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Bovine milk lactoferrin induces synthesis of the angiogenic factors VEGF and FGF2 in osteoblasts via the p44/p42 MAP kinase pathway

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Abstract Lactoferrin (LF) belongs to the transferrin family and is present in several physiological fluids, including milk and colostrum. LF has recently been identified as an anabolic factor for bone. Here we investigated whether bovine LF (bLF) induces synthesis of angiogenic factors by osteoblasts. If so, we examined the underlying mechanism. We found that bLF purified from milk increased the mRNA expression of vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF2) in murine osteoblast-like MC3T3-E1 cells and primary murine osteoblasts in a time- and dose-dependent manner. Furthermore, bLF increased VEGF and FGF2 protein levels in MC3T3-E1 cells. In addition, treatment of MC3T3-E1 cells with bLF rapidly induced phosphorylation of p44/p42 mitogen-activated protein (MAP) kinase. The bLF-mediated increases in VEGF and FGF2 mRNA and protein were inhibited by U0126, a specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase (MEK). Taken together, our results strongly suggest that bLF induces VEGF and FGF2 synthesis in a p44/p42 MAP kinase-dependent manner in MC3T3-E1 cells.

Keywords FGF2 · Lactoferrin · Osteoblasts · p44/p42 MAP kinase · VEGF

Abbreviations

LF	Lactoferrin
bLF	Bovine LF
VEGF	Vascular endothelial growth factor
FGF	Fibroblast growth factor
MAP	Mitogen-activated protein
FCS	Fetal calf serum
hLF	Human LF

Introduction

Lactoferrin (LF) is an iron-binding glycoprotein that belongs to the transferrin family (Anderson et al. 1989). LF is found in many physiological fluids including milk and colostrum (Steijns and van

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Hooijdonk 2000) and is believed to be an important part of the mammalian host defense mechanism because of its potent antimicrobial, antiviral, and immunomodulatory activities (Hasegawa et al. 1994; Caccavo et al. 2002). Experiments in animals and clinical studies in humans have demonstrated that oral administration of bovine LF (bLF) increases resistance to infections (Yamauchi et al. 2006). In addition, it has been reported that oral administration of bLF cured iron deficiency anemia in pregnant women (Paesano et al. 2010) and decreased cancer development in rats (Tsuda et al. 2002).

LF has recently been demonstrated to be an anabolic factor for bone both in vivo and in vitro. Local administration of bLF over the calvariae of adult male mice increased bone formation (Cornish et al. 2004), and oral administration of bLF to ovariectomized rodents improved bone density (Blais et al. 2009; Guo et al. 2009). Two types of functional bone cells, osteoblasts and osteoclasts, regulate bone metabolism: osteoblasts are responsible for bone formation, whereas osteoclasts reabsorb bone (Nijweide et al. 1986). Interestingly, bLF has been reported to stimulate proliferation and differentiation of osteoblasts (Cornish et al. 2004; Blais et al. 2009; Takayama and Mizumachi 2009; Takayama and Mizumachi 2008) and to inhibit the differentiation of osteoclasts (Cornish et al. 2004; Blais et al. 2009; Lorget et al. 2002). Thus, bLF may be a promising therapeutic agent for treatment of osteoporosis.

Development of the vascular system is necessary for bone tissue growth (Erlebacher et al. 1995). Cross-talk between endothelial cells and osteoblasts has been demonstrated, and the factors produced by these cells influence the functions of other cells (Guenther et al. 1986; Villanueva and Nimni 1990; Villars et al. 2000; Fiorelli et al. 1994). Because bLF does not directly stimulate endothelial cell growth (Norrby et al. 2001), angiogenesis, accompanied by bone growth, is believed to occur due to paracrine actions of angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF2).

