

Interaction of Human Lactoferrin with Cell Adhesion Molecules through RGD Motif Elucidated by Lactoferrin-binding Epitopes*

Received for publication, May 24, 2006 Published, JBC Papers in Press, June 19, 2006, DOI 10.1074/jbc.M604974200

Kotaro Sakamoto[‡], Yuji Ito^{‡,1}, Toshiyuki Mori^{§,2}, and Kazuhisa Sugimura[‡]

From the [‡]Faculty of Engineering, Kagoshima University, Korimoto, Kagoshima 890-0065, Japan and the [§]Molecular Targets Development Program, Center for Cancer Research, NCI-Frederick, National Institutes of Health, Frederick, Maryland 21702

Lactoferrin (LF) is an iron-binding secretory protein, which is distributed in the secondary granules of polynuclear lymphocytes as well as in the milk produced by female mammals. Although it has multiple functions, for example antimicrobial, immunomodulatory, antiviral, and anti-tumor metastasis activities, the receptors responsible for these activities are not fully understood. In this study, the binding epitopes for human LF were first isolated from a hexameric random peptide library displayed on T7 phage. Interestingly, two of the four isolated peptides had a representative cell adhesion motif, Arg-Gly-Asp (RGD), implying that human LF interacts with proteins with the RGD motif. We found that human LF bound to the RGD-containing human extracellular matrix proteins, fibronectin and vitronectin. Furthermore, human LF inhibited cell adhesion to these matrix proteins in a concentration-dependent manner but not to the RGD-independent cell adhesion molecule like laminin or collagen. These results indicate that a function of human LF is to block the various interactions between the cell surface and adhesion molecules. This may explain the multifunctionality of LF.

Lactoferrin (LF)³ is an 80-kDa secretory glycoprotein that is contained in the secretory granules of polynuclear leukocytes as well as in the milk produced by female mammals. This protein is thought to be important in transporting iron to cells or providing iron to infants because of the features it has in common (with respect to structure and iron binding ability) with a representative iron-transporting protein, transferrin (TF). However, LF has recently been recognized as a multifunctional protein because it has various biological activities. LF has antimicrobial activities against bacteria, fungi, and viruses (1).

The antibacterial activity of LF is due to its N-terminal Arg-rich region, which binds to lipopolysaccharides (LPS) on Gram-negative bacteria, and this region is also responsible for binding lysozyme, heparin, and DNA (2, 3). With respect to its antiviral activity, LF acts as an inhibitor for viral entry to target cells but does not repress the intracellular replication of viruses (4). For example, LF prevents viral infection by binding to the envelope proteins E1 and E2 of the hepatitis C virus (5) or the VP1 protein in enterovirus (6). Several reports have also described the immunoregulatory and anti-tumor metastasis activities of LF. However, the reasons for these activities have not been well established. It was found that orally administrated human LF (hLF) inhibited the growth of established tumors to the same extent as the chemotherapeutic agent *cis*-platinum (7). It is thought that this anti-tumor activity is derived from induction of secretion of interleukin-18 in the intestinal tract (8), subsequent systemic NK cell activation, and increased numbers of CD8⁺ T cells. However, in another report, LF was found to exert its anti-tumor activity by inhibiting the angiogenesis induced by the tumor cells (9).

To account for these various activities, several LF receptors have been reported. Legrand *et al.* (3) noted that a 105-kDa hLF receptor expressed on Jurkat cells binds to hLF with a dissociation constant (K_d) of 70 nM. Another research group isolated a hLF-specific receptor that forms a 110–120-kDa complex, which was purified from brush-border membrane in the intestinal tract (10). Furthermore, the same group identified the associated hLF receptor gene (10). This receptor bound to hLF with a K_d of 1 μ M. A nucleolar protein called nucleolin is also a potential hLF receptor (11). In this case, both the N- and C-lobes of LF contributed to the receptor binding, and the K_d was 240 nM. However, nucleolin is known to function as a receptor of various other protein ligands. Therefore, the crucial receptor that is specifically directed to LF with high affinity has not been found.

