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Human lactoferrin suppresses TNF-α-induced intercellular adhesion molecule-1 expression via competition with NF-κB in endothelial cells

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ABSTRACT

Lactoferrin (Lf) is known to have anti-inflammatory activity, but the mechanisms of action by Lf remain to be elucidated. Here, we demonstrated that TNF-α-induced expression of intercellular adhesion molecule-1 (ICAM-1) was down-regulated by Lf in a DNA-binding dependent manner at transcriptional level in endothelial cells. Our results showed that Lf bound to a DNA region in the ICAM-1 promoter in vitro as well as in chromatin context. Lf inhibited binding of NF-κB to a proximal NF-κB site in ICAM-1 promoter. This type of repression represents an additional mechanism for the action of Lf in regulation of gene expression.

1. Introduction

Lactoferrin (Lf) is a multifunctional glycoprotein which is present in breast milk [1–3] and also found in high concentration in most exocrine secretions and in the secondary granules of neutrophils [4,5]. Lf was shown to exert a number of biological responses such as primary defense against microbial infection, inflammation, angiogenesis, and inhibition of tumor growth [6–10]. Regulation of these processes by Lf is not understood clearly, although some of these functions are thought to be independent of its ability to bind ferric ions. Lf is known to be internalized and translocated into the nucleus, where it may regulate expression of target genes [11–13]. Previous reports have documented that Lf and delta-Lf, an Lf isoform, regulate a number of genes via activation of signaling pathways or via binding to DNA [14–19].

Intercellular adhesion molecule-1 (ICAM-1) is a cell surface glycoprotein that plays important roles in cell adhesion and transendothelial migration of leukocytes and Ag-specific activation of T lymphocytes [20,21]. ICAM-1 is constitutively expressed in a wide variety of cell types including leukocytes, endothelial and epithelial cells [22]. ICAM-1 gene can be induced to high levels by stimulation with external stimuli such as oxidative stresses and inflammatory cytokines. NF-κB activation has been shown to play a predominant role in induction of ICAM-1 gene expression by TNF-α and thrombin in endothelial cells [23,24]. In the present study, we demonstrated that Lf repressed the expression of TNF-α-induced ICAM-1 in endothelial cells at transcriptional level and investigated the molecular mechanism responsible for its inhibition.

2. Materials and methods

2.1. Cell culture

Human umbilical vascular endothelial cells (HUVECs) were obtained from American Tissue Culture Collection and passages 5–8 were used. HUVECs were grown in M199 supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 3 ng/ml bFGF, and 5 units/ml heparin at 37 °C under a humidified 95% and 5% (v/v) mixture of air and CO_2, respectively.

2.2. Plasmid constructions, transfection, and luciferase reporter assay

Different regions of 5′-flanking sequence were fused with promoterless luciferase gene in pGL3-basic (Promega). The plasmid pLf-T was constructed previously by inserting lactoferrin cDNA into pRcCMV plasmid [16]. Multiple mutations were introduced into the putative DNA elements within the pICAM-1(-1357)/Luc

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plasmid using mutagenic primers and a Quick-change site-directed mutagenesis II kit (Stratagene). The mutagenic primers used in this study are as follows: Mut1, forward 5′-CCC GAT TGC TTT AGC TTG Gcc ATT ggG GAG CTG AAG CGG CCA G-3′ and reverse 5′-CTG GCC GCT TCA GCT CCc cAA Tgg CCA AGC TAA AGC AAT CGG G-3′; Mut2, forward 5′-CCC GAT TGC TTT AGC TTG cAA gTT CCG GAG CTG AAG CGG CCA G-3′ and reverse 5′-CTG GCC GCT TCA GCT CCG GAA cTT gCA AGC TAA AGC AAT CGG G-3′. Transfection was performed according to the manufacturer's protocol using Lipofectamine 2000 (Invitrogen). The cell extracts were prepared 24 h after transfection for further analysis. The luciferase assays were performed using the Luciferase Assay System (Promega).

2.3. Reverse transcription and real time polymerase chain reaction

Total RNA was isolated from HUVECs using a commercially available RNA-Bee isolation kit (Tel-Test) as previously described [10]. The following primers were used in PCR reactions: ICAM-1, forward 5′-CGA CCT TGG TTG TGG CTG ACT-3′ and reverse 5′-CCC TTC TGG TTG GTG GCT TTG-3′; β-actin, forward 5′-ATC TGG CAC CAC ACC TTC TA-3′ and reverse 5′-CGT CAT ACT CCT GCT TGC TG-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 real-time PCR were as follows: ICAM-1, forward 5′-CTC CAA TGT GCC AGG CTT G-3′ and reverse 5′-CAG TGG GAA AGT GCC ATC CT-3′. GAPDH, forward 5′-GTC TTC ACC ACC ATG GAG AA-3′ and reverse 5′-AGG AGG CAT TGC TGA TGA TC-3′. A negative control without cDNA template was performed to assess the overall specificity.

