, the cells were cultured overnight and then mechanically stressed at the following time points: 6, 12, 24 and 48 h. The experimental regimens used delivered 10% elongation at 30 cycles/min (0.5 Hz) in a humidified atmosphere of 95% air and 5% CO₂ (28). The control culture was grown under the same conditions without mechanical stress.

Reverse transcription-polymerase chain reaction. Total RNA was extracted using the TRIzol reagent isolation kit (Invitrogen). cDNA was amplified by Rever TraAcea FSK-101/Toyobo, Japan). Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed as previously described (28). The primers used were: M-CSF, VEGF-A, VEGFR-1, VEGFR-2, RANKL, OPG, alkaline phosphatase (ALP), collagen type I and MMP-13. Primer sequences are shown in Table I. Amplified products were distributed on a 1% agarose gel attained with ethidium bromide. The same cDNA was amplified using primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in order to equalize the amount of total RNA.

Measurement of VEGF and M-CSF concentrations. Cultured medium was collected from MC3T3-E1 cells that had or had not been exposed to mechanical stress. Protein concentrations of VEGF and M-CSF were measured using Enzyme Linked Immunosorbent Assay (Quantikine M mouse VEGF Immunoassay kit, Quantikine M mouse M-CSF Immunoassay kit; R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Neutralizing effect of anti-VEGF antibody. Anti-mouse VEGF neutralizing antibody (rmVEGF) (AF-493-NA; R&D Systems Inc.) was added to the culture medium 24 h before the application of the Flexercell Strain Unit. As the maximum dose,

Table I. Primer sequences.

Gene	Primer sequence (5'-3')
M-CSF	F: CCCATATTGCGACACCGAA R: AAGCAGTAACTGAGCAACGGG
VEGF-A	F: GGACCCTGGCTTTACTGC R: CGGGCTTGGCGATTTAGOPG
Flt-1 VEGFR-1	F: GGTGCCCCGCTCTTTG R: TGTCTCAGTGGGGATTGC
Flk-1 VEGFR-2	F: CCTGGCTGACCCGATTCC R: TCCCGCTTTGTTGATGGC
RANKL	F: TATGATGGAAGGCTCATGGT R: TGTCTGAACTTTGAAAGCC
OPG	F: GAGGAAGGAAAGGGCCTATG R: TGGGAATGAAGATCCTCCAG
ALP	F: CGGGGACATGCAGTATGA R: AAAGATGGAGTTGCCCGG
MMP-13	F: CTTCTGGTCTTCTGGCACAC R: CCCCACCCCATACATCTGAA
Collagen type I	F: ACTCCCCAGAGTTTGGAACCTTACTG R: GTCGGAGCAGACGGGAGTTTCTCCT
GAPDH	F: CGGAGTCAACGGATTTGGTCGTAT R: AGCCTTCTCCATGGTGGTGAAGAC

F, forward; R, reverse.

~500 pg/ml of rmVEGF was measured in one of the 48-h mechanical stress groups. Antibody (20 ng/ml) was applied to neutralize ~100% of rmVEGF bioactivity according to the manufacturer's dose-response curve.

Results

Effect of mechanical stress on mRNA expression. VEGFR-2 mRNA expression was not detected in the osteoblastic MC3T3-E1 cells (data not shown). VEGF-A and RANKL mRNA expression in the mechanical stress group was significantly increased at all time points compared with the control group. VEGF-A and RANKL mRNA expression resulted in a 2.7- and 3.6-fold increase at 48 h (Fig. 1A and C). VEGFR-1 and OPG mRNA expression markedly increased at 24 and 48 h, and resulted in a 2.0-fold increase at 48 h (Fig. 1B and D). M-CSF and collagen type I mRNA expression remained unchanged by mechanical stress at all time points (Fig. 1E and G). ALP mRNA expression was time-dependently decreased, culminating in a 0.65-fold decrease at 48 h (Fig. 1F). MMP-13 mRNA expression resulted in a 1.5-fold increase at 48 h only (Fig. 1H).

VEGF and M-CSF protein concentration. VEGF protein secretion significantly increased in the mechanical stress group compared with the control group at all time points (Fig. 2A).