VEGF is a critical mediator of angiogenesis in various physiological and pathological conditions including embryonic development, wound healing, and solid tumor growth (Ferrara 1999; Neufeld et al. 1999). It is a multifunctional cytokine that stimulates proliferation of endothelial cells and enhances microvascular permeability. In VEGF gene knockout studies, disruption of even one VEGF allele leads to embryonic lethality due to multiple defects in the development of the cardiovascular system (Ferrara et al. 1996; Carmeliet et al. 1996). Many tissues and cell types, including osteoblasts, express VEGF. VEGF expression is regulated at the transcriptional and post-transcriptional levels (Forsythe et al. 1996; Shima et al. 1995). On the other hand, FGFs comprise a family of more than 20 structurally related polypeptides. FGF2 (also known as basic FGF) is one of the well-characterized members of this family, and it elicits various biological effects in different physiological situations including embryonic development, tumorigenesis, and angiogenesis (Powers et al. 2000). Bone cells produce both FGF1 and FGF2 in a ratio of approximately 1:10 (Globus et al. 1989). Unlike VEGF, FGF2 lacks a signal peptide sequence for secretion (Friesel and Maciag 1999). Interestingly, VEGF and FGF2 potently and synergistically stimulate neovascular formation both in vivo and in vitro (Pepper et al. 1992; Asahara et al. 1995; Goto et al. 1993).

In this study, we determined whether bLF alters the synthesis of specific angiogenic factors in osteoblasts. If so, we characterized the mechanisms underlying the synthesis of these factors.

Materials and methods

Materials

bLF purified from milk was obtained from Wako (Osaka, Japan). Endotoxin contamination in the purified bLF used in this study was confirmed not to affect the status of VEGF and FGF2 expression in osteoblasts. The MEK inhibitor U0126 was obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). Phospho-specific p44/p42 mitogen-activated protein (MAP) kinase antibody and p44/p42 MAP kinase antibody were obtained from New England BioLabs, Inc. (Beverly, MA, USA). FGF2 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The mouse VEGF ELISA kit was obtained from R&D systems Inc. (Minneapolis, MN, USA).

Cell culture

Primary osteoblasts derived from mice calvariae were obtained as previously described (Suda et al. 1997). Primary osteoblasts and osteoblast-like MC3T3-E1

cells were cultured in plastic dishes containing alpha-MEM and 10% fetal calf serum (FCS) under 5% CO_2 at 37°C and were subcultured every 3 days. For real-time quantitative RT-PCR and ELISA, the cells were seeded in 40-mm plastic dishes containing alpha-MEM with 10% FCS and cultured for 5 days until nearly confluent. The medium was then replaced with alpha-MEM containing 0.5% FCS, and the cells were incubated for 24 h before analysis.

Real-time quantitative RT-PCR

Total RNA was extracted from MC3T3-E1 cells using Isogen (Nippon Gene Co., Toyama, Japan), according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized with M-MLV reverse transcriptase (Sigma Chemical Co.) using random primers. VEGF and FGF2 mRNA expression was measured by real-time quantitative RT-PCR amplification using SYBR Premix Ex Taq II (Takara Bio, Inc., Shiga, Japan). The thermal cycling conditions included an initial denaturation step at 95°C for 30 s followed by 40 cycles at 95°C for 5 s, at 58°C for 15 s, and at 72°C for 34 s. mRNA levels were normalized to β-actin mRNA levels. The PCR primers used for VEGF (M95200) mRNA amplification were forward, 5′-ATCTTCAAGCCGTCCTG TGT-3′ and reverse, 5′-GCATTCACATCTGCTGTG CT-3′. The PCR primers used for FGF2 (NM_ 008006) mRNA amplification were forward, 5′-AG CGGCTCTACTGCAAGAAC-3′ and reverse, 5′-GC CGTCCATCTTCCTTCATA-3′. The PCR primers used for β-actin (M12481) mRNA amplification were forward, 5′-AGCCATGTACGTAGCCATCC-3′ and reverse, 5′-CTCTCAGCTGTGGTGGTGAA-3′. Semi-quantitative RT-PCR analysis of VEGF, FGF2, and β-actin was also performed. cDNA fragments of test genes were amplified by PCR within the linear range using GoTaq DNA Polymerase (Promega, Madison, WI, USA). The primer sets were the same as those used for real-time quantitative RT-PCR except for VEGF. The primers used for semi-quantitative VEGF mRNA amplification were forward, 5′-TGCACCCAC GACAGAAGGAGA-3′ and reverse, 5′-TCACCG CCTTGGCTTGTCACAT-3′. Semiquantitative amplification conditions included an initial denaturation step at 94°C for 5 min followed by 23 cycles (for VEGF) at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s or 27 cycles (for FGF2) at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s. Aliquots of the PCR products were separated by 1.5% agarose gel electrophoresis.