2.4. Western blot analysis

Western blot analysis was performed using whole cell extracts prepared from HUVECs, as previously described [10]. Cell extracts were fractionated by electrophoresis on 10% SDS–PAGE gel and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by soaking the membrane in Tris buffered saline-Tween 20 (TBS-T) buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20) containing 3% nonfat milk. The membrane was incubated with polyclonal rabbit anti-human ICAM-1 (Santa Cruz). Peroxidase activity on the membrane sheet was visualized on X-ray films by a standard enhanced chemiluminescence procedure.

2.5. Determination of ICAM-1 mRNA stability

Briefly, 0–24 h after HUVEC cells were treated with or without Lf, 1 μg/ml of actinomycin D (Sigma) was then added to each culture to stop the further synthesis of ICAM-1 transcript. HUVEC cells were harvested from the cultures at the indicated time points following the addition of actinomycin D, and total RNA was isolated. The level of ICAM mRNA was quantified for each time point by semiquantitative RT-PCR and a real-time RT-PCR as described above.

2.6. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared and EMSA were performed as described previously with slight modification [24]. The deoxyoligomers used for EMSAs were as follows: Wild type oligomer containing NF-κB site of ICAM-1 promoter, 5′-TTA GCT TGG AAA TTC CGG AGC T-3′; NF-κB mutant oligomer (Mut1) generating Lf binding site (LBS) with high affinity (13), 5′-TTA GCT TGG cAC TTg CAG C T-3′; NF-κB mutant oligomer (Mut2) with no LBS, 5′-TTA GCT TGG ccA TTg gGG AGC T-3′. Oligomers were annealed and radiolabeled by phosphorylation with [γ-^{32}P] ATP using T4 polynucleotide kinase and purified on sodium Tris–EDTA (STE)-10 columns (BD Biosciences). The reaction products were separated on non-denaturing 6% polyacrylamide gels in 0.25X Tris–borated EDTA buffer. After electrophoresis, the gels were dried and subjected to autoradiography.

2.7. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed to detect in vivo association of Lf and NF-κB to ICAM-1 promoter according to the methods previously described [25]. A portion of the lysate was saved for subsequent purification of input genomic DNA. The remaining supernatant was pre-cleared with protein A Sepharose beads and sheared herring sperm DNA, and followed by incubation with anti-lactoferrin rabbit polyclonal antibody at 4 °C overnight. The primers for amplification of NF-κB region were as follows: ICAM-1 forward 5′-CGA TTG CTT TAG CTT GGA AA-3′; ICAM-1 reverse 5′-TTT ATA GCG CTA GCC ACC TG-3′. The primers for amplification of random sequence located upstream region of ICAM-1 promoter were also used: forward 5′-GCG TCT CTG GAT GGC CAG TG-3′; reverse 5′-TCT TAA GTC TCC GAG CAC CA-3′.

2.8. Avidin–biotin-complex DNA (ABCD) assay

ABCD assay was performed as described previously [25]. This assay is based on the immobilization of protein–DNA complexes via binding of a biotinylated oligonucleotide to a streptavidin. 200 μl HUVECs cell extract (5 mg/ml of protein concentration) was incubated with 200 μl buffer H (100 mM KCl, 20 mM HEPES pH 7.8–7.9, 20% glycerol, 1 mM DTT, 0.1% NP-40), 2 μg biotinylated oligonucleotide, and Lf for 1 h on ice. After the addition of 40 μl equilibrated streptavidin agarose beads (Amersham Pharmacia Biotech), incubation was continued for 30 min at 4 °C on a rotator. Beads were washed repeatedly with buffer H (containing 50 mM KCl), boiled in Laemmli sample buffer, and separated by SDS–PAGE under reducing conditions. Enhanced chemiluminescence (Amersham Pharmacia Biotech) was used for detection. Biotinylated oligos ordered from Bioneer (Seoul, Korea) comprise the following sequences: wild type, 5′-TTA GCT TGG AAA TTC CGG AGC T-3′; Mut1(high affinity LBS), 5′-TTA GCT TGG CAC TTG CGG AGC T-3′; Mut2(no LBS), 5′-TTA GCT TGc AAg TTC CGG AGC T-3′.