To search for proteins that interact with hLF, we first screened a 6-mer random peptide library displayed on T7 phage and isolated four hLF-specific peptides by biopanning with hLF. Interestingly, two of the four peptides had the typical cell adhesion motif Arg-Gly-Asp (RGD), suggesting that hLF may interact with RGD-containing proteins. Binding experiments using ELISA and surface plasmon resonance (SPR) analysis demonstrated that hLF bound to human fibronectin (hFN) and vitronectin (hVN) with relatively high affinity. Furthermore, the cell adhesion of Chinese hamster ovary (CHO), HeLa,

* This work was supported in part by the Intramural Research Program of the National Institutes of Health, NCI, Center for Cancer Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ To whom correspondence should be addressed. Tel.: 81-99-285-8346; Fax: 81-99-258-4706; E-mail: yito@be.kagoshima-u.ac.jp.

² Present address: Biomedical Research Laboratories, Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, 2-17-85, Jusohonmachi, Yodogawa-ku, Osaka 532-8686, Japan.

³ The abbreviations used are: LF, lactoferrin; TF, transferrin; FN, fibronectin; LN, laminin; CN, collagen; h, human; LPS, lipopolysaccharide(s); ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; CHO, Chinese hamster ovary; HEL, hen egg white lysozyme; BSA, bovine serum albumin; PBS, phosphate-buffered saline; pfu, plaque-forming unit; SA, streptavidin.

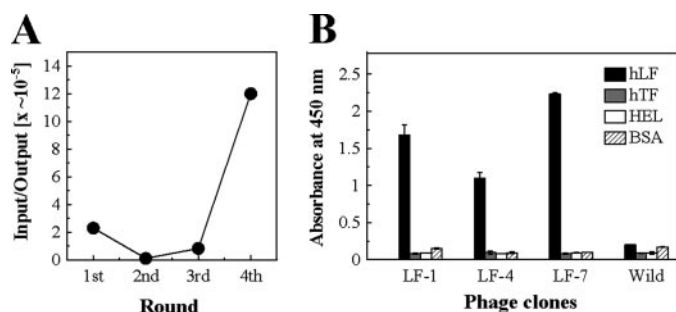


FIGURE 1. *A*, enrichment of hLF-specific phages by biopanning. Output/input ratios of phages by titration was indicated in each round of biopanning. *B*, human LF binding specificity of isolated phage clones in ELISA. The binding of isolated phage clones (LF-1, -2, -4, and -7) was examined using plates coated with hLF, hTF, HEL, or BSA, as described under "Experimental Procedures." The wild type phage was included as a control. Data represent the mean $\pm$ S.D. of duplicate measurements.

Cell Adhesion Inhibition Assay—The cell adhesion activity of the extracellular matrix protein was evaluated by using a method described by Zhao and Newman (12). A 96-well cell culture plate (Iwaki, Tokyo) coated with hFN, hLN, or hCN (300 ng/100 μ l/well) was preincubated with hLF, hTF, or HEL (1.5, 3, 6, 12.5, 25, 50, and 100 μ g/ml) and washed three times with PBS. CHO (ATCC CCL-61; America Type Culture Collection), murine macrophage cell line RAW264.7 (ATCC TIB-71), and human epithelial cell line HeLa (ATCC CCL-2) (10^5 cells/100 μ l/well) were added to the wells and cultured at 37 $^{\circ}$ C for 2 h in serum-free RPMI 1640 medium. For the control experiments, the cells were pretreated with hLF (1.5, 3, 6, 12.5, 25, 50, and 100 μ g/ml), washed, and added to the wells coated with hFN, hLN, or hCN. After the plate was agitated on a bioshaker at 500 rpm for 10 min, the adherent cells on the plate were counted. The cell adhesion ratio was represented as a percentage value relative to the number of adherent cells in the absence of hLF.

