FEBS Letters 580 (2006) 4332–4336

Human lactoferrin upregulates expression of KDR/Flk-1 and stimulates VEGF-A-mediated endothelial cell proliferation and migration

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Received 26 April 2006; revised 16 June 2006; accepted 30 June 2006

Available online 7 July 2006

Edited by Beat Imhof

Abstract Lactoferrin (LF) is a multifunctional iron-binding glycoprotein, which plays a variety of biological processes including immunity. In this study, we demonstrate that human LF upregulates KDR/Flk-1 mRNA and protein levels in HUVECs at an optimal concentration of 5 μg/ml, which subsequently promotes the VEGF-induced proliferation and migration of the endothelial cells. Exposure of HUVECs to LF significantly increased VEGF-induced ERK MAP kinase phosphorylation. The maximal stimulation of KDR/Flk-1 expression by LF was correlated with LF-induced increase in cell proliferation and migration. These findings suggest that LF may stimulate in vivo angiogenesis via upregulation of KDR/Flk-1 expression in endothelial cells.

Keywords: Lactoferrin; KDR/Flk-1; VEGF; Migration; Proliferation

1. Introduction

Lactoferrin (LF) is an iron-binding glycoprotein belonging to the transferrin family. LF is present in breast milk, epithelial secretions, and the secondary granules of neutrophilic leukocytes [1,2]. Serum LF levels are derived primarily from neutrophils and in healthy adults LF circulates at concentrations of 2–7 μg/ml, but its local concentrations tend to be far higher during inflammation [3]. A number of biological roles are ascribed to LF, including host defenses against microbial infection, bone formation, modulation of the inflammatory response, and angiogenesis [1–7].

Angiogenesis performs essential functions in a variety of physiological and pathological processes such as wound healing, tumor growth, metastasis and hypoxia [8,9]. Among numerous proangiogenic mediators [10–13], VEGF-A is best characterized as a VEGF family member which is an endothelial cell specific mitogen and induces angiogenesis [14]. VEGF has two known receptors, KDR/Flk-1 and Flt-1 [15–17]. Recently, human and bovine LF were shown to modulate VEGF-A-mediated angiogenesis *in vivo* [5–7]. However, the molecular mechanism underlying this process has yet to be elucidated. In this study, we showed that human LF potentiates VEGF-induced endothelial cell migration and proliferation via the upregulation of KDR/Flk-1 expression in endothelial cells.

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Abbreviations: VEGF, vascular endothelial growth factor; KDR/Flk-1, kinase insert domain-containing receptor/fms-like tyrosine kinase 1; Flt-1, fetal liver kinase-1; ERK, extracellular signal regulated kinase; MAPK, mitogen activated protein kinase; PMA, phorbol-12-myristate 13-acetate

2. Materials and methods

2.1. Reverse transcription and real time polymerase chain reaction

Total RNA was isolated from HUVECs by using RNA-Bee isolation kit (Tel-Test Inc.). The following primers were used in this study: KDR/Flk-1, 5′-CGACCTTGGTTGTGGCTGACT-3′ and 5′- CCCTTCTGGTTGGTGGCTTTG-3′; Flt-1, 5′-AACAAAGTCGGGAGAGGA-3′ and 5′-TGACAAGAAGTAGCCAGAAGA-3′; β-actin, 5′-ATCTGGCACCACACCTTCTA-3′ and 5′-CGTCATACTCCTGCTTGCTG-3′. Real time PCRs were performed using the real-time fluorescence detection method using the LightCycler System with a First-Start DNA Master SYBR Green I kit (Roche Diagnostics). The primer sequences for KDR/Flk-1 were as follows: 5′-TCTCAATGTGGTCAACCTTQCTAGG-3′; 5′- TTAAACGTCTTAAGGGTGTTAGTGG-3′. The primer sequences for Flt-1 were as follows: 5′-CGACGTGTGGTCTTACGGAGTA-3′; 5′-CTTCCCTCAGGCGACTGC-3′. The cycling conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 amplification cycles of 95 °C for 15 s, 56 °C for 5 s and 72 °C for 15 s. After amplification, the temperature was slowly elevated above the melting temperature of the PCR product to measure the fluorescence and thereby to determine the melting curve. A negative control without cDNA template was performed to assess the overall specificity.