2.9. Confocal laser scanning microscope for translocation of Lf

To observe the fate of Lf added to living cells, Lf (25 μg/ml) was conjugated to FITC (PIERCE) for 1 h in PBS and dialyzed against PBS according to manufacturer's instruction. HUVECs were incubated with FITC-labeled Lf for 12 h, fixed with 3.7% paraformaldehyde, and then washed in PBS. To examine the distribution of Lf, the fixed cells were mounted with mounting solution containing DAPI (4′-6-diamidino-2-phenylindole), and photographed using a laser-scanning confocal microscope (Carl Zeiss).

2.10. Statistical analysis

The data are presented as means ± SD and statistical comparisons between groups were performed by Student's *t*-test.

3. Results

3.1. Human Lf down-regulates TNF-α-induced expression of ICAM-1

To examine whether Lf regulates expression of TNF-α-induced ICAM-1, HUVECs were pretreated without or with various concentrations of Lf for 12 h and then stimulated with TNF-α for 12 h. TNF-α-induced ICAM-1 gene expression was significantly

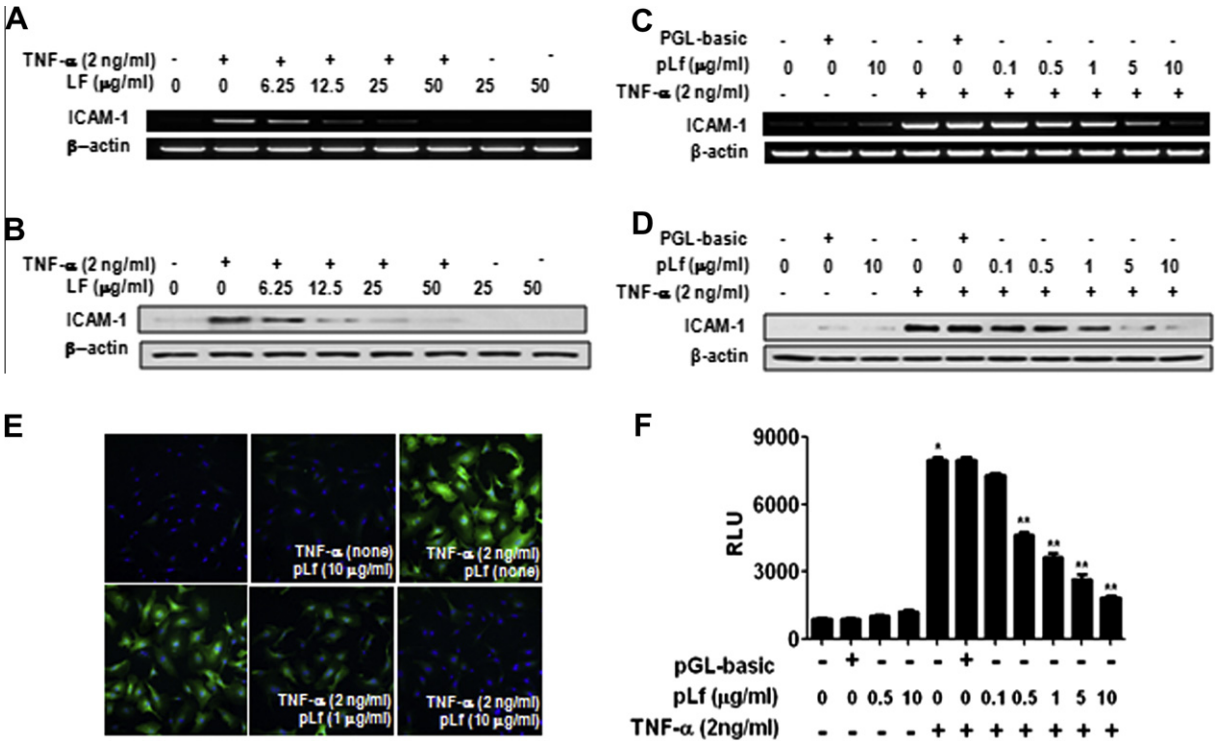

Fig. 1. Down-regulation of TNF-α-induced ICAM-1 expression in endothelial cells by Lf. (A) HUVECs were pretreated with various concentrations of Lf for 12 h and then stimulated with TNF-α (2 ng/ml) for 12 h. ICAM-1 mRNA levels were measured by RT-PCR analysis using specific primers. β-Actin mRNA was used as control. (B) ICAM-1 protein levels were measured by Western blot analysis using anti-ICAM-1 antibody. β-Actin protein was used as control. (C) HUVECs were transfected with various concentrations of pLf for 4 h and then stimulated with TNF-α (2 ng/ml) for 12 h. ICAM-1 mRNA levels were measured by RT-PCR analysis using specific specific oligonucleotide primers for ICAM-1. (D) ICAM-1 protein levels were measured by Western blot analysis using anti-ICAM-1 antibody. (E) ICAM-1 expression levels were detected by fluorescence microscopy using anti ICAM-1-FITC. (F) ICAM-1 proteins were measured by cell based ELISA using ICAM-1 antibody. $^{**}P < 0.05$ versus TNF-α alone. The data are mean values ± SD from quadruplicates and are representative of at least three experiments.

down-regulated in response to Lf treatment as shown by RT-PCR (Fig. 1A). The decreased expression of ICAM-1 protein was shown by Western blot analysis in TNF-α-stimulated endothelial cells after treatment with Lf (Fig. 1B). Our data showed that ICAM-1 expression was not affected by treatment with Lf alone in HUVECs.

