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Possible involvement of PI3K-dependent pathways in the increased $VEGF_{120}$ release from osteoblastic cells preloaded with palmitate *in vitro*

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ABSTRACT

It have been reported that abnormal bone metabolism often occurs in patients with type 2 diabetes, but the underlying mechanisms remain to be elucidated. In recent years dyslipidemia (hyperlipidemia) has been presumed to have an influence on bone metabolism. In addition, the involvements of VEGF and MCP-1 derived from osteoblasts in bone abnormal metabolism were also observed. Thus, we investigated the pathogenic mechanism of this abnormal bone metabolism, which is included in the regulation of VEGF and MCP-1 secretions from osteoblasts, by using UMR-106 osteosarcoma cells as an osteoblast cell model and treating them with palmitate in order to mimic a state of hyperlipidemia.

Palmitate-preloaded cells showed the significant increase of $VEGF_{120}$ release (1.8-fold vs. control cells, $p < 0.01$). Moreover, the treatment with palmitate significantly increased VEGF-A mRNA with the maximal 2.5-fold upregulation at 12 h after the treatment ($p < 0.01$). However, MCP-1 release was not affected by palmitate. Moreover, the amplified $VEGF_{120}$ secretion with palmitate was significantly decreased by the treatment with TLR4 antagonist or PI3K pathway inhibitors, LY294002 and wortmannin ($p < 0.01$, respectively). On the other hand, the stimulation with TNF-α, which osteoclasts were able to release, significantly enhanced MCP-1 secretion ($p < 0.01$), but had no effect on $VEGF_{120}$. On the contrary IL-1β amplified $VEGF_{120}$ release ($p < 0.01$), but not MCP-1.

These results suggest that palmitate can increase $VEGF_{120}$ release from UMR-106 osteosarcoma cells, which is accelerated at the transcriptional level, and this increase of $VEGF_{120}$ release may be mediated though, at least partly, TLR4 and the PI3K pathways. In addition, we also verified that TNF-α and IL-1β, which are considered to be derived from osteoclasts, amplified the secretions of MCP-1 and $VEGF_{120}$ from UMR-106 cells, respectively.

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1. Introduction

Recently, cases of osteoporosis have increasingly been reported in patients with type 2 diabetes, and abnormal bone metabolism has drawn attention as a serious complication in diabetes [1–3]. Bone metabolism comprises bone formation, mainly by osteoblasts, and bone resorption, by osteoclasts, which are balanced in healthy individuals, resulting in a stable bone metabolic turnover. However, we believe that this balance is disrupted in patients with type 2 diabetes, shifting toward bone resorption, thereby inducing abnormal bone metabolism. The presumed mechanisms underlying reduced bone formation under a diabetic state include reduced expression of Runx2 and osteocalcin, which are transcription factors required for osteoblast cell differentiation [1,3]; however, these mechanisms alone are not enough to explain the onset and progression of abnormal bone metabolism in type 2 diabetes. Increased bone resorption is considered to be more closely associated with abnormal bone metabolism than is reduced bone formation in patients under diabetic conditions [1–3], and therefore, the underlying mechanism urgently needed to be elucidated, but the details have remained unclear.

It has recently been reported that dyslipidemia (hyperlipidemia), which is frequently associated with diabetes, affects bone metabolism by reducing bone density in the bone cortex and cancellous bone [4], and that the blood lipid level is reported to be negatively associated with bone density [4]. Thus, hyperlipidemia may result in a disproportionate increase in bone resorption, disrupting the balance in bone metabolic turnover and triggering abnormal bone metabolism in patients with type 2 diabetes. In addition, vascular endothelial growth factor (VEGF), an angiogenic factor, was shown to cause abnormal activation of osteoclasts and

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increased bone resorption [5,6]. Moreover, MCP-1, a primary chemokine, promotes the fusion of hematopoietic stem cell-derived monocytes/macrophage precursor cells and further induces differentiation to osteoclasts, which could be another risk factor for osteoporosis [3].

Accordingly, in this study, we elucidated the pathogenic mechanism of abnormal bone metabolism in diabetic patients by using UMR-106 osteoblast cells derived from rat osteosarcoma cells and treating them with palmitate, a representative saturated fatty acid, to mimic a state of hyperlipidemia. The main aim of our study was to elucidate the kinetics of the secretion of VEGF (with a focus on $VEGF_{120}$, a freely secreted isoform without the heparin-binding domain [7,8]) and MCP-1 and their regulatory mechanisms in osteoblastic cells.