2.2. Cell based ELISA

For enzyme-linked immunosorbent assay (ELISA), flat-bottom 96-well plates were coated with 0.1% gelatin. HUVECs were cultured in 96-well plate (2×10^4 cells/well) for 24 h. Cells were washed with PBS and fixed with 3.7% formaldehyde in PBS for 15 min. After blocking with 3% BSA/PBS, cells were incubated with 0.2 mg/ml of anti-KDR/Flk-1 monoclonal antibody in 1% BSA/PBS for 1 h at room temperature and washed three times with PBS. After 1 h incubation with a 1:5000 dilution of peroxidase-labeled goat anti-mouse IgG in 1% BSA in PBS, wells were washed three times 0.5% Tween 20 in PBS. The bound peroxidase was detected using the chemiluminescense ELISA reagent.

2.3. Western blot analysis

Western blot analysis was performed as described previously [18]. Antibodies against KDR/Flk-1, Flt-1 (Research Santa Cruz, Santa Cruz, CA), and ERK1/2 (Cell Signaling Technology, Beverly, MA) were utilized as primary antibodies and peroxidase-conjugated antibody was used as a secondary antibody.

0014-5793/$32.00
doi:10.1016/j.febslet.2006.06.091

2.4. Confocal laser scanning microscope

HUVECs were grown with or without LF on chamber slides (Nalge Nunc, IL). Cells were fixed with 4% paraformaldehyde, washed in PBS, and incubated overnight with polyclonal anti-KDR/Flk-1 antibody. Cells were then washed and incubated for 1 h with FITC-conjugated anti-rabbit antibodies. Fluorescence image analyses were performed by using confocal microscope (Carl Zeiss, Germany).

2.5. Cell proliferation assay

The assay was conducted by using BrdU and a Detection Kit (Roche, Indianapolis, IN). In brief, HUVECs were seeded in 0.1% gelatin-coated 96-well plates. After 12 h of LF treatment, VEGF (10 ng/ml) was added. The BrdU-integrated DNA was quantitated by the relative luminescence unit (RLU) of each well using a Wallace Victor^2 1420 Multilabel counter (Perkin–Elmer, Norwalk, CT).

2.6. Endothelial cell migration and scratch wound assays

Cell migration assay [19] and scratch wound assays [13] were conducted as previously described. In brief, the lower chamber of a Boyden chamber (Neuro Probe Inc., Cabin John, MD) was loaded with cells, and the membrane was laid over the cells. The upper chamber was loaded with or without VEGF containing 0.1% BSA/M199. The chamber was inverted and incubated for 3 h at 37 °C and the migrated cells were counted.

Scratch wounds were generated in confluent HUVEC cultures with a sterile 200-μl pipette tip. Cells were treated with LF (Sigma–Aldrich, St. Louis, MO) for 12 h prior to VEGF (10 ng/ml) treatment. After 24 h, HUVECs were stained with Diff-Quick (Baxter Healthcare Corp., McGraw Park, IL) and photographed.

2.7. Chick chorioallantoic membrane assay

Modified chick CAM assays were performed as previously described [18]. In brief, test samples in rat tail type I collagen (Collaborative Biomedical Products) were applied onto Thermonox disks (Nalge Nunc, IL). The disks were loaded onto the CAM of 10-day-old embryos. After 72 h of incubation, the areas around the loaded disks were photographed with a Nikon digital camera and the newly formed vessels were counted. Each assay was conducted twice, and each experiment contained 15 eggs per sample.