We next investigated whether the endogenously expressed Lf could inhibit the expression of TNF-α-induced expression of ICAM-1. HUVECs were transfected with various amounts of pLf-T for 4 h and then stimulated with TNF-α. The levels of TNF-α-induced ICAM-1 mRNAs were reduced in dose-dependent manner in HUVECs expressing Lf (Fig. 1C). The ICAM-1 protein levels were similarly decreased in the transfected endothelial cells, as demonstrated by Western blot analysis (Fig. 1D), fluorescent staining of cells using FITC-labeled anti-ICAM-1 (Fig. 1E) and cell-based ELISA (Fig. 1F).

3.2. Lf inhibits TNF-α-induced ICAM-1 expression at transcriptional level

We analyzed whether Lf regulates ICAM-1 gene expression at the transcriptional level using ICAM-1/luciferase reporter gene constructs. High levels of induction with TNF-α were observed with the reporter constructs containing the 5′-flanking sequence up to −468 bp from the transcription initiation site and the induced ICAM-1 promoter activities were inhibited in Lf-treated cells significantly (Fig. 2A). The 5′-deletion up to −112 resulted in a complete loss of TNF-α-induced promoter activity. The promoter region contains a NF-κB binding sequence, 5′-GGAAATTCC-3′ at the position −187 to −178 from the transcription initiation site of ICAM-1 gene, which is known to play a critical role in TNF-α-induced activation of ICAM-1 gene expression [26,27]. Sequence comparison of Lf binding sequences to the ICAM-1 promoter region revealed that the sequence GGAAATTCC at the position −187 to −178 in ICAM-1 promoter was found in be very similar to GGCACTTGC, one of Lf binding sequences previously reported [13], indicating that Lf may bind to the proximal NF-κB site. We constructed wild type promoter construct, pICAM-1(-1357)/Luc and also two different NF-κB mutations in pICAM-1(-1357)/Luc, and analyzed transcription in HUVECs. Our results showed that mutation of NF-κB site to GGccATTgg (Mut2) abolished induction of ICAM-1 promoter activity by TNF-α (Fig 2A). In contrast, mutation of NF-κB site to Mut1 with high affinity LBS retained low level of inducibility of ICAM-1 promoter by TNF-α, which was strongly inhibited by treatment with Lf.

We examined whether Lf affects ICAM-1 mRNA stability. Actinomycin D inhibition assays showed that the half-lives of ICAM-1 mRNA appeared to be similar between mock- and Lf-treated cells as shown by RT-PCR (Fig. 2B upper) and by quantitative real-time PCR (Fig. 2B lower). The results suggest that a stability mechanism appeared not to be responsible for the decrease in ICAM-1 expression by Lf.