Measurement of VEGF in conditioned medium

Cultured cells were stimulated with 100 μg/ml bLF in 1 ml of alpha-MEM containing 0.5% FCS for 24 h. Where indicated, the cells were pretreated with various doses of U0126 for 60 min. Stimulation was terminated by collecting the conditioned medium, and VEGF concentration in the medium was determined by ELISA; absorbance was measured at 450 nm with a microplate reader (MTP-800Lab, Corona Electric, Ibaraki, Japan).

Western blot analysis of p44/p42 MAP kinase

Cells were washed twice with PBS, resuspended in sampling buffer (62.5 mM Tris/HCl, pH 6.8, 2% SDS, 50 mM dithiothreitol, and 10% glycerol), and boiled for 10 min. Equal amount of total protein from each cell lysate was subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred on PVDF membranes (Millipore, Bedford, MA, USA) by semi-dry blotting. Western blotting was performed using phospho-specific p44/p42 MAP kinase antibody (1:1000; New England Biolabs), followed by stripping and reblotting with the total p44/p42 MAP kinase antibody (1:1000; New England Biolabs). The signal intensities were quantified using Image J software (National Institutes of Health). The intensities of bLF-induced phosphorylation signals in each lane were normalized to total p44/p42 MAP kinase expression.

Western blot analysis of FGF2

Western blot analysis of FGF2 was performed as described previously with a few modifications (Hurley et al. 1994). Cultured cells were stimulated with 100 μg/ml bLF in alpha-MEM containing 0.5% FCS for 16 h. Where indicated, cells were pretreated with 30 μM U0126 for 60 min. The cells were washed twice with PBS and lysed in lysis buffer (10 mM Tris-HC1, pH 7.4, 150 mM NaCl, 1% Nonidet P-40). Lysates were incubated for 30 min on ice and clarified by centrifugation at 15,000×g for 20 min. Heparin-Sepharose was then added, and the samples were mixed by rotation for 16 h at 4°C. After

washing twice with wash buffer (10 mM Tris-HC1, pH 7.4, 500 mM NaCl), heparin-bound proteins were eluted, subjected to 15% polyacrylamide gel electrophoresis, and transferred on PVDF membranes by semi-dry blotting. Western blotting was performed using FGF2 antibody (1:200; Santa Cruz).

Statistical analysis

The data were analyzed by ANOVA followed by Dunnett's or Tukey's test for multiple comparisons between pairs. $P < 0.05$ was considered significant. Data from real-time quantitative RT-PCR and ELISA are presented as means ± S.D. from triplicate determinations.

Results

Analysis of VEGF and FGF2 mRNA levels in MC3T3-E1 cells and primary murine osteoblasts following treatment with bLF

We investigated the effect of bLF on VEGF and FGF2 mRNA levels in MC3T3-E1 cells. bLF stimulated VEGF and FGF2 mRNA expression in a time-dependent manner (Fig. 1). VEGF and FGF2 mRNA expression peaked at 120–180 and 180 min of bLF stimulation, respectively. In addition, the stimulatory effects of bLF on VEGF and FGF2 mRNA expression were dose-dependent (Fig. 2). The effect of bLF on