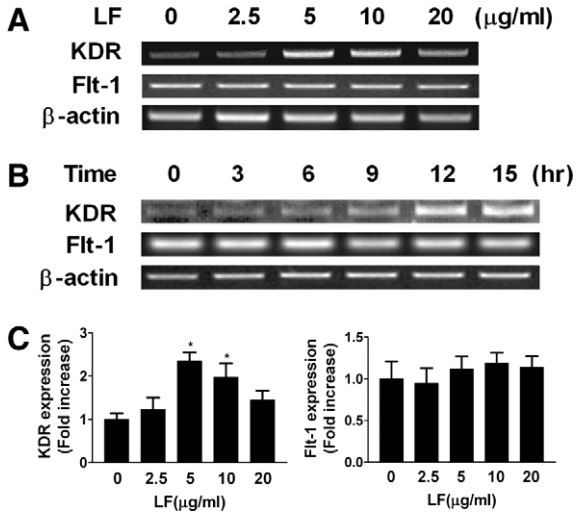

Fig. 1. Human LF upregulates mRNA expression of KDR/Flk-1 in HUVECs. (A) HUVECs were treated with various concentrations of LF for 12 h. RT-PCR was performed with the same samples using primer sets as described in Section 2. LF increased KDR/Flk-1 mRNA levels but not Flt-1 levels. (B) HUVECs were treated with 5 μg/ml of LF for the indicated times. LF increased KDR/Flk-1 mRNA after 12 h of treatment. (C) Real-time quantitative RT-PCR of KDR/Flk-1 and Flt-1 expression. The data are mean values ± S.D. from triplicates and are representative of at least four experiments. *, $P < 0.01$ versus vehicle.

2.8. Statistical analysis

The data are presented as means ± S.D. and statistical comparisons between groups were performed using 1-way ANOVA followed by Student's *t* test.

3. Results and discussion

3.1. Human LF upregulates mRNA and protein expression of KDR/Flk-1 in HUVECs

It was previously reported that the human iron-unsaturated form of LF enhanced VEGF-A-mediated angiogenesis *in vivo*