2. Materials and methods

2.1. Reagents

Palmitate and rat recombinant TNF-α and IL-1β were purchased from Wako (Osaka, Japan) and Lipopolysaccharide (LPS) was from Wako. LY294002 and SB203580 were obtained from Calbiochem (La Jolla, CA, USA) and Wortmannin was from Sigma (St. Louis, MO, USA) and SP600125 was from AG Scientific, Inc. (San Diego, CA, USA). Hydrogen peroxide (H_2O_2) was from Sigma (St. Louis, MO, USA) and Toll-like receptor 4 (TLR4) peptide inhibitor was from IMGENEX (San Diego, CA, USA). Antibodies against $VEGF_{120}$ and IL-10 were from R&D Systems (Minneapolis, MN, USA) and antibodies against MCP-1 was from GeneTex (Irvine, CA, USA). Anti-β-actin antibody from Sigma was used.

2.2. Preparation and treatment of UMR-106

UMR-106 osteosarcoma cells were obtained from the cell bank of the Japanese Collection of Research Bioresources (Tokyo, Japan). UMR-106 cells were seeded and fed in DMEM containing 25 mmol/L glucose supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 100 mmol/L MEN sodium pyruvate, and 10% FCS. Then, the cells were grown under 5% CO_2 at 37 °C. At the confluent condition, the cells were used for experiments. 0.03 mmol/L palmitate was added to the culture medium. At 48 h after the addition of palmitate, $VEGF_{120}$, MCP-1 and IL-10 release were quantified by immunoblotting, and $VEGF_{120}$ secretion was also measured at 48 h after the treatment with 100 ng/ml LPS. VEGF-A mRNA was analyzed at 0, 3, 6, 12 and 24 h by real-time RT-PCR after palmitate treatment.

In the same manner, UMR-106 cells were treated with 100 ng/ml TNF-α or 10 ng/ml IL-1β for 24 h, and $VEGF_{120}$ and MCP-1 release were quantified by immunoblotting, respectively.

In other experimental series, UMR-106 cells were pre-treated with either 5 mmol/L TLR4 peptide inhibitor, 50 μmol/L LY294004, 10 nmol/L Wortmannin, 1 μmol/L SP600125, 1 μmol/L SB203580 or 2 μmol/L PD98059, and then 0.03 mmol/L palmitate was added to the culture medium for 48 h. $VEGF_{120}$ released into the culture medium was then quantified by immunoblotting.

2.3. Immunoblotting

Cultured UMR-106 osteosarcoma cells were lysed in SDS sample buffer, sonicated, and centrifuged. Resulting supernatants were boiled in the presence of 50 mmol/L dithiothreitol. To measure secreted proteins, the cultured medium of the cells was also boiled in SDS sample buffer with 50 mmol/L dithiothreitol. Boiled samples were subjected to SDS–PAGE, and transferred onto polyvinylidene difluoride membranes (Bio Craft, Tokyo, Japan). Membranes were incubated with primary antibodies as described in Reagents, and thereafter with horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized with chemiluminescence reagents according to the manufacturer's protocol (Amersham, Little Chalfont, Buckinghamshire, UK). Bands were then scanned and analyzed with NIH Image software. Protein band intensities under basal conditions were set as 100% for normalization purposes. $VEGF_{120}$, MCP-1 and IL-10 proteins were confirmed from observations that the strong bands appeared at 20.0, 13.8, 18.0 and 19.0 kDa with their corresponding antibodies, respectively.

2.4. Quantitative real-time RT-PCR

Total RNA was extracted from UMR-106 using the RNAqueous®-4PCR kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Quantitative real-time RT-PCR was conducted using the 7300 real-time RCR system (Applied Biosystems, Foster City, CA, USA). The following primers and probes were ordered from Applied Biosystems: VEGF-A (Rm01511601_m1). The mRNA signal was normalized over 18S rRNA signal. A mean value of triplicates was used for relative mRNA level.

2.5. Hydroperoxides measurement

UMR-106 cells were incubated with 0.03 mM/L palmitate for 48 h. Supernatants were then removed and cells were washed three times with PBS. Cells were lysed in 0.5 mmol/L Tris–HCl (pH 7.4), 1.5 mmol/L NaCl, 2.5% deoxycholic acid and 10% Nonidet P-40. Lysates were centrifuged for 10 min at 15,000*g* and 4 °C; supernatants were assayed for intracellular endogenous hydroperoxides by the Free Radical Elective Evaluator system (Diacron, Grosseto, Italy) according to the manufacturer's protocol. Hydroperoxides units of Carratelli units were adjusted to intracellular total protein contents.

2.6. Statistical analysis

Statistical analysis was performed by unpaired *t*-test or by analysis of variance (ANOVA) using Stat View computer software (Abacus, Berkeley, CA, USA). Results are expressed as the means ± SEM and $p < 0.05$ was considered significant.

3. Results

3.1. The effects of palmitate on cytokine secretions from UMR-106 osteosarcoma cells

Immunoblotting results showed that $VEGF_{120}$, which is lacked a heparin-binding domain, secretion were increased significantly by 1.8-fold in 48 h palmitate-stimulated UMR-106 osteosarcoma cells relative to control cells ($p < 0.01$; Fig. 1A). However, no significant effect of palminate was elicited by stimulating for 48 h on either MCP-1 or IL-10 release from osteoblast-like UMR-106 cells (Fig. 1C and D).