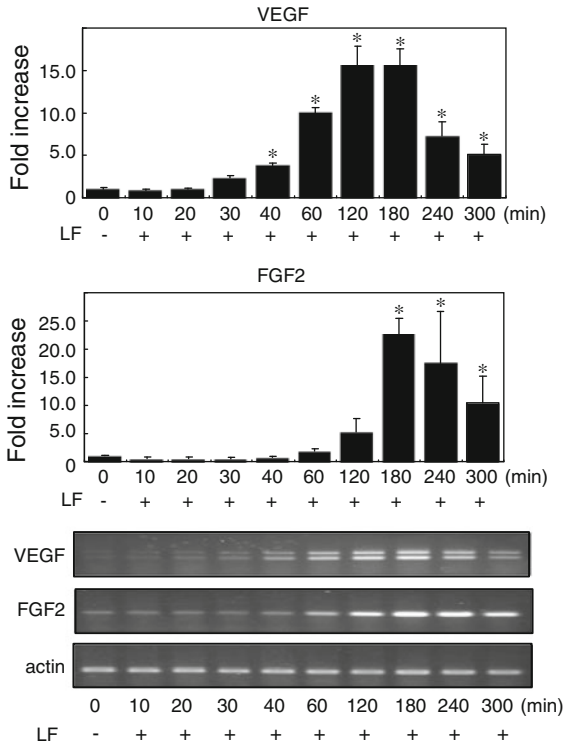

Fig. 1 bLF stimulates VEGF and FGF2 mRNA expression in MC3T3-E1 cells in a time-dependent manner. Cultured cells were stimulated with 100 μg/ml bLF for the indicated times. Total RNA was isolated from samples prepared in triplicate, and relative VEGF and FGF2 mRNA expression was determined by real-time quantitative RT-PCR analysis. Histograms represent means ± S.D. of triplicate plates. *$P < 0.05$ compared with 0 min (ANOVA followed by Dunnett multiple comparisons test)

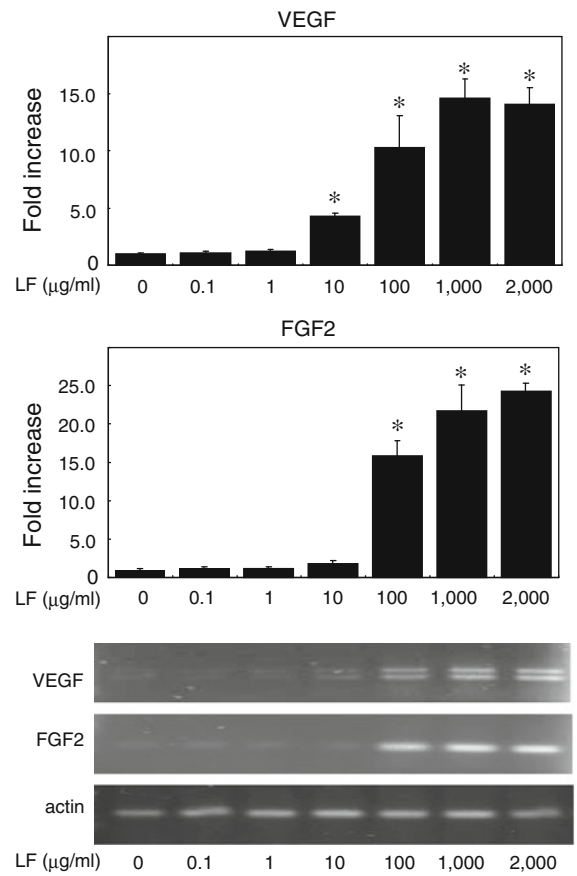

Fig. 2 bLF stimulates VEGF and FGF2 mRNA expression in MC3T3-E1 cells in a dose-dependent manner. Cultured cells were stimulated with the indicated doses of bLF for 3 h. Total RNA was isolated from samples prepared in triplicate, and relative VEGF and FGF2 mRNA expression was determined by real-time quantitative RT-PCR analysis. Histograms represent means ± S.D. from triplicate plates. *$P < 0.05$ compared with vehicle (ANOVA followed by Dunnett multiple comparisons test)