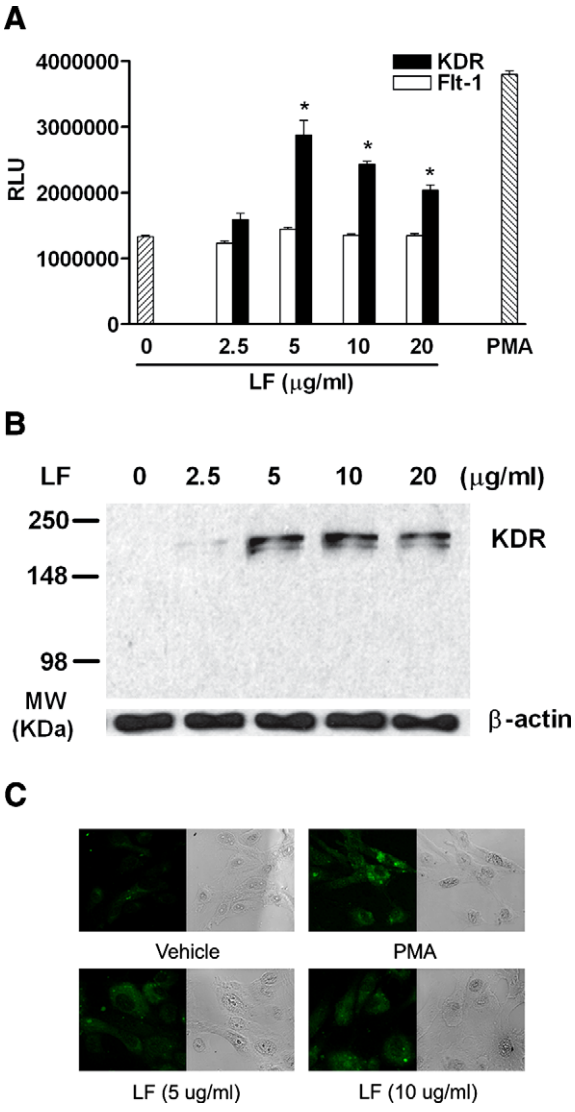

Fig. 2. Human LF upregulates protein expression of KDR/Flk-1 in HUVECs. HUVECs were treated with various concentrations of LF for 12 h. (A) Cell based ELISA showed that KDR/Flk-1 protein levels were increased in LF-treated HUVECs, but Flt-1 protein levels were not changed. *, $P < 0.01$ versus vehicle. PMA (100 nM): Phorbol-12-myristate 13-acetate, RLU: relative luminescence unit. The data are mean values ± S.D. from quadruplicates and are representative of at least three experiments. (B) Western blot analysis showed that LF increased KDR/Flk-1 protein levels in HUVECs. Molecular size markers are indicated on the left. (C) KDR/Flk-1 protein levels were increased in HUVECs treated with LF in immunofluorescent staining of cells with anti-KDR/Flk-1 antibodies. The photo figures in the left panels are immunofluorescence-stained cells, and those in the right panels are photomicrographs. Magnification: ×800.

[6]. To elucidate the molecular mechanisms underlying the effects of LF on VEGF-A-mediated angiogenesis, we first investigated the possibility that the LF might increase VEGF receptor expression, which would potentiate VEGF action, as it was known that LF is internalized, and translocated into the nucleus, where it regulates gene expression [20–23]. We performed RT-PCR and real time PCR analysis to evaluate the mRNA levels for the KDR/Flk-1 and Flt-1 VEGF receptors. As shown in Fig. 1A and C, LF stimulated KDR/Flk-1 mRNA expression at maximal levels of approximately 5 μg/ml, but did not affect Flt-1 expression. LF-induced stimulation of KDR/Flk-1 mRNA expression was maximal at approximately 12 h of treatment (Fig. 1B). We next measured KDR/Flk-1 protein levels by Western blot analysis and cell based ELISA, determining that LF increased KDR/Flk-1 protein levels with maximal levels at 5 μg/ml (Fig. 2A and B). KDR/Flk-1 proteins were also detected in endothelial cells by confocal microscopic analysis of immunofluorescent-stained cells. As expected, the fluorescence intensity of KDR/Flk-1 expression increased in LF-treated HUVECs compared with the controls (Fig. 2C). The increase in KDR/Flk-1 protein levels coincided with that of the mRNA levels. These findings suggested that LF upregulates the expression of the KDR/Flk-1 gene, which in turn enhances the action of VEGF in HUVECs.

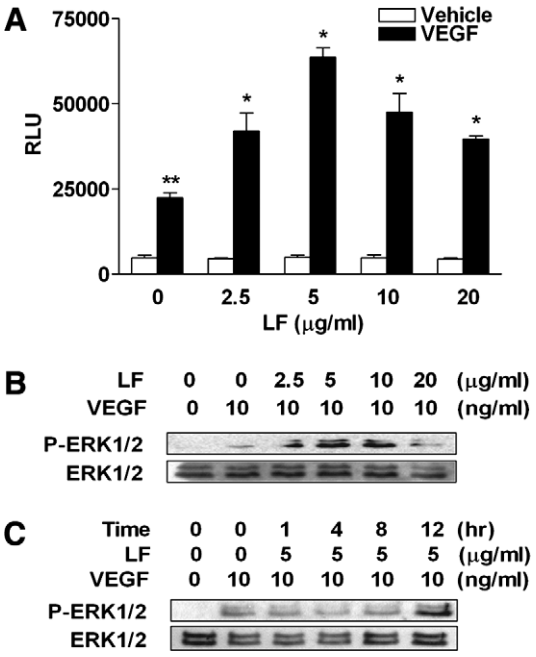

Fig. 3. Human LF stimulates endothelial cell proliferation and phosphorylation of MAPK mediated by VEGF-A. (A) HUVECs were pretreated with various concentrations of LF for 12 h and then treated with or without VEGF (10 ng/ml). HUVECs proliferation at different LF concentrations was compared with that observed in the presence of VEGF alone. *, $P < 0.01$ versus VEGF alone; **, $P < 0.01$ versus vehicle. RLU: relative luminescence unit. The data are mean values ± S.D. from quadruplicates and are representative of at least three experiments. (B) HUVECs were pretreated with various concentrations of LF for 12 h and then treated with or without VEGF (10 ng/ml). Pretreatment of HUVECs with LF increased the VEGF-induced phosphorylation of ERK1/2 in a dose-dependent manner. (C) HUVECs were pretreated with 5 μg/ml of LF for the indicated times and then treated with VEGF. The VEGF-induced phosphorylation of ERK1/2 was increased in HUVECs after 12 h of LF pretreatment.