Immunostaining of hLF—Biotinylated hLF (600 nM) was added to human monocytic cell line THP-1 (ATCC TIB-202) (10^5 cells/100 μ l/well) and cultured at 37 $^{\circ}$ C for 1 h in RPMI 1640 medium containing 10% fetal bovine serum in the presence of LFpep-1 peptide (250 μ M) or hLF (6 μ M). After the cells were washed with fluorescence-activated cell sorter buffer, the cells were subjected to permeabilization and fixation by employing the Cytofix Cytoperm plus kit (Pharmingen). After fixation, the cells were reacted with SA-fluorescein isothiocyanate (Pharmingen) for 30 min and observed by fluorescence microscopy (IX71 fluorescence microscope equipped with a DP30BW digital camera; Olympus).

RESULTS

Isolation of hLF-specific Phage Clones from a T7 Phage-displayed Random 6-mer Peptide Library—Using a T7 phage library displaying hexameric random peptides, hLF-specific binding phages were isolated by a total of four rounds of biopanning against hLF. Fig. 1*A* shows the ratio of the output versus input phages (output/input ratio) in each round of panning. Enrichment for hLF-binding phages was observed at the fourth round.

After the fourth round of panning, the cloned phages were subjected to the hLF-binding assay in an ELISA format. Forty

TABLE 1

Amino acid sequences of hLF-binding peptide

Phage clone	Sequence	Frequency
LF-1	RGDDWA	20/40 (50%)
LF-4	RGDDWE	12/40 (30%)
LF-7	NRASNR	4/40 (10%)
LF-2	KKILH*	4/40 (10%)

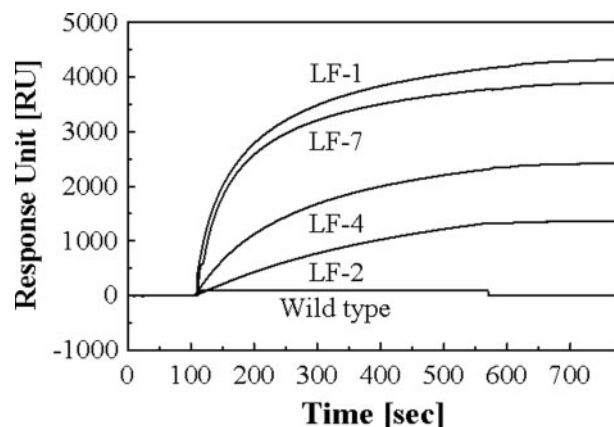


FIGURE 2. **SPR analysis of hLF binding activity of isolated phage clones.** The binding of isolated phage clones to hLF was examined using a BIAcore 2000 biosensor as described under "Experimental Procedures." LF-1, LF-2, LF-4, LF-7, and the wild type phage clones were injected onto hLF-immobilized sensorchips for association reaction and subsequently the washing procedure with HBS buffer started for dissociation reaction after 560 s.

out of the 50 isolated phages bound to hLF. All 40 clones were subjected to DNA sequence analysis, and the peptide sequences displayed on the phages were identified. A total of four sequences (LF-1, -2, -4, and -7) were found, as listed in Table 1. Among them, LF-1 and LF-4 were highly enriched and possessed the RGD motif, a representative cell adhesion motif. These phage clones clearly showed specific binding to hLF but not to other proteins such as hTF (Fig. 1*B*). These binding specificities were confirmed by SPR analysis. All the four phage clones showed responsive curves with respect to SPR signal to the hLF immobilized on the sensorchip (Fig. 2), but no signals were observed for hTF and HEL (data not shown). The sensorgrams for the dissociation phase of all the phage clones were very slow ($K_d < 10^{-5}$ s⁻¹). This slow dissociation may be related to the multivalent interactions between the hLF immobilized on the matrix of the sensorchip and the peptides on the phages.