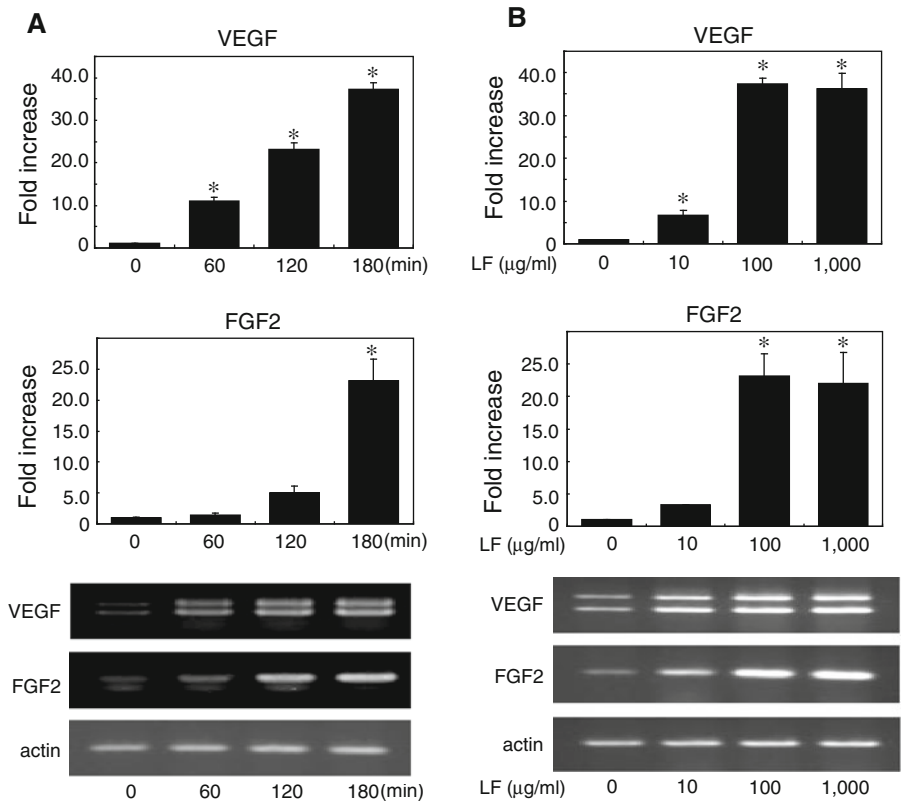

Fig. 3 bLF stimulates VEGF and FGF2 mRNA expression in primary osteoblasts. Cultured cells were stimulated with 100 μg/ml bLF for the indicated times (**a**) or with the indicated doses of bLF for 3 h (**b**). Total RNA was isolated from samples prepared in triplicate, and relative VEGF and FGF2 mRNA expression was determined by real-time quantitative RT-PCR analysis. Histograms represent means ± S.D. from triplicate plates. *$P < 0.05$ compared with vehicle (ANOVA followed by Dunnett multiple comparisons test)

VEGF and FGF2 mRNA expression was significant at a dose greater than 10 and 100 μg/ml, respectively. The maximum effect of bLF was observed at 1000 μg/ml for VEGF and 2000 μg/ml for FGF2. We confirmed that bLF also stimulated VEGF and FGF2 mRNA expression in primary osteoblasts in a time- and dose-dependent manner (Fig. 3).

Analysis of p44/p42 MAP kinase phosphorylation in MC3T3-E1 cells following treatment with bLF

We investigated the effect of bLF on phosphorylation of p44/p42 MAP kinase in MC3T3-El cells. As shown in Fig. 4a, bLF induced rapid phosphorylation of p44/p42 MAP kinase in these cells after 5–10 min; the phosphorylation level at 5 min of bLF stimulation was approximately 5-fold higher than that of unstimulated cells. bLF did not alter total p44/p42 MAP kinase levels. In addition, we examined the effect of U0126, a specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase, on bLF-induced phosphorylation of p44/p42 MAP kinase. As shown in Fig. 4b, bLF-induced phosphorylation of p44/p42 MAP kinase decreased in the presence of 1 μM U0126.

Effect of U0126 on bLF-mediated stimulation of VEGF and FGF2 mRNA expression in MC3T3-E1 cells

To investigate whether p44/p42 MAP kinase is involved in bLF-mediated increases in VEGF and FGF2 mRNA expression in MC3T3-El cells, we examined bLF-mediated VEGF and FGF2 mRNA expression after treatment with U0126. U0126 alone did not affect VEGF and FGF2 mRNA levels, but inhibited bLF-mediated increases in VEGF and FGF2 mRNA expression (Fig. 5). The inhibitory effects of