3.2. Human LF stimulates endothelial cell proliferation and phosphorylation of MAPK

Mitogenic activity of endothelial cells can be stimulated by a variety of growth factors including VEGF [10–12]. We tested the mitogenic effect of LF on HUVECs and observed that LF alone did not promote endothelial cell growth significantly. However, when HUVECs were pretreated with LF at various concentrations for 12 h and subsequently exposed to VEGF, LF stimulated VEGF-induced cell proliferation further. Cell proliferation increased approximately 3-fold over VEGF alone at 5 μg/ml of LF (Fig. 3A), which was also correlated with the LF-induced increase in the KDR/Flk-1 mRNA and protein levels.

KDR/Flk-1 transduces the VEGF signal via MAPK activation [19,24,25]. VEGF induced ERK1/2 phosphorylation, which increased markedly when cells were pretreated with LF (Fig. 3B). It is noteworthy that ERK1/2 phosphorylation was increased after 12 h (Fig. 3C), which coincided with an increase in KDR/Flk-1 protein levels. The results indicate that the cell proliferation induced by VEGF and LF was associated with the sensitization of the MAPK pathway in the HUVECs.

3.3. Human LF stimulates endothelial cell migration and angiogenesis in vivo

We first investigated the effects of LF on HUVEC migration using Boyden chamber assays. As shown in Fig. 4A, LF stimulated HUVEC migration in the presence of VEGF-A, but did not induce endothelial cell migration by itself. We observed

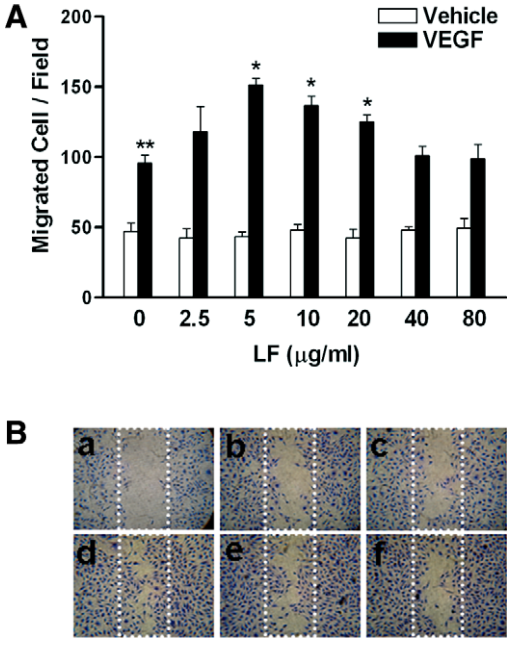

Fig. 4. Human LF stimulates endothelial cell migration mediated by VEGF-A. (A) HUVECs were pretreated with various concentrations of LF for 12 h and then treated with or without VEGF (10 ng/ml). Human LF significantly stimulated the VEGF-mediated migration of HUVECs over the presence of VEGF alone. *, $P < 0.01$ versus VEGF alone; **, $P < 0.01$ versus vehicle. The data are mean values ± S.D. from quadruplicates and are representative of at least three experiments. (B) HUVECs were pretreated with various concentrations of LF for 12 h and then treated with or without VEGF (10 ng/ml). LF enhanced endothelial cell migration in scratch wound healing assay. ((a) medium alone; (b) VEGF alone; (c–f), LF 2.5, 5, 10, 20 μg/ml in the presence of 10 ng/ml VEGF). Magnification: ×20.

that iron-loaded form of LF did not enhance HUVEC migration, even in the presence of VEGF-A (data not shown), which was consistent with the previous report that human iron-unsaturated form of LF but not the iron-loaded forms of LF enhanced VEGF-A-mediated angiogenesis *in vivo* [6]. The enhancement of VEGF-mediated endothelial cell migration by LF was also demonstrated by scratch wound assays (Fig. 4B). The stimulatory activity of LF was maximal at a concentration of approximately 5 μg/ml.