3.2. Palmitate enhanced VEGF-A mRNA revel in UMR-106 cells

In addition, we assessed mRNA revel of VEGF-A including $VEGF_{120}$ in palmitate-stimulated UMR-106 cells. The treatment with palmitate significantly increased VEGF-A mRNA with 1.8-fold upregulation first at 6 h ($p < 0.05$), and the maximal 2.5-fold induction was seen at 12 h after the treatment ($p < 0.01$), and this significant increase continued up to 12 h (Fig. 1B). These results show that the increased $VEGF_{120}$ expression occurred at transcriptional level.

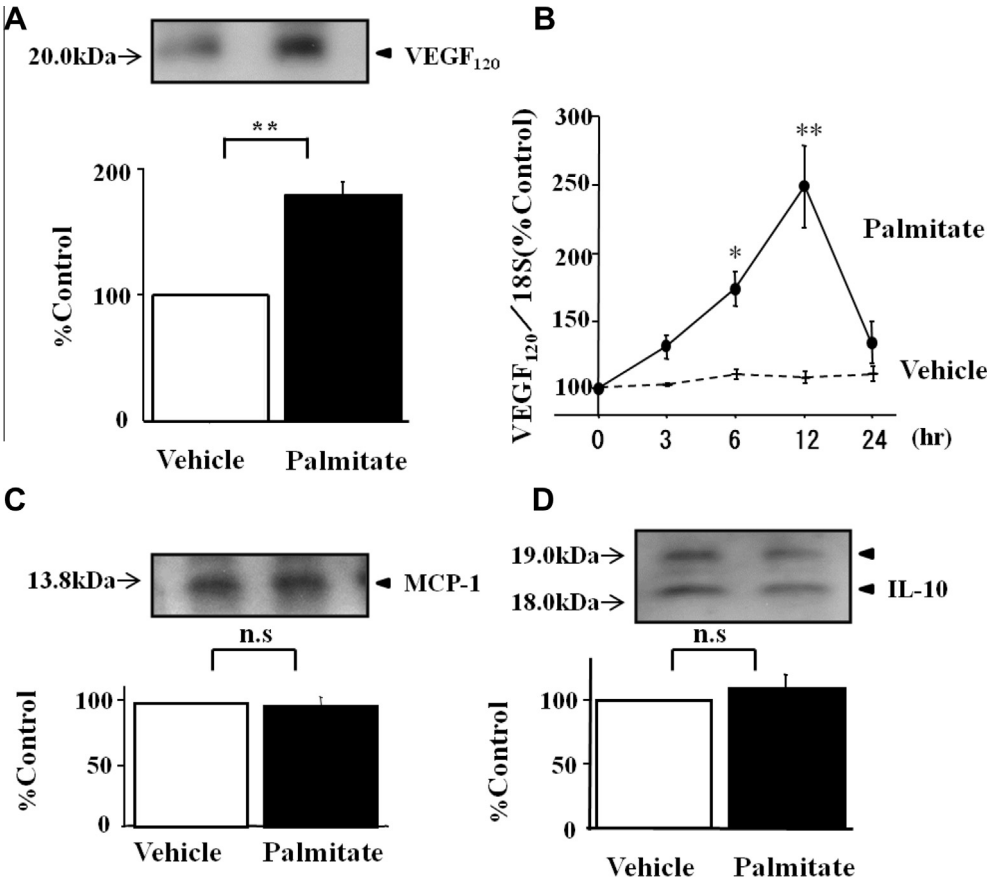

Fig. 1. Cytokine releases from 24 h palmitate-stimulated UMR-106 cells. VEGF-A mRNA expressions on UMR-106 osteosarcoma cells treated with palmitate. UMR-106 osteosarcoma cells were stimulated with 0.03 mmol/L palmitate [■] or ethanol vehicle alone [□] for 48 h. $VEGF_{120}$ (A), MCP-1 (C) and IL-10 (D) secretions from 3T3-L1 adipocytes were quantified by immunoblot analysis. (A–C) top: representative pictures of immunoblotting that was quantified. Results are means ± SE ($n = 4$). $^{**}p < 0.01$ compared to vehicle. NS; no significant difference compared to vehicle. UMR-106 cells were stimulated with 0.03 mmol/L palmitate [—] or vehicle alone [---] over 24 h (B). The mRNA levels of VEGF-A including $VEGF_{120}$ was measured by quantitative real-time RT-PCR at 0, 3, 6, 12 and 24 h after stimulation. The mRNA signal for each gene was normalized over 18S rRNA signal. Results are means ± SE ($n = 4$). $^{*}p < 0.05$; $^{**}p < 0.01$ compared to the corresponding controls.