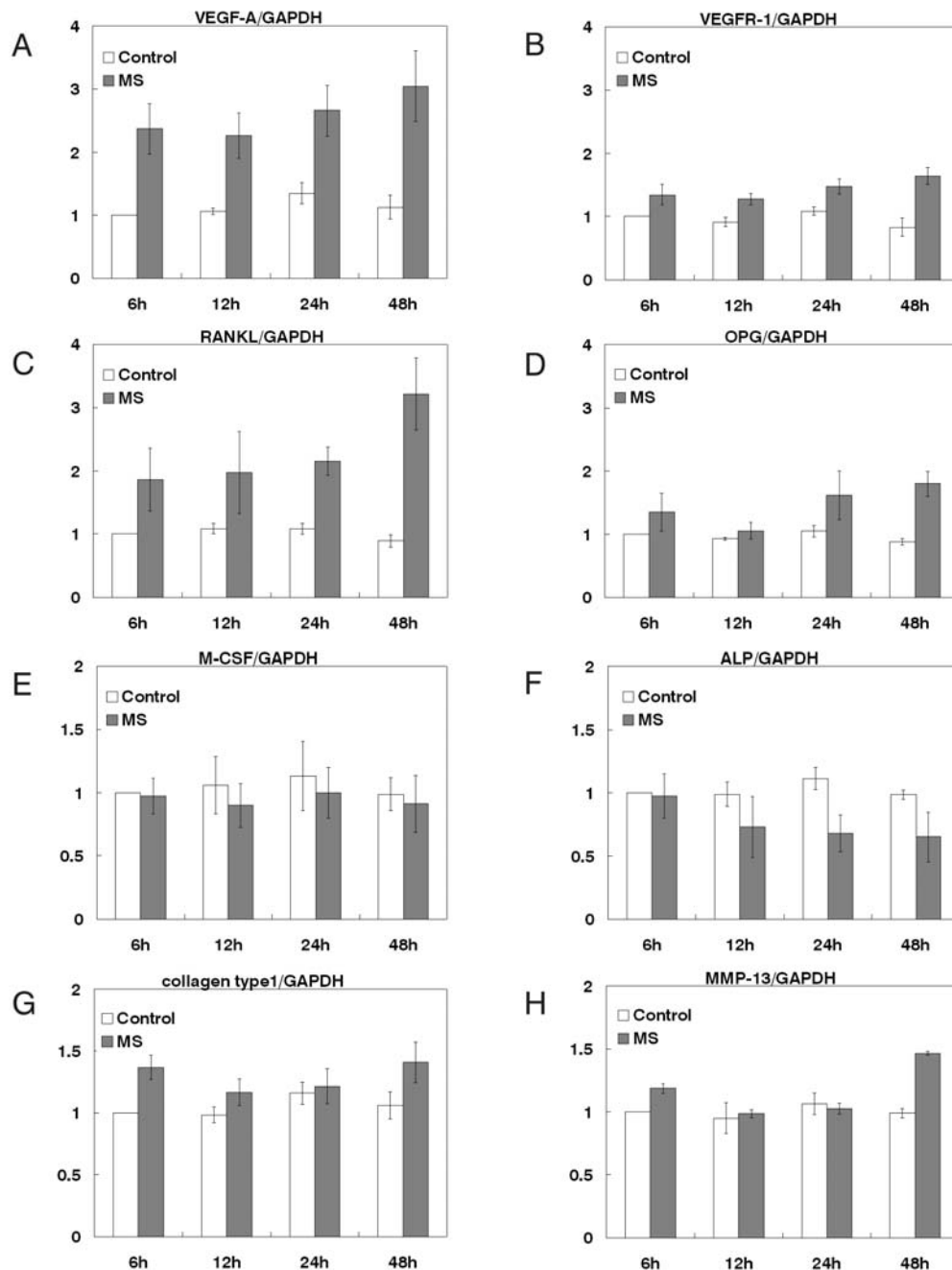


Figure 1. The effect of mechanical stress on mRNA expression of VEGF-A, VEGFR-1, RANKL, OPG, M-CSF, ALP, collagen type I and MMP-13. (A) VEGF-A mRNA expression increased time-dependently from 6 h, and resulted in a 2.7-fold increase at 48 h. (B) VEGFR-1 mRNA expression increased slightly at 6, 12 and 24 h, and resulted in a 2.0-fold increase at 48 h. (C) RANKL mRNA expression increased time-dependently from 6 h, and resulted in a 3.6-fold increase at 48 h. (D) OPG mRNA expression increased at 24 and 48 h, and resulted in a 2.0-fold increase at 48 h. (E) M-CSF mRNA expression was unchanged at the 6, 12, 24 and 48 h time points. (F) ALP mRNA expression decreased time-dependently, and resulted in a 0.65-fold decrease at 48 h. (G) Collagen type I mRNA expression remained unchanged. (H) MMP-13 mRNA expression resulted in a 1.5-fold increase at 48 h only. Results are shown as the means \pm SD (n=4). The control group at 6 h is defined as the standard (=1). Control, control group; MS, mechanical stress group.