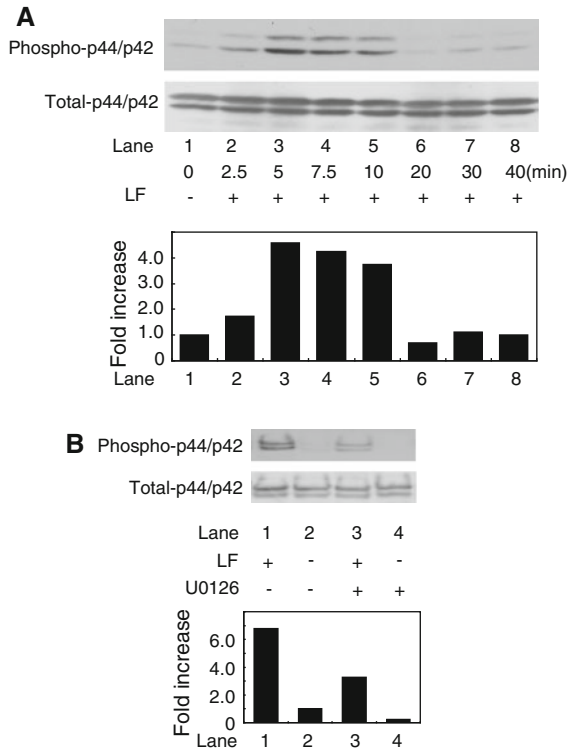

Fig. 4 bLF stimulates phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. **a** Time course of bLF-induced p44/p42 MAP kinase phosphorylation in MC3T3-E1 cells. Cultured cells were incubated with 100 μg/ml bLF for the indicated times. Cell extracts were subjected to western blot analysis using phospho-specific p44/p42 MAP kinase antibody and p44/p42 MAP kinase antibody. Histograms show the fold-increase in the relative levels of phospho-p44/p42 MAP kinase over total p44/p42 MAP kinase compared to that of the unstimulated cells. Similar results were obtained with two different cell preparations. **b** Effect of U0126 on bLF-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 1 μM U0126 or vehicle for 60 min, and then stimulated by 100 μg/ml bLF or vehicle for 10 min. Western blot analysis was performed described as above

U0126 were dose-dependent and significant between 1 and 30 μM.

Effect of U0126 on bLF-mediated stimulation of VEGF secretion from MC3T3-E1 cells

To confirm the involvement of p44/p42 MAP kinase-mediated signaling in bLF-mediated enhancement of VEGF secretion from MC3T3-E1 cells, we examined the effect of U0126 on VEGF secretion levels from these cells. As shown in Fig. 6a, bLF significantly stimulated VEGF secretion, and U0126 decreased this effect in a dose-dependent manner. The maximum effect of U0126 was observed at 30 μM, resulting in approximately 80% reduction of bLF-mediated stimulation of VEGF secretion.

Effect of U0126 on bLF-mediated stimulation of FGF2 protein expression in MC3T3-E1 cells

We examined the effect of bLF on FGF2 protein levels in MC3T3-El cells and involvement of p44/p42 MAP kinase-mediated signaling in this effect. Western blot analysis of heparin-bound proteins from cell lysates revealed that bLF stimulated FGF2 protein expression, and treatment with U0126 (30 μM) decreased bLF-mediated stimulation (Fig. 6b).

Discussion

In this study, we showed that bLF significantly stimulated the expression of the angiogenic factors VEGF and FGF2 in MC3T3-E1 cells and primary osteoblasts in a time- and dose-dependent manner. VEGF and FGF2 transcripts increased maximally approximately 2–3 h after bLF stimulation. Similar phenomena have been observed in MC3T3-E1 cells, in which well-known anabolic factors for bone stimulate expression of VEGF and FGF2. TGF-β-stimulated VEGF mRNA expression peaks at 2 h (Chua et al. 2000), and parathyroid hormone (PTH)-stimulated FGF2 mRNA expression peaks at 2 h (Hurley et al. 1999).

LF is known to bind to the low-density lipoprotein receptor-related protein (LRP) (Willnow et al. 1992; Ji and Mahley 1994; Vash et al. 1998; Neels et al. 1999), and various types of osteoblastic cells, including MC3T3-E1 cells, express LRP1 mRNA (Grey et al. 2004). LF stimulates osteoblast mitogenesis by enhancing the LRP1-mediated p44/p42 MAP kinase pathway signaling (Grey et al. 2004). We showed that bLF stimulated phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells as reported in primary rat osteoblastic cells (Grey et al. 2004). Furthermore, we showed that VEGF and FGF2 synthesis was regulated by p44/p42 MAP kinase in MC3T3-E1 cells. However, bLF-stimulated VEGF secretion and mRNA expression were incompletely blocked by U0126. Therefore, some additional