3.3. Lf binds to a DNA region in ICAM-1 promoter

Electromobility shift assays showed that purified Lf was bound to a DNA fragment containing the proximal NF-κB site of human ICAM-1 promoter (Fig. 3A). Specific binding of Lf to the ICAM-1 promoter probe was demonstrated by competition with an excess of unlabeled probe and by supershift assay using an antibody against human Lf. The LBS (Mut1) with high affinity for Lf competed more efficiently than wild type ICAM-1 oligomer. Competition with Mut2 oligomer did not affect the formation of the Lf-DNA complex

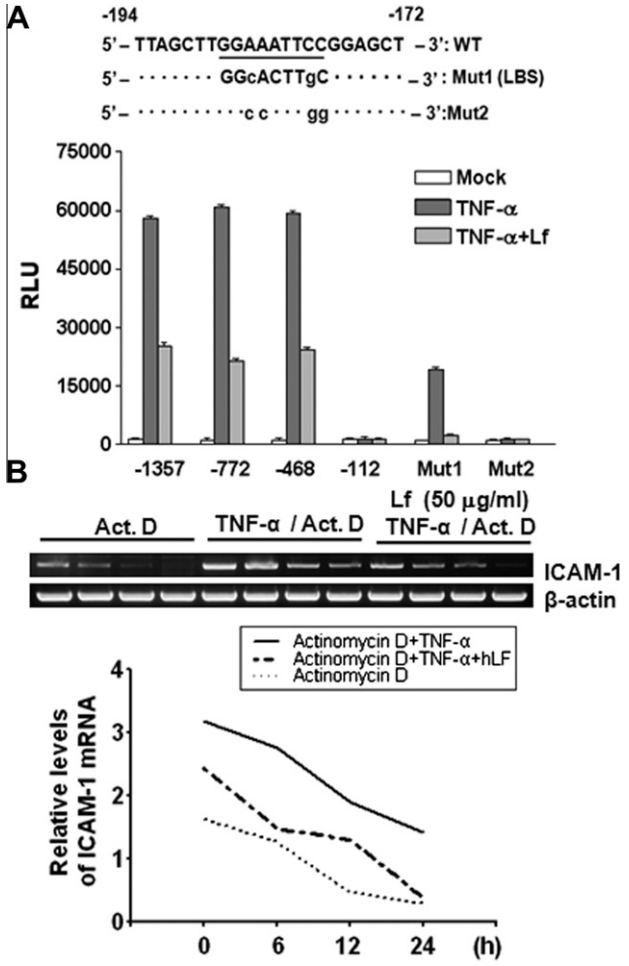

Fig. 2. Inhibition of TNF-α-induced expression by Lf at transcriptional level. (A) Analysis of ICAM-1 promoter/Luc expression in endothelial cells. Various ICAM-1 promoter fragments and ICAM-1 promoter containing Mut1 or Mut2 sequence in the proximal NF-κB site were fused to luciferase reporter gene. The plasmids were transfected into HUVEC using Lipofectamine. After transfection, cells were treated with Lf (20 μg/ml) for 12 h, and stimulated with TNF-α (2 ng/ml) for 12 h. Cell extracts were analyzed for luciferase activities using luminometer. Values are the average of three separate experiments in triplicate samples. RLU: relative luminescence unit. (B) ICAM-1 mRNA levels in the absence or presence of Lf. HUVECs were preincubated for 12 h in the absence or presence of Lf (50 μg/ml) and stimulated with TNF-α (2 ng/ml). Actinomycin D (1 μg/ml) was then added and total cellular RNA was harvested at the indicated time points (0–24 h). RT-PCR (top panel) or a real-time RT-PCR (lower panel) were performed. Results are means of three different experiments.