The protein concentration of M-CSF in the two groups increased time-dependently. M-CSF protein concentration in the mechanical stress group was reduced compared with the control group at 12, 24 and 48 h (Fig. 2B).

Neutralizing effect of anti-VEGF antibody on mRNA expression. VEGF-A mRNA expression in the mechanical stress group remained unchanged at 6 and 12 h, but increased at 24 and 48 h compared with the control group (Fig. 3A). VEGFR-1 mRNA expression in the two groups remained

unchanged at all time points (Fig. 3B). RANKL mRNA expression was unchanged at 6 h, but increased at 12, 24 and 48 h (Fig. 3C). OPG mRNA expression remained unchanged at 6 and 12 h and slightly increased at 24 and 48 h (Fig. 3D). Treatment with anti-VEGF antibody reduced VEGF-A and RANKL mRNA expression by 0.53- and 0.56-fold compared with the non-mechanical stress group. VEGFR-1 mRNA expression was completely suppressed by the effect of the anti-VEGF antibody compared with the non-mechanical stress group. Neutralizing VEGF did not affect OPG mRNA

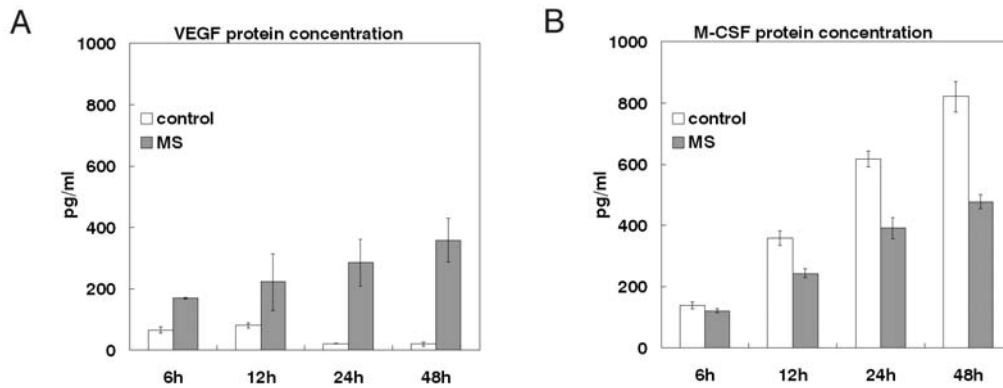


Figure 2. Effect of mechanical stress on the protein concentration of VEGF and M-CSF. (A) VEGF protein concentration in the mechanical stress group increased time-dependently, showing significant differences compared with the control group at 6, 12, 24 and 48 h, respectively. (B) M-CSF protein concentration in the two groups increased time-dependently, and was lower in the mechanical stress group than in the control group at 12, 24 and 48 h. The means and standard deviations of the VEGF and M-CSF protein concentration are shown (n=4). Control, control group; MS, mechanical stress group.

expression under mechanical stress. M-CSF mRNA expression remained unchanged at all time points (Fig. 3E). ALP mRNA expression decreased time-dependently (Fig. 3F). Collagen type I and MMP-13 mRNA expression remained unchanged at all time points (Fig. 3G and H). Neutralizing VEGF did not affect M-CSF, ALP and collagen type I mRNA expression under mechanical stress. MMP-13 mRNA expression was partially inhibited by the effect of anti-VEGF antibody compared with the non-mechanical stress group.

Discussion

It has been reported that VEGF is detected in periodontal tissues during experimental tooth movement, and that the local injection of VEGF enhances the number of osteoclasts and increases the rate of tooth movement (29,30). The effect of anti-VEGF polyclonal antibody markedly decreased the number of osteoclasts and suppressed the amount of tooth movement and the relapse of moved teeth (31). Osteoblast activation requires cell-to-cell association between osteoblasts (RANKL) and osteoclasts (RANK) (32). It has been reported that osteoblasts may up-regulate VEGF expression in response to mechanical stress through the SA channel (23). Thus, we examined the effect of anti-VEGF antibody on the VEGF-A, VEGFR, RANKL and M-CSF mRNA expression of osteoblasts under conditions of mechanical stress. The results suggest that osteoblastic MC3T3-E1 cells up-regulate VEGF/VEGFR-1 as a reaction to mechanical stress. As VEGF supports the differentiation and migration of osteoclasts and osteoblasts (9,10,17-19), the VEGF/VEGFR-1 autocrine pathway is thought to play an important role in bone resorption. A recent report suggested that the VEGF signalling pathway through the VEGFR-1 tyrosine kinase domain is implicated in osteoblast development (33). The VEGF/VEGFR-1 autocrine loop in osteoblasts may activate its function in response to mechanical stress.