3.3. TLR4 is involved in the increased $VEGF_{120}$ secretion by palmitate stimulation

We next analyzed the induction of oxidative stress in osteoblast-like UMR-106 cells stimulated with palmitate, because in our previous study, palmitate caused oxidative stress in adipocyte [7]. The concentration of hydroperoxides, a marker for endogenous oxidative stress, in palmitate-stimulated cells was not observed any differentiations relative to non-stimulated UMR-106 (Fig. 2A). This data suggests that palmitate can induce no oxidative stress in UMR-106.

Since it was reported that TLR4 was concerned in palmitate-activated intracellular signal transduction [9–16], we investigated the effects of TLR4 on $VEGF_{120}$ release from UMR-106 osteosarcoma cells treated with palmitate. Addition of 5 mmol/L TLR4 inhibitor significantly suppressed the increased $VEGF_{120}$ secretion from palmitate-stimulated UMR-106 cells ($p < 0.01$; Fig. 2B). Moreover, the administration of LPS (100 ng/ml), a potent agonist of TLR4, to UMR-106 cells amplified $VEGF_{120}$ release by 1.6-fold compared to control cells with vehicle alone ($p < 0.01$; Fig. 2C).

3.4. Palmitate augmented $VEGF_{120}$ release via PI3K pathways

We investigated the pathway involved in $VEGF_{120}$ release from palmitate-preloaded UMR-106 osteosarcoma cells. Addition of 50 μmol/L LY294002, an inhibitor of phosphatidylinositol 3-kinase (PI3K), significantly suppressed the increase of palmitate-stimulated $VEGF_{120}$ secretion by 63% compared to the cells stimulated with palmitate alone ($p < 0.01$; Fig. 3A). But the treatment with LY294002 alone showed no significant effects on $VEGF_{120}$ secretion from non-stimulated UMR-106 cells (Fig. 3A). In addition, 10 nmol/L wortmannin, another PI3K inhibitor, was also diminished the increased $VEGF_{120}$ secretion by 56% ($p < 0.01$; Fig. 3B), while there were no changes on $VEGF_{120}$ release from UMR-106 cells treated wortmannin only. These results indicate that PI3K pathways participate in $VEGF_{120}$ release.

We examined other pathways implicated in the regulation of $VEGF_{120}$ secretion from osteoblast-like UMR-106 cells stimulated with palmitate. Treatment with any agents of PD98059 (2 μmol/L), an inhibitor of mitogen-activated protein kinase kinase/extracellular signal regulated protein kinase kinase 1/2 (MEK1/2), SP600125 (1 μmol/L), an inhibitor of c-Jun NH_2-terminal protein kinase (JNK), and SB203580 (1 μmol/L), an inhibitor of p38 mitogen-activated protein kinase (MAPK), failed to attenuate the amplified $VEGF_{120}$ secretion seen in palmitate-stimulated UMR-106 cells (Fig. 3C–E), suggesting that the intracellular mechanism concerning enhanced $VEGF_{120}$ secretion with palmitate from osteoblast-like UMR-106 cells is independent of MAPK pathways activation.

3.5. The actions of cytokines to $VEGF_{120}$ and MCP-1 secretion

The results by immunoblotting revealed that $VEGF_{120}$ secretion was not significantly changed in 24 h TNF-α-stimulated UMR-106 osteosarcoma cells relative to control cells (Fig. 4A), whereas