We confirmed that LF promoted VEGF-mediated angiogenesis *in vivo* by CAM assays [6]. LF clearly enhanced angiogenesis *in vivo* in the presence of VEGF with no visible effects on the pre-existing blood vessels, compared with that of VEGF alone (Fig. 5A). Quantitative analysis revealed that LF increased the number of newly-formed blood vessels as compared with that of VEGF alone, in a dose-dependent manner (Fig. 5B).

Recent papers indicate that apo-bovine LF inhibits VEGF-mediated angiogenesis [5,7], which is contrast to the stimulatory effects of apo-human LF *in vivo* [6]. It has been suggested that human LF and bovine LF play specific but contrary roles in angiogenesis. The difference in modulating angiogenesis between human and bovine LF still remains controversial. In this report, we demonstrated that human LF upregulates KDR/Flk-1 mRNA expression and protein levels, and subsequently stimulates VEGF-mediated endothelial cell migration and proliferation. Our results may provide some explanation for previously observed effect of subcutaneously infused apo-human LF which significantly enhanced VEGF-mediated angiogenesis *in vivo* [6]. The indirect effects of human LF in angiogenesis may perform functions in processes including bone formation and neonatal development. Our findings could further serve to understand the mechanisms underlying LF's effects on VEGF-mediated angiogenesis, and to raise the possibility of potential uses for LF in the prevention and treatment of diseases including ischemic diseases and hypoxia.

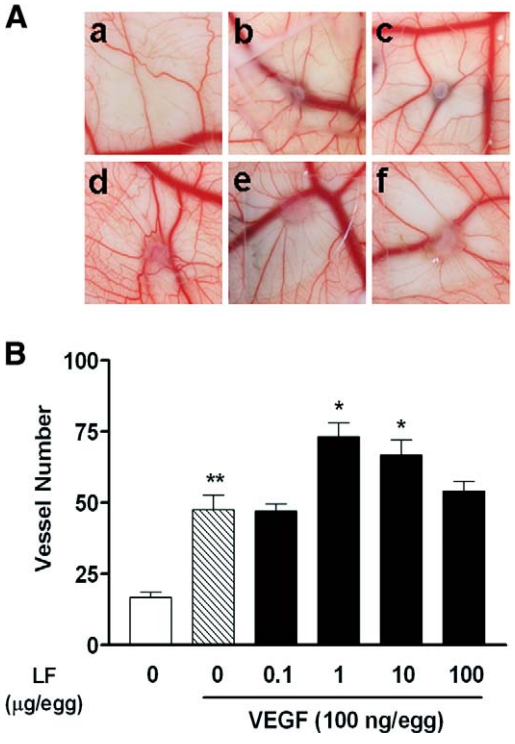

Fig. 5. Human LF stimulates angiogenesis mediated by VEGF-A *in vivo*. (A) LF or LF with VEGF was loaded on the CAMs of day 10 chick embryos. After 72 h incubation, eggs were processed as described in Section 2. Photographs of vehicle, VEGF, and different LF concentration in the presence of VEGF. ((a) Vehicle; (b) VEGF alone; (c–f), LF 0.1, 1, 10, 100 μg/egg in the presence of VEGF 100 ng/egg). (B) Quantification of newly formed blood vessels in the presence of different LF concentrations was compared with that observed in the presence of either medium or VEGF alone. *, $P < 0.01$ versus VEGF alone; **, $P < 0.01$ versus vehicle. The data are mean values ± S.D. from 15 eggs and are representative of at least two experiments.

Acknowledgement: This work was supported by Grant No. R01-2004-000-10481-0 from the Basic Research Program of the Korea Science & Engineering Foundation.

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