Suppression of RANKL mRNA expression by neutralizing VEGF indicates that this expression is assisted by the VEGF/VEGFR-1 autocrine pathway. However, this study shows that mechanical stress up-regulates RANKL mRNA expression despite the interception of VEGF/VEGFR-1. It was reported that angiotensin II type 1 receptor (AT1), a known mechano-

receptor in cardiomyocytes, up-regulates RANKL mRNA expression to some extent in mechanically stressed osteoblasts (34). Such receptors of mechanical stimulation are also expected to control RANKL mRNA expression.

As OPG is the decoy receptor for RANKL and inhibits osteoclastogenesis, the bone resorption rate is controlled by the balance of RANKL and OPG (5,6). OPG mRNA expression was slightly up-regulated, thereby delaying RANKL mRNA expression. It appears that this negative feedback suppressed RANKL overexpression in osteoblasts.

The protein concentration of M-CSF in the two groups increased time-dependently. However, the protein concentration of VEGF in the control group did not. These results may be due to the fact that osteoblasts express VEGF/VEGFR-1 but not the M-CSF receptor c-Fms (35,36). Since no significant difference was observed in M-CSF mRNA expression, the induction of osteoclast precursor cells was expected to be accelerated by VEGF acting as a substitute for M-CSF.

It has been reported that ALP probably withdraws inorganic pyrophosphate (PPi), known as the inhibition of bone mineralization (37). Extracellular phosphate concentration is expected to be necessary and sufficient to induce bone mineralization (38). In this study, ALP mRNA expression was time-dependently down-regulated by mechanical stress. The down-regulation of ALP expression may help in the promotion of bone resorption.

Bone matrix is composed of collagen type I and non-collagenous protein. Osteoblasts participate in bone formation by producing collagen type I (39). In this study, mechanical stress and neutralizing VEGF did not affect collagen type I mRNA expression. It has been reported that MMP-13 can degrade type I, II and III collagens (40), and can be utilized as a marker of osteoblastic differentiation (41). Acting in cooperation with cathepsin K and MMP-9 produced by osteoclasts, MMP-13 produced by osteoblast-lineage cells may engage in bone matrix degradation in the process of bone resorption. MMP-13 from osteoblasts plays an important role in the initiation and progress of bone resorption (42). MMP-13 mRNA expression was partially inhibited by the effect of anti-VEGF antibody, raising the possibility that VEGF/VEGFR-1 partially supports MMP-13 mRNA expression.