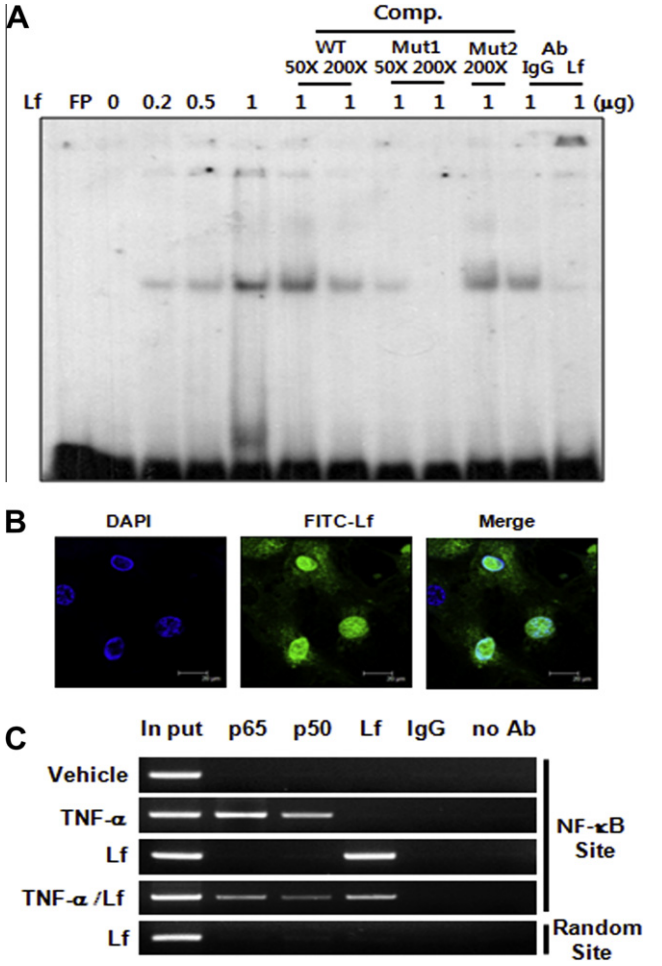

Fig. 3. Lf binds to ICAM-1 promoter region and nuclear translocation of Lf in HUVECs. (A) Human Lf (Sigma) was incubated with ^{32}P-end-labeled oligonucleotides in the absence or presence of excess of unlabeled competitors. Mut competitors are DNA fragments as shown in Fig. 2A. Supershift experiments were performed using antibody against Lf. (B) The nuclear translocation of Lf in HUVECs. HUVECs were incubated with 25 μg/ml FITC-conjugated Lf for 12 h and fixed with paraformaldehyde. The fixed cells were mounted with mounting solution containing DAPI and photographed using a laser-scanning confocal microscope. The nucleus is represented by the blue color in left panel, Lf represented by the green color in middle panel, and the merged image is shown in right panel. (C) ChIP assays were performed in HUVECs pre-treated with Lf for 12 h and/or treated with TNF-α for 2 h as indicated on the left of the Fig 3C. Antibodies used for precipitation are shown on the top. The DNA region comprising the proximal NF-κB site or upstream non-specific region was amplified by PCR using specific primers. Input represents PCR amplification of the unprecipitated DNA.

(Fig. 3A). Our results demonstrated that Lf directly binds to a DNA fragment containing the NF-κB site of ICAM-1 promoter.

We determined that exogenous Lf could be internalized and translocated into the nucleus, using confocal laser microscopic analysis of HUVECs treated with FITC-labeled Lf. The co-labeling of HUVECs showed that the nucleus (blue color, Fig. 3B, left) and FITC-labeled Lf (green color, Fig. 3B, middle) appear to be colocalized. The merged image (Fig. 3B, right), obtained by overlaying two images from Fig. 3B (left, middle), demonstrates that Lf is localized inside the nucleus of the endothelial cells.

DNA binding analysis in vivo by ChIP showed that NF-κB was bound to the proximal region of ICAM-1 promoter in endothelial cells treated with TNF-α. Pretreatment of cells with Lf resulted in reduction of p50 and p65 subunits of NF-κB binding as well as in substantial binding of Lf to the region, indicating that Lf binds to ICAM-1 promoter in vivo (Fig. 3C). Lf did not bind to an upstream non-specific region of ICAM-1 promoter, indicating that binding of Lf to the proximal site is specific. The results suggest that Lf may down-regulate transcription of ICAM-1 gene via competition of binding to ICAM-1 promoter of specific LBS with NF-κB.

3.4. Lf inhibits binding of NF-κB to the ICAM-1 promoter

We further investigated whether treatment of HUVECs with Lf affects TNF-α-stimulated NF-κB binding to the proximal NF-κB site in the ICAM-1 promoter. DNA binding activities of proteins in nuclear extracts of HUVECs incubated in the presence Lf were analyzed using ^{32}P-labelled oligonucleotides corresponding to the NF-κB binding site (Fig. 4A). Formation of NF-κB-DNA complexes was prominent in nuclear extracts from TNF-α-stimulated cells. When HUVECs were treated with Lf at 12.5 μg/ml and stimulated with TNF-α, a band of NF-κB-DNA complexes was reduced slightly comparing to only TNF-α treated endothelial cells, although we were not able to