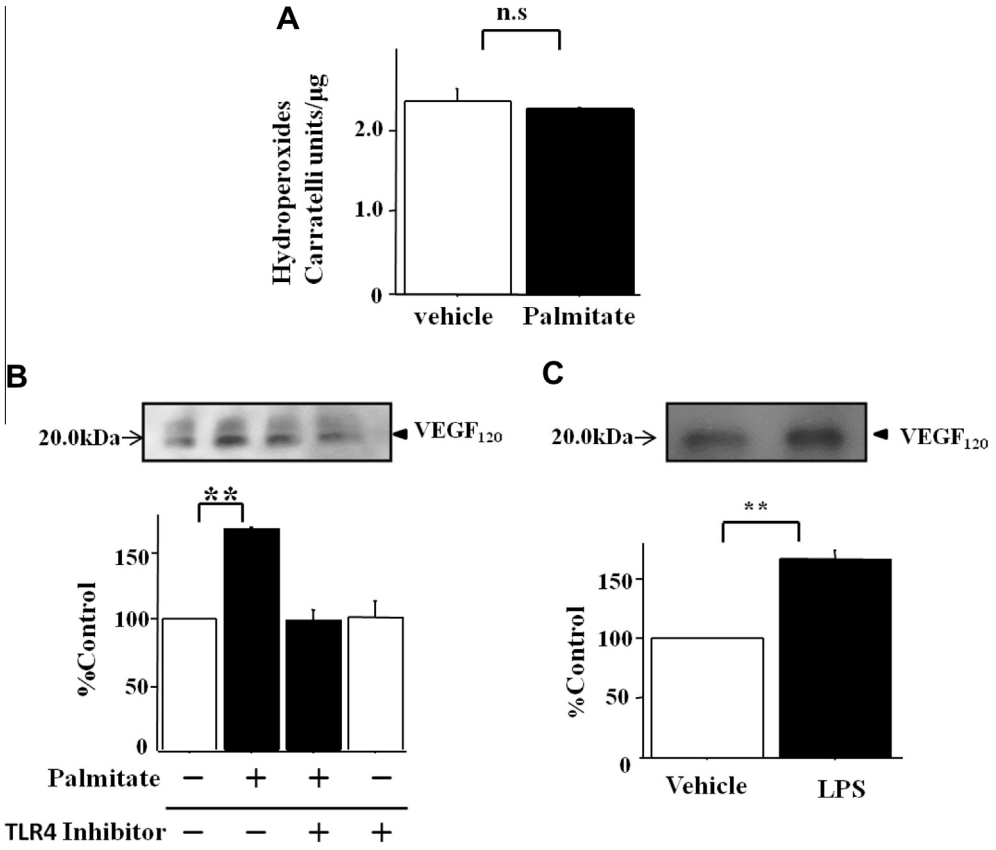

Fig. 2. The involvement of TLR4 on $VEGF_{120}$ secretion from palmitate-stimulated UMR-106 cells. (A) UMR-106 cells were treated with 0.03 mmol/L palmitate [■] or vehicle alone [□] for 48 h. Hydroperoxides content in UMR-106 cells was assayed by the Free Radical Elective Evaluator system. Results are means ± SE ($n = 4$). NS; no significant difference compared to vehicle. (B) UMR-106 cells were pretreated with 5 mmol/L TLR4 peptide inhibitor or vehicle (dimethyl sulfoxide) alone for 30 min. Then, the cells were incubated with 0.03 mmol/L palmitate [■] or vehicle alone [□] for 48 h in the presence or absence of this inhibitor. $VEGF_{120}$ secretion was analyzed by quantitative immunoblots. (C) UMR-106 cells were treated with 100 ng/ml LPS [■] or vehicle alone [□] for 48 h. $VEGF_{120}$ release was quantified by immunoblot analysis. B and C, top: representative pictures of immunoblotting that was quantified. Results are means ± SE ($n = 4$). $^{**}p < 0.01$ compared to the corresponding controls. NS, no significant difference compared to vehicle.

MCP-1 release from the osteoblast-like UMR-106 cells treated with TNF-α exhibited a marked increase of 1.5-fold ($p < 0.01$; Fig. 4B). In contrast, $VEGF_{120}$ secretion from UMR-106 cells stimulated with IL-1β over 24 h was clearly augmented by 1.8-fold relative to the control cells receiving vehicle alone ($p < 0.01$; Fig. 4C). However, any differentiation was not able to be confirmed in the release of MCP-1 from IL-1β-stimulated UMR-106 osteosarcoma cells (Fig. 4D).

4. Discussion

It have been reported that abnormal bone metabolism often occurs in patients with type 2 diabetes [1–3], but the underlying pathogenic mechanisms remain to be elucidated. However, in recent years dyslipidemia, otherwise hyperlipidemia, which frequently accompanies in type2 diabetes has been presumed to have an influence on bone metabolism [4]. On the other hand, VEGF is shown to induce abnormal activation of osteoclasts and to consequently enhance bone absorption [5,6]. In addition, MCP-1 is also identified as being able to induce differentiation to osteoclasts by promoting the fusion of hematopoietic stem cell-derived monocyte/macrophage precursor cells, suggesting that MCP-1 may be a risk factor for osteoporosis [3]. Therefore, in this study, we investigated the pathogenic mechanism of abnormal bone metabolism by using UMR-106 rat osteosarcoma cells as an osteoblast cell model and treating them with palmitate, a representative saturated fatty acid, in order to mimic a state of hyperlipidemia.

Treatment with palmitate resulted in a significant increase in $VEGF_{120}$ secretion from UMR-106 cells. It was further confirmed that the expression of VEGF-A mRNA including $VEGF_{120}$ significantly increased, showing that the increase in palmitate-induced $VEGF_{120}$ secretion is regulated on the transcriptional level. In contrast, palmitate had no effect on the release of MCP-1 and also IL-10, an anti-inflammatory cytokine, from UMR-106 osteosarcoma cells. These results suggest that $VEGF_{120}$ may play a central role in abnormal bone metabolism in diabetes which is induced by hyperlipidemia.