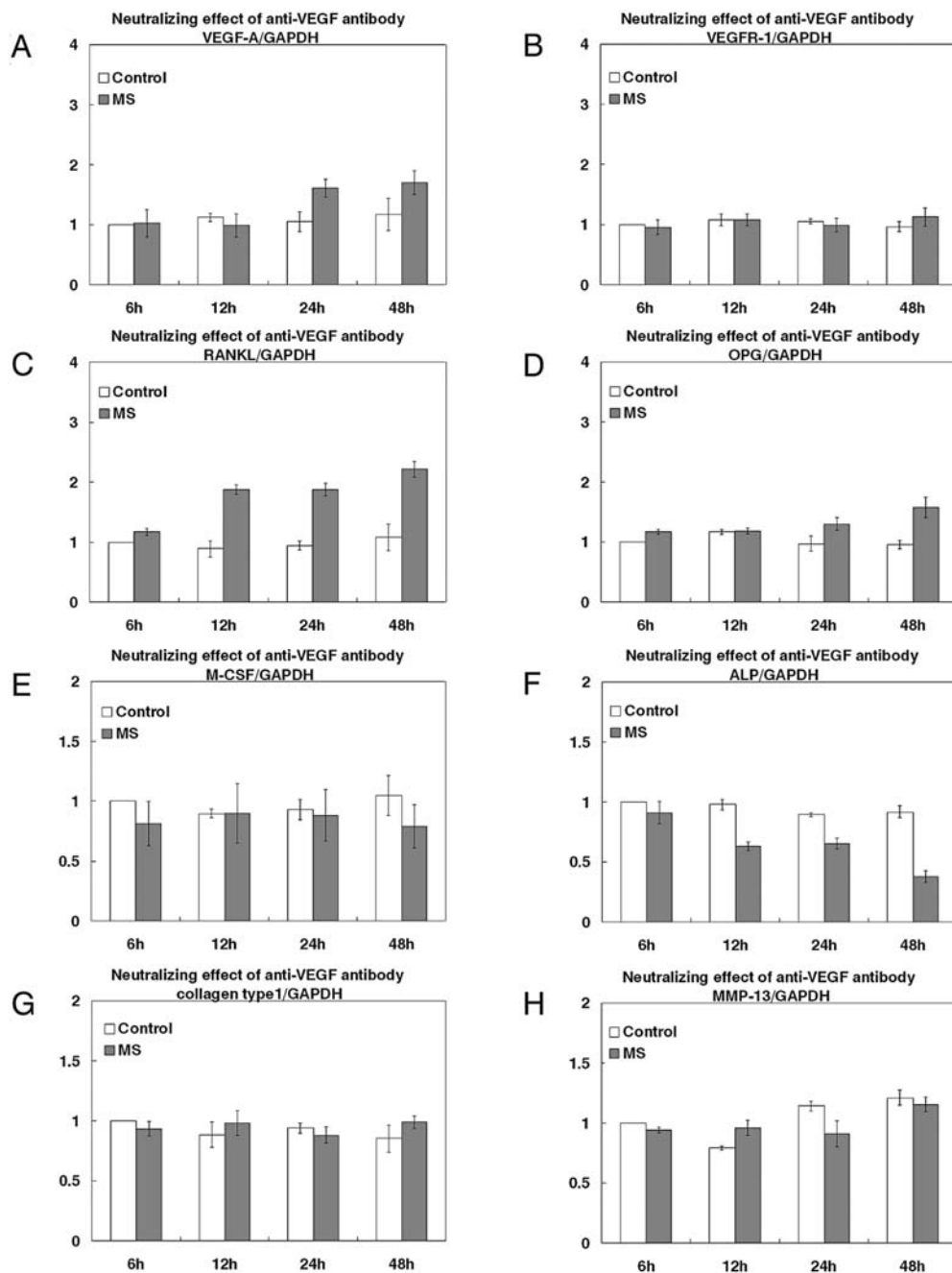


Figure 3. Neutralizing effect of anti-VEGF antibody on mRNA expression of VEGF-A, VEGFR-1, RANKL, OPG, M-CSF, ALP, collagen type I and MMP-13. VEGF-A mRNA expression was nearly equal to the control at 6 and 12 h and elevated at 24 and 48 h. (A) VEGF-A mRNA expression resulted in a 1.5-fold increase at 48 h. (B) VEGFR-1 mRNA expression was equivalent to the control at 6, 12, 24 and 48 h. (C) RANKL mRNA expression was nearly equal to the control at 6 h and elevated at 12, 24 and 48 h, and resulted in a 2.0-fold increase at 48 h. (D) OPG mRNA expression increased slightly at 24 and 48 h, and resulted in a 1.7-fold increase at 48 h. (E) M-CSF mRNA expression remained unchanged at 6, 12, 24 and 48 h. (F) ALP mRNA expression decreased time-dependently, and resulted in a 0.4-fold decrease at 48 h. (G and H) Collagen type I and MMP-13 mRNA expression was equivalent to control levels at 6, 12, 24 and 48 h. Results are shown as the means \pm SD (n=4). The control group at 6 h is defined as the standard (=1). Control, control group; MS, mechanical stress group.

In summary, since there are few reports on how the VEGF/VEGFR system functions and affects bone remodeling under mechanical stress *in vitro*, we aimed to investigate the VEGF/VEGFR system of osteoblasts with mechanical stress according to the flexercell tension system. We found that osteoblasts promote VEGF/VEGFR-1 expression and that the VEGF/VEGFR-1 autocrine pathway is involved in the reinforcement of RANKL expression in response to mechanical stress. Although no significant difference in M-CSF expression was observed, the induction of osteoclast precursor cells was apparently accelerated by VEGF acting as a substitute for

M-CSF. Therefore, mechanical stress up-regulates VEGF/VEGFR-1 and RANKL expression, promotes osteoclast differentiation and induces bone resorption.

Acknowledgements

We are grateful to Dr N. Suzuki for technical advice and support. This study was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan [Kakenhi (B): grant no. 17390549 to J.I., Kakenhi (C): grant nos. 18592225 and 19592344 to Y.Y.].

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