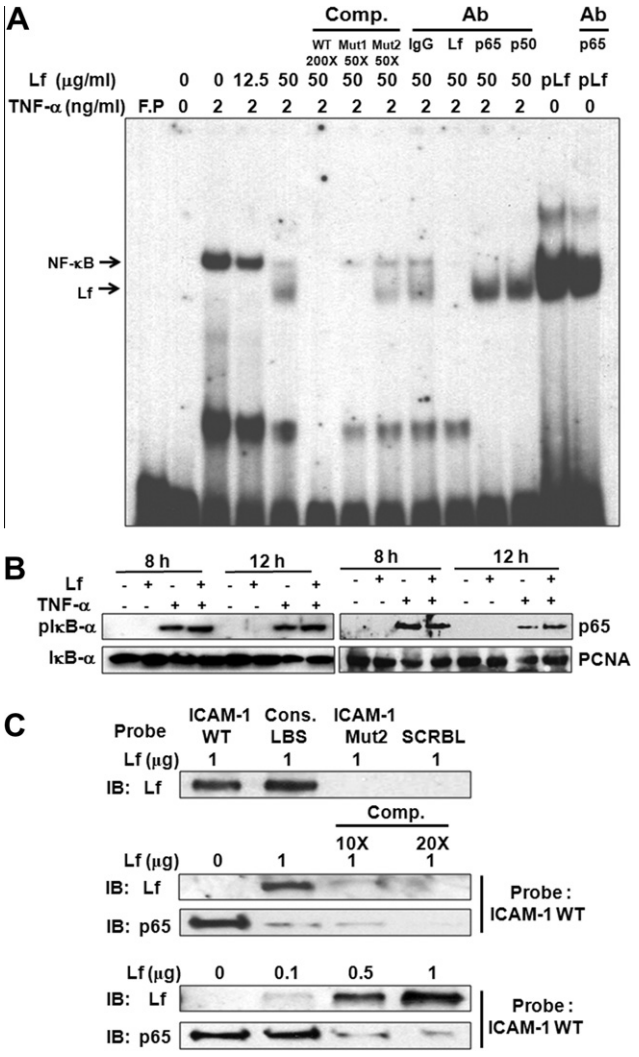

Fig. 4. Lf reduces binding of NF-κB to the proximal NF-κB site of ICAM-1 promoter. (A) 50 μg of nuclear extracts were incubated on ice for 30 min with ^{32}P-end-labeled oligonucleotides in a binding buffer containing 3 μg of poly(dI–dC) in the absence or presence of excess of unlabeled competitors. One microgram of purified Lf (pLf) was incubated under the same condition as a positive control. Supershift experiments were performed by preincubating nuclear extracts with 1 μg of antibody against p65 or p50. Upper arrows indicates NF-κB/DNA complexes and lower arrows indicate the position of Lf/DNA complexes. Mut1 and Mut2 are described in Fig. 2A. (B) Effects of Lf on TNF-α-induced phosphorylation of IκB-α and nuclear translocation of NF-κB. HUVECs were pre-treated with Lf at 50 μg/ml concentration at the indicated time and then treated with TNF-α (2 ng/ml) for 2 h. Cell lysates were analyzed by Western blot analysis using specific antibodies against IκB-α or phosphorylated IκB-α. Nuclear extracts were prepared and analyzed by Western blot analysis using specific antibody against p65 subunit of NF-κB. PCNA was used as control. (C) Binding of Lf to a DNA region containing the proximal NF-κB was shown by avidin–biotin-complex DNA (ABCD) assay. Biotinylated oligonucleotides of containing the proximal NF-κB in ICAM-1 promoter were used in an ABCD assay with TNF-α induced HUVECs lysates. Oligomers of wild type ICAM-1 promoter region containing the proximal NF-κB, consensus LBS [13], Mut2 and scrambled oligomer (SCRBL) were used as probe (upper panel). The sequences of the oligomers are shown in Fig. 2A. Addition of non-biotinylated oligonucleotides in large excess of molar ratios reduced binding of Lf as well as NF-κB to wild type oligomer (middle panel). Addition of increasing amounts of Lf to the wild type oligomer attenuated NF-κB binding to NF-κB site in ICAM-1 promoter (lower panel).

see appearance of a band of Lf-DNA complexes. However, when HUVECs were treated with Lf at 50 μg/ml and stimulated with TNF-α, NF-κB-DNA complexes diminished and Lf-DNA complexes were formed instead, which was migrated to the same position as those of purified Lf-DNA complexes on the gel. Cold wild type (WT) oligomer at 50× molar excess inhibited formation of Lf-DNA complexes rather weakly (Data not shown). WT oligomer at 200× and the Mut1 (mutated to Lf consensus sequence) oligomer at 50× excess inhibited the formation of Lf-DNA complexes completely, indicating that the Mut1 competed more efficiently than WT oligomer for Lf-DNA complexes, which is consistent with the result observed in Fig. 3A. Mut2 (mutated to random sequence) oligomer did not inhibit formation of Lf-DNA complexes. The antibody of Lf inhibited formation of the complexes, while antibodies directed against p65 or p50 subunit of NF-κB did not inhibit formation of Lf-DNA complexes. Inhibition of NF-κB binding by Lf was not due to reduction of nuclear translocation of NF-κB, because Lf alone did not affect phosphorylation of IκB-α and nuclear translocation of NF-κB (Fig. 4B). TNF-α-activated phosphorylation of IκB-α was not changed by Lf up to 24 h. Our results demonstrate that Lf inhibits binding of NF-κB to the site in ICAM-1 promoter via competition for the overlapped site.