We confirmed in our previous study that palmitate can enhance endogenous oxidative stress in 3T3-L1 adipocytes, which is involved in increased secretion of $VEGF_{120}$ [7]. Thus, we examined the state of oxidative stress in UMR-106 osteosarcoma cells treated with palmitate. Interestingly, we failed to confirm significant changes in the intracellular content of hydroperoxide, a primary oxidative stress marker, in UMR-106 osteosarcoma cells stimulated with palmitate as compared with control cells. Accordingly, unlike 3T3-L1 adipocytes, endogenous oxidative stress is negated to be correlated with the increased $VEGF_{120}$ secretion from UMR-106 cells. The difference in the condition of palmitate-induced oxidative stress between UMR-106 osteosarcoma cells and 3T3-L1 adipocytes might be attributable to the ability of each cell type to eliminate oxidative stress; however, further study is required to elucidate this difference. Since the involvement of oxidative stress was considered unlikely, we next evaluated the involvement of TLR4 on the augmented $VEGF_{120}$ release from UMR-106 cells

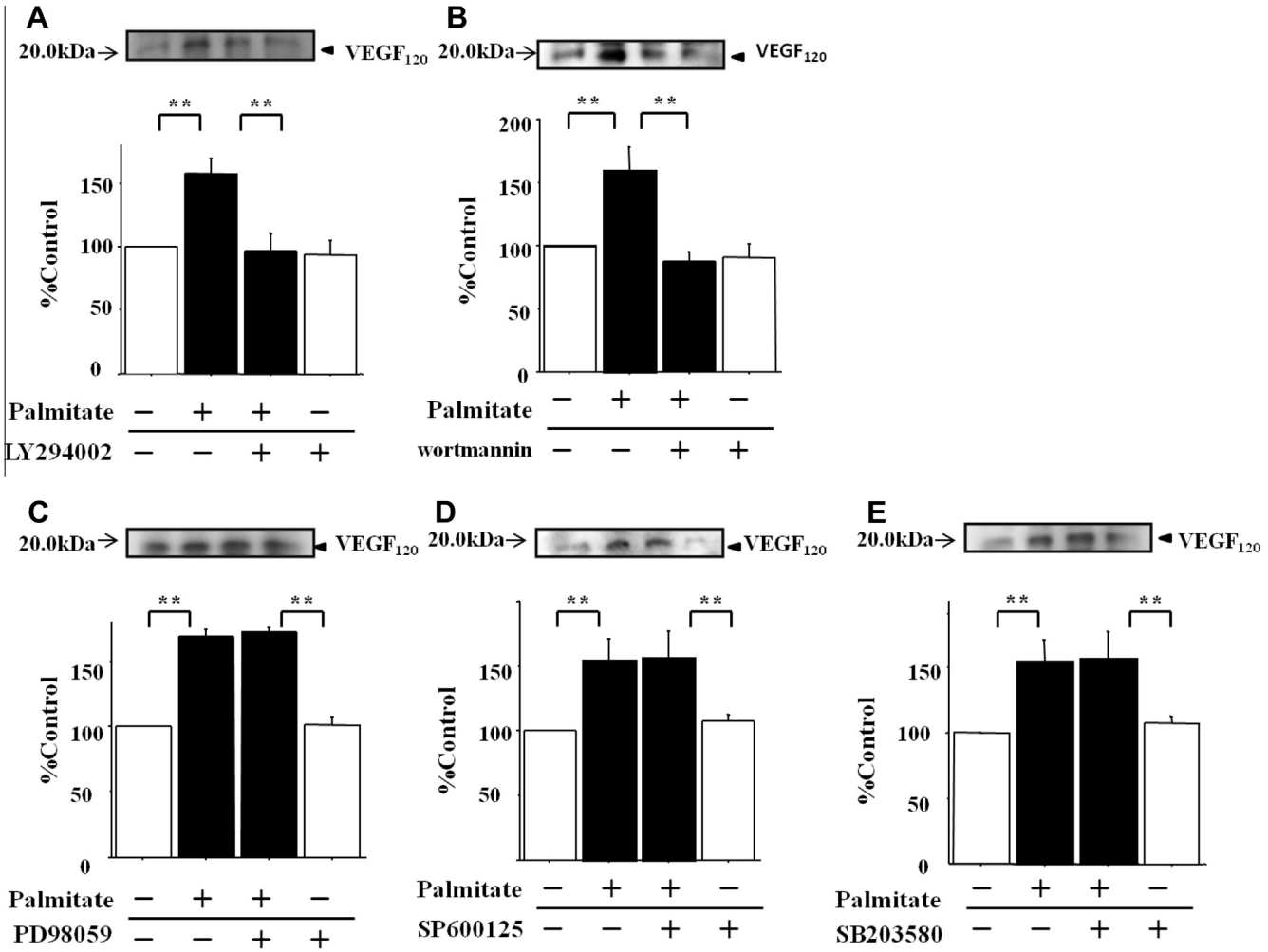

Fig. 3. $VEGF_{120}$ secretion from palmitate-stimulated UMR-106 osteosarcoma cells is enhanced through the activation of PI3K pathways. UMR-106 osteosarcoma cells were pretreated with 50 μmol/L LY294002 (an inhibitor of PI3K) (A), 10 nmol/L Wortmannin (an inhibitor of PI3K) (B), 20 μmol/L PD98059 (an inhibitor of MEK1/2) (C), 10 μmol/L SP600125 (an inhibitor of JNK) (D) and 10 μmol/L SB203580 (an inhibitor of MAPK) (E), or vehicle (dimethyl sulfoxide) alone for 30 min. Then, the cells were incubated with 0.03 mmol/L palmitate [■] or vehicle alone [□] for 48 h in the presence or absence of these inhibitors. $VEGF_{120}$ (A–E) release was analyzed by quantitative immunoblots. (A–E) top: representative pictures of immunoblotting that was quantified. Results are means ± SE ($n = 4$). $^{**}p < 0.01$ compared to the corresponding controls.

