

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

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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.

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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.

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'-CCC-TTCTGGTTGGTGGCTTTG-3'; Flt-1, 5'-AACAAAGTCGGGAGAGGA-3' and 5'-TGACAAGAAGTAGCCAGAAGA-3'; β-actin, 5'-ATCTGGCACCACCTTCTA-3' and 5'-CGTCATACCTCTGCTTGCTG-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'-TCTCAAGTGGTCAACCTTQCTAGG-3'; 5'-TTAAACGTCTTAAGGGTGTTAGTGG-3'. The primer sequences for Flt-1 were as follows: 5'-CGACGTGTGGTCTTACGGAGTA-3'; 5'-CTTCCCTCAGGC-GACTGC-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 chemiluminescence 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.

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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

2.4. 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 Victor² 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- μ l 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 Thermanox 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.

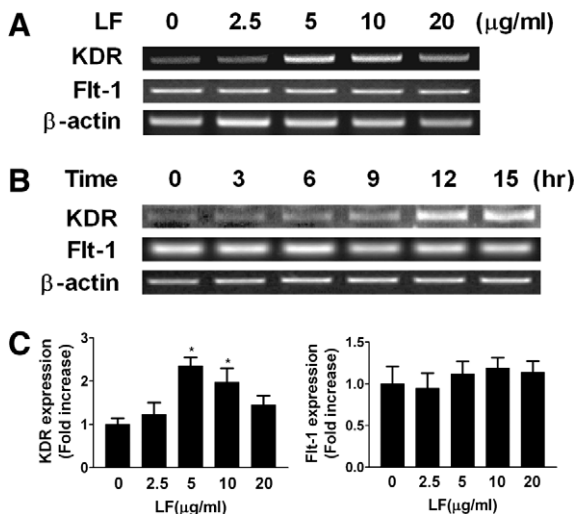


Fig. 1. Human LF upregulates mRNA expression of KDR/Flk-1 in HUVECs. (A) HUVECs were treated with various concentrations of LF for 12 h. RT-PCR was performed with the same samples using primer sets as described in Section 2. LF increased KDR/Flk-1 mRNA levels but not Flt-1 levels. (B) HUVECs were treated with 5 μ g/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 \pm 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 \pm 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*

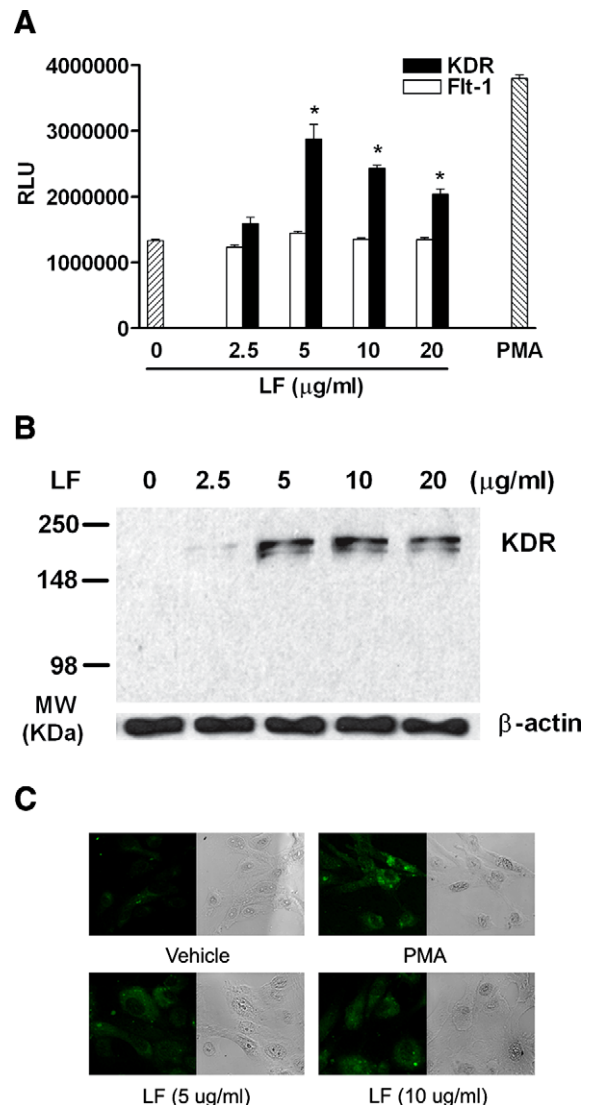


Fig. 2. Human LF upregulates protein expression of KDR/Flk-1 in HUVECs. HUVECs were treated with various concentrations of LF for 12 h. (A) Cell based ELISA showed that KDR/Flk-1 protein levels were increased in LF-treated HUVECs, but Flt-1 protein levels were not changed. *, $P < 0.01$ versus vehicle. PMA (100 nM): Phorbol-12-myristate 13-acetate, RLU: relative luminescence unit. The data are mean values \pm 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: $\times 800$.

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