ABCD assays demonstrated that Lf bound to the DNA region containing the proximal NF-κB site in ICAM-1 promoter and to LBS sequence (Mut1). However, Lf did not bind to the Mut2 sequence used in Fig. 2A or to the scrambled sequence. When non-biotinylated competitor DNA was added to the assays, the competitor attenuated not only Lf binding but also NF-κB binding to the DNA fragment (Fig. 4B middle panel). Addition of increasing amounts of Lf attenuated NF-κB binding to the DNA region in the ICAM-1 promoter (Fig. 4B lower panel). Our results suggest that Lf inhibited binding of NF-κB to the proximal binding site.

4. Discussion

The present study aimed at investigating effects of Lf on ICAM-1 gene expression and the underlying mechanism of gene regulation by human Lf in endothelial cells. Our results showed that the inhibitory effect of Lf on TNF-α-induced ICAM-1 mRNA steady state levels in cells was primarily due to inhibition of transcription of the ICAM-1 gene, rather than promoting degradation of ICAM-1 mRNA. Inhibition of TNF-α-induced ICAM-1 expression in endothelial cells by Lf was DNA binding-dependent. It has been reported that TNF-α-induced activation of ICAM-1 gene expression is primarily regulated through NF-κB activation [27], which mediates a wide range of transcriptional responses, including IL-1β, thrombin and LPS responses [22]. Analysis of ICAM-1 promoter showed that the region upstream of the proximal NF-κB site is not functionally important for NF-κB activation as well as Lf-responsiveness.

Sequence comparison of the ICAM-1 promoter region revealed that the sequence GGAAATTCC at the position −187 to −178 in ICAM-1 promoter was found in be very similar to GGCACTTGC, one of Lf binding sequences. Indeed, we showed that Lf is able to bind directly to the region containing the proximal NF-κB site in vitro and in vivo. We found that pretreatment of cells with Lf inhibited binding of TNF-α-stimulated NF-κB binding to the ICAM-1 promoter by EMSA and ABCD assays. ChIP assay demonstrated that Lf also reduced TNF-α-stimulated NF-κB binding to its promoter elements in vivo.

The present results demonstrated that exogenous Lf was translocated into nucleus of endothelial cells by confocal laser scanning microscopic analysis of FITC-conjugated Lf, where Lf was detected in the nucleus within 1 h of administration. Other investigators previously reported that exogenous Lf was transported to the nucleus of the cells such as human K562 and Caco-2 cells [11,12].

Other investigators reported that upstream components of NF-κB, TNFR-associated factors, IKKβ, NIK or MEKK1 participated in NF-κB activation by Lf signaling [15,28]. However, our results showed that Lf alone did not activate phosphorylation of IκB, and thereby phosphorylation of IκB and translocation of NF-κB in TNF-α-activated endothelial cells was not increased any further by pretreatment with Lf. The discrepancy may be explained by different

cell types or by differences of Lf concentrations used for the studies. Transfection of endothelial cells with plasmid expressing human Lf showed that endogenously expressed Lf was able to inhibit TNF-α-stimulated ICAM-1 expression, suggesting that Lf may not require signaling pathways of NF-κB for inhibition of ICAM-1 expression by Lf in endothelial cells.

Up-regulation of adhesion molecules on the surface of the endothelium plays a key role in recruitment and infiltration of polymorphonuclear leukocyte (PMN) at inflammation sites. We observed that U937 monocytoid cell adhesion to endothelial cells was reduced significantly by Lf (data not shown), which was reported by other group recently [29]. Serum Lf are primarily released from secondary granules of neutrophils and circulate at concentrations of 0.1–3 μg/ml in healthy adults and the local concentrations of Lf tend to be far higher in inflammatory regions [30]. Our results showed that Lf, at concentration encountered at the inflammatory site, strongly inhibits TNF-α-stimulated expression of ICAM-1 via competing with NF-κB in endothelial cells. Taken together, our results suggest one of underlying mechanisms by which Lf reduces inflammatory events and development of inflammatory diseases such as atherosclerosis.

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