treated with palmitate. It has been reported that palmitate can activate the signaling pathway downstream of TLR4 *in vitro* [9–13] and that the TLR4 signaling pathway is related to the inflammatory pathway induced by lipids *in vivo* [14–16]. The pretreatment with a TLR4 antagonist to UMR-106 cells clearly reduced $VEGF_{120}$ secretion which had been significantly amplified by palmitate stimulation. Moreover, stimulation with LPS, a potent agonist of TLR4, significantly increased the secretion of $VEGF_{120}$ from UMR-106 cells. These results suggest that the palmitate-induced increase of $VEGF_{120}$ release from UMR-106 cells may be mediated through, at least partly, TLR4.

We conducted further studies to manifest the intracellular signaling pathway functioning downstream of TLR4. Our previous study verified that the palmitate-induced increase of $VEGF_{120}$ secretion from 3T3-L1 adipocytes is regulated by the activation of the PI3K pathways [8]. In addition, another group has confirmed that the PI3K signaling pathways operate downstream of TLR4 [12,13]. Therefore, we analyzed the activation state of the PI3K pathways in UMR-106 osteosarcoma cells treated with palmitate. Treatment with LY294002 and wortmannin, PI3K pathway inhibitors, significantly attenuated the palmitate-induced amplification of $VEGF_{120}$ release. Moreover, as palmitate has been shown to increase the secretion of cytokines and chemokines such as MCP-1 via MAPK pathways in adipocytes [9,17], we also analyzed the activation of the MAPK pathways in UMR-106 cells treated with palmitate. However, in UMR-106 cells, there were no effects to the palmitate-stimulated augmentation of $VEGF_{120}$ release by pretreatment with an ERK pathway inhibitor (PD98059), a JNK pathway inhibitor (SP600125), or a p38 MAPK pathway inhibitor (SB203580). This indicates that palmitate, a saturated fatty acid, may increase the expression and secretion of $VEGF_{120}$ through, mainly, the PI3K pathways in UMR-106 cells. This also suggests that the ERK, JNK, and p38 MAPK pathways might have, if any, only slight involvement. Furthermore, saturated fatty acids have been observed to be able to activate the TLR4/PI3K/Akt pathways, whereas unsaturated fatty acids do not [12]. Considering these results, the essential fatty acid resulting in abnormal bone metabolism in diabetes is presumed to be saturated, not unsaturated, fatty acid.

We also evaluated the effects of humoral factors derived from osteoclasts activated by osteoblast-secreted $VEGF_{120}$. As the representative osteoclast-derived factors, we analyzed pro-inflammatory cytokines that have been reported to have effects on osteoblast function, particularly focusing on TNF-α and IL-1β [2,3,18–21]. TNF-α stimulation was significantly able to increase the secretion of MCP-1 from UMR-106 osteosarcoma cells; however, it had no effect on the release of $VEGF_{120}$. Interestingly, IL-1β markedly augmented the secretion of $VEGF_{120}$ from