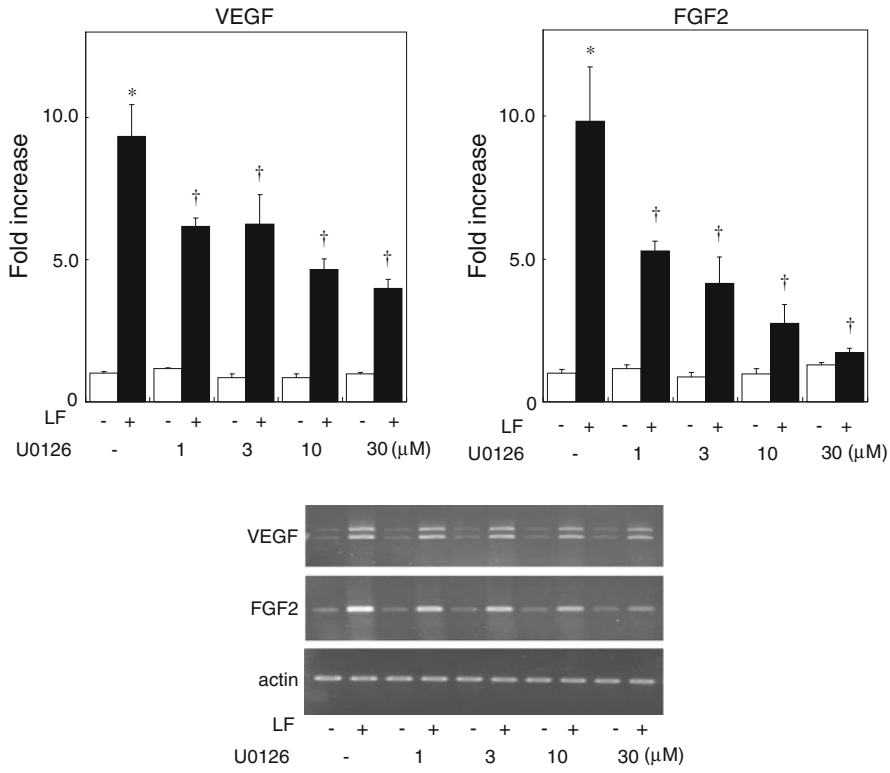

Fig. 5 U0126 decreases bLF-mediated stimulation of VEGF and FGF2 mRNA expression in MC3T3-E1 cells. Cultured cells were pretreated with the indicated doses of U0126 for 60 min, and then stimulated with 100 μg/ml bLF for 3 h. Total RNA was isolated from samples prepared in triplicate, and relative VEGF and FGF2 mRNA expression was determined by real-time quantitative RT-PCR analysis. Histograms represent means ± S.D. of triplicate plates. $^{*}P < 0.05$ compared with vehicle. $^{\dagger}P < 0.05$ compared with bLF alone (ANOVA followed by Tukey multiple comparisons test)

signaling pathways are likely to be involved in LF-induced VEGF synthesis in these cells. Grey et al. (2006) reported that the anti-apoptosis effect of LF is mediated by a p44/p42 MAP kinase-independent pathway in osteoblasts. VEGF expression is known to be regulated at several levels, and multiple signaling pathways are involved in its expression (Berra et al. 2000). Prostaglandin E2 induces VEGF and FGF2 mRNA expression in cultured rat Müller cells, and this induction is inhibited by PKA inhibitors (Cheng et al. 1998). In fact, LF stimulates PKA activity via LRP in M21 human melanoma cells and HepG2 human hepatocellular carcinoma cells (Goretzki and Mueller 1998). Therefore, it will be interesting to further examine the involvement of additional signaling pathways, including the PKA pathway, in VEGF and FGF2 expression in osteoblasts.

Recently, it was reported that human LF (hLF) and bLF exert opposite effects on angiogenesis in vivo. Ingested iron-unsaturated hLF enhanced VEGF-mediated angiogenesis in a rat mesenteric angiogenesis assay (Norrby 2004). In addition, hLF promoted VEGF-mediated angiogenesis in a chick chorioallantoic membrane assay (Kim et al. 2006). On the other hand, ingested iron-unsaturated bLF inhibited VEGF-mediated angiogenesis in a rat mesenteric angiogenesis assay (Norrby et al. 2001). Similarly, in mice, both oral and intraperitoneal administrations of bLF inhibited tumor-induced angiogenesis in a dorsal air sac assay (Shimamura et al. 2004). Although hLF and bLF have 69% amino acid sequence identity, species-specific functional differences exist between them (Pierce et al. 1991). The mechanism underlying the opposing effects of hLF and bLF on angiogenesis in vivo remains unclear. LF-mediated induction of VEGF and FGF2 synthesis and the resulting effect on angiogenesis in bone has not been examined in vivo. Both hLF and bLF promote proliferation and differentiation of osteoblasts (Cornish et al. 2004; Blais et al. 2009; Takayama and Mizumachi 2009; Grey et al. 2004), and may therefore, have similar effects on angiogenesis in bone in vivo. In the present study, VEGF concentration after 24 h of bLF stimulation in the conditioned medium of MC3T3-E1 cells was approximately 300 pg/ml. It has been reported that approximately 200 pg/ml or 450 pg/ml of serum VEGF increased angiogenesis in animal experiment or clinical study (Li et al. 2008; Füreder et al. 2006),