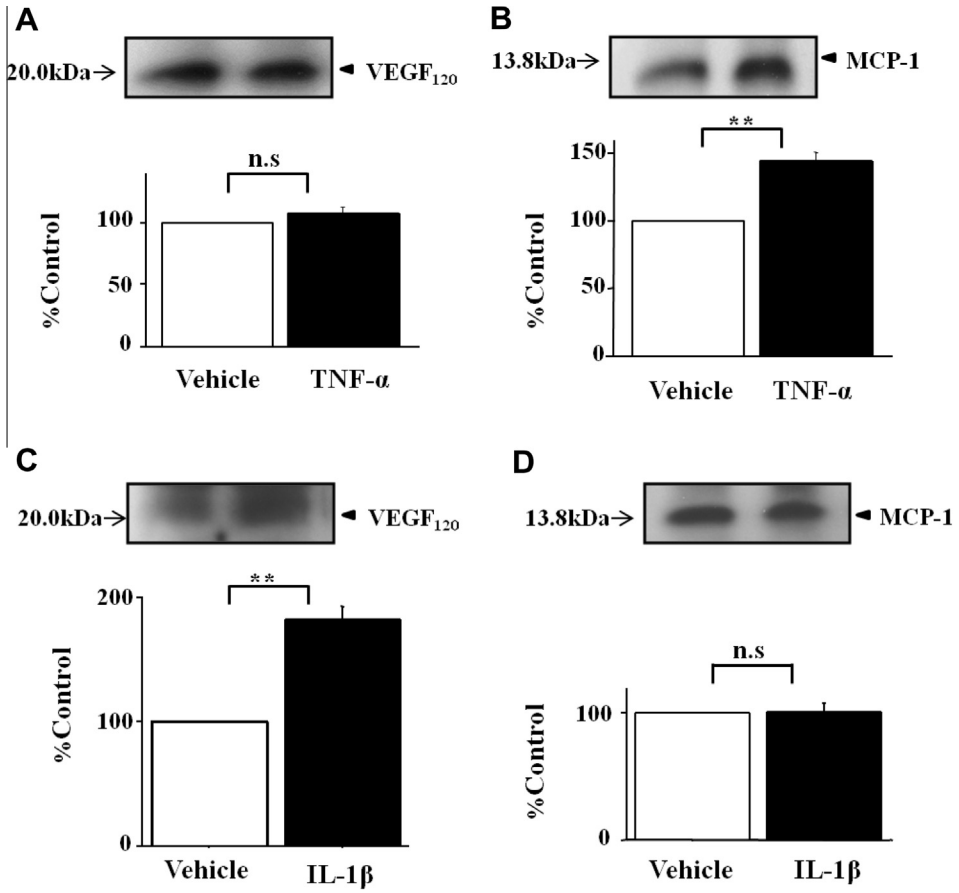

Fig. 4. $VEGF_{120}$ and MCP-1 releases from UMR-106 cells treated with cytokines. (A and B) UMR-106 cells were treated with 100 ng/ml TNF-α [■] or vehicle (sterile distilled water) alone [□] for 24 h. $VEGF_{120}$ (A) and MCP-1 (B) releases were quantified by immunoblot analysis. (C and D) UMR-106 cells were treated with 10 ng/ml IL-1β [■] or vehicle (sterile distilled water) alone [□] for 24 h. $VEGF_{120}$ (C) and MCP-1 (D) secretions were assayed by quantitative immunoblots. (A–D) top: representative pictures of immunoblotting that was quantified. Results are means ± SE ($n = 4$). $^{**}p < 0.01$ compared to vehicle. NS; no significant difference compared to vehicle.

UMR-106 cells, although it was unable to affect the MCP-1 release. On the basis of these results, we could establish the following hypothesis. $VEGF_{120}$, whose secretion from osteoblasts keep increasing during hyperlipidemia, activates osteoclasts, leading to increasing TNF-α and IL-1β secretions from osteoclasts. Then, the stimulation of TNF-α enhances MCP-1 releases from osteoblast, so that hematopoietic stem cell-derived monocyte/macrophage precursor cells can fuse and their differentiation to osteoclasts is induced, resulting in an increased number of osteoclasts. On the other hand, IL-1β stimulation amplifies $VEGF_{120}$ secretion from osteoblast to activate the osteoclasts increased by MCP-1 stimulation. Hence, the TNF-α and IL-1β secretions derived from osteoclasts are further, and more potently, enhanced by this mechanism, ultimately causing a vicious cycle that further amplifies the abnormal activation of osteoclasts. However, with regard to this difference in the secretory movements between MCP-1 and $VEGF_{120}$ resulting from TNF-α and IL-1β stimulations, the crosstalk occurs in the signaling pathways downstream of TNF-α and IL-1β receptors [3,21–23]; therefore, accurate elucidation appears to be difficult. This difference might be attributable to temporal and spatial changes in the intracellular signaling pathways, affected by the functioning of various scaffold proteins and negative regulatory factors.

In summary, we have confirmed for the first time in an *in vivo* system that palmitate directly increases the expression and secretion of $VEGF_{120}$ on UMR-106 osteoblast cells. While endogenous oxidative stress is unlikely to be involved, the expression and secretion of $VEGF_{120}$ may be mediated via, at least partly, the activation of the TLR4 receptor. Moreover, increased secretion of $VEGF_{120}$ is regulated by the activation of the PI3K pathways, whereas the ERK, JNK, and p38 MAPK pathways can scarcely impact. We also verified that TNF-α and IL-1β, which are considered to be derived from osteoclasts activated by $VEGF_{120}$ from palmitate-stimulated osteoblasts, increase the secretions of MCP-1 and $VEGF_{120}$, respectively. Accordingly, in regard of the bone metabolism in patients with type 2 diabetes, since the increased levels of fatty acids are common in the hyperlipidemia condition to frequently accompany in type 2 diabetes, it can become an initiation that $VEGF_{120}$, whose secretion from osteoblast is enhanced by the stimulations, especially with saturated fatty acids, induce abnormal activation of osteoclasts, terminally leading to the development of abnormal bone metabolism to decrease bone mass in type 2 diabetes. Therefore, we firmly believe that the improvement of hyperlipidemia (i.e., decreasing $VEGF_{120}$ secretion from osteoblast cells) can suppress the onset and progression of abnormal bone metabolism in diabetes, which would allow for the establishment of a promising treatment strategy.

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