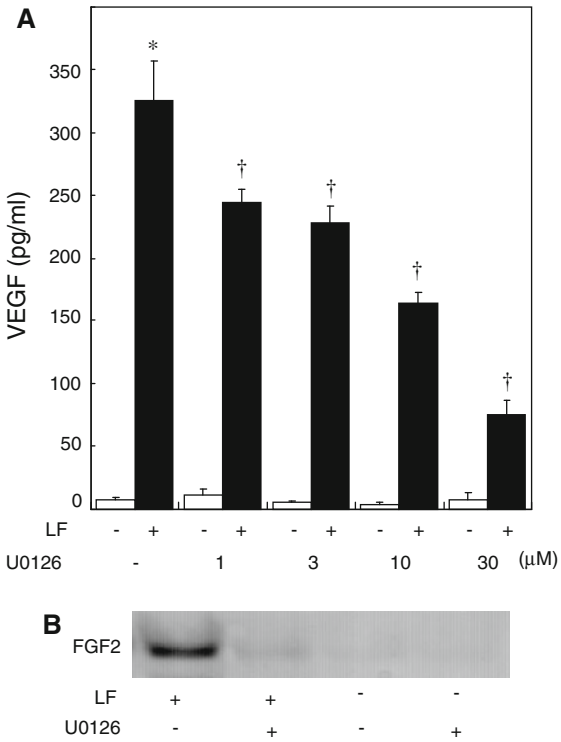

Fig. 6 U0126 decreases bLF-mediated stimulation of VEGF and FGF2 protein expression in MC3T3-E1 cells. **a** Cultured cells were pretreated with the indicated doses of U0126 for 60 min, and then stimulated with 100 μg/ml bLF for 24 h. VEGF concentration in conditioned medium was measured by ELISA. Data represent means ± S.D. of triplicate plates. $^{*}P < 0.05$ compared with vehicle. $^{\dagger}P < 0.05$ compared with bLF alone (ANOVA followed by Tukey multiple comparisons test). **b** Cultured cells were pretreated with U0126 (30 μM) for 60 min, and then stimulated with 100 μg/ml bLF for 16 h. Heparin-bound proteins from cell lysates were subjected to western blot analysis using FGF2 antibody

implicating that bLF-stimulated osteoblasts may secrete sufficient VEGF to induce angiogenic events in vivo. Thus, our results may provide some explanation for the anabolic effects of bLF on bone observed in vivo. On the other hand, FGF2 is known to accelerate the angiogenic effect of VEGF (Pepper et al. 1992; Asahara et al. 1995; Goto et al. 1993), however, it remains to be clarified whether bLF-stimulated osteoblasts secrete sufficient FGF2 to accelerate the VEGF-induced angiogenesis in bone in vivo.

Osteoblasts express functional receptors for VEGF and FGF2, and these growth factors directly regulate cellular functions of osteoblasts in an autocrine manner in vitro (Globus et al. 1989; Street and Lenehan 2009). As previously reported (Cornish et al. 2004; Blais et al. 2009), bLF significantly simulated the proliferation of MC3T3-E1 cells and primary osteoblasts (data not shown). However, we did not clarify whether bLF-induced stimulation of osteoblast proliferation was mediated by upregulation of FGF2 or VEGF expression. Intriguingly, U0126 significantly decreased the bLF-induced proliferative effect on osteoblasts (data not shown), indicating that bLF-induced FGF2 and VEGF may participate in stimulating proliferation of osteoblasts through their p44/p42 MAP kinase-dependent signaling. Further investigations would be required to clarify the details.

In conclusion, our results strongly suggest that milk-purified bLF stimulates VEGF and FGF2 synthesis via p44/p42 MAP kinase in osteoblasts.

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