Binding Specificity of Synthetic Peptide LFpep-1 to hLF—To evaluate the specificity and the essential residues for the binding of the LF-1, LF-1 synthetic peptide (LFpep-1: GGSRGD-DWAG) and its Ala-substituted peptides were prepared. Fig. 3*A* shows the results of their binding to hLF and control proteins. LFpep-1 peptide specifically bound to hLF. On the other hand, the binding of Ala-substituted peptides (where Arg¹, Asp³, Asp⁴, or Trp⁵ in LF-1 was replaced with Ala) resulted in significant decrease in affinity. These results indicate that not only cell adhesion motif (RGD) contributes to LF-1 binding to hLF but also that Asp⁴ and Trp⁵ play the role in LF-1 binding to hLF.

To examine the binding constant between the LFpep-1 peptide and hLF, SPR analysis was carried out by injecting the synthetic peptide into the sensorchip immobilized with hLF (Fig.

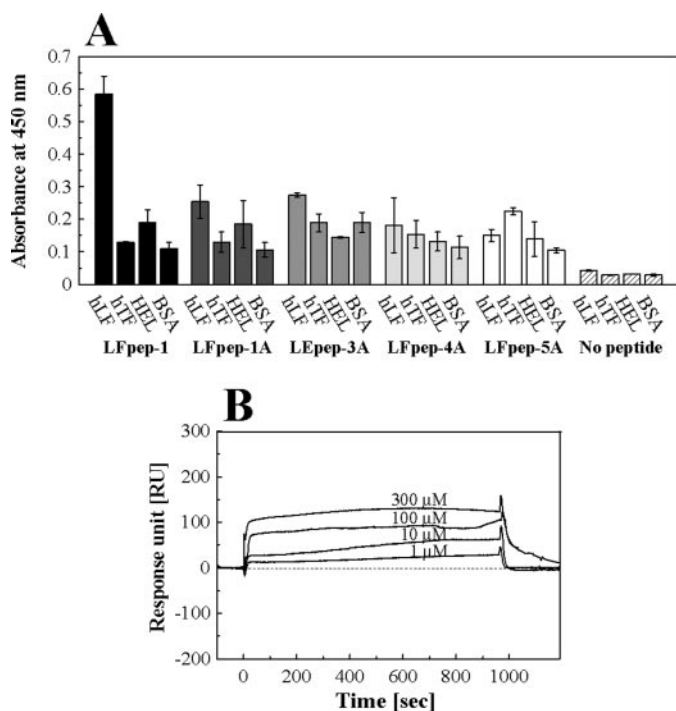


FIGURE 3. A, binding experiments of the LFpep-1 peptide and its Ala-substituted peptides to hLF. ELISA was performed to examine the binding of the LFpep-1, and its Ala-substituted peptides (LFpep-1A, LFpep-3A, LFpep-4A, and LFpep-5A) with hLF, hTF, HEL, or BSA, as described under "Experimental Procedures." Data represent the mean $\pm$ S.D. of duplicate measurements. B, SPR analysis of the binding of the LFpep-1 peptide to hLF on BIAcore 2000 biosensor. The sensorgrams represent the data in injecting the different concentration of LFpep-1 peptide (1, 10, 100, and 300 μ M) to immobilized hLF. At time = 970 s, the dissociation phase started by eluting with HBS buffer, as described under "Experimental Procedures."

3B). A concentration-dependent binding of LFpep-1 was observed on the sensorgram. However, the K_d was not able to be calculated because the affinity was extremely weak.

Interaction of hLF and Extracellular Matrix Proteins—The finding that the cell adhesion motif (RGD) was in human LF-binding peptides suggested the possibility that extracellular matrix proteins such as FN or VN, which interact with integrin receptors on the cell surface through the RGD motif, could be acceptors for hLF. Therefore, we first confirmed this hypothesis by using ELISA. As shown in Fig. 4, A and B, concentration-dependent binding of hLF with hFN and VN was observed. On the other hand, RGD-independent extracellular adhesion proteins such as hLN and hCN did not show the hLF binding.

To examine the affinity and binding specificity between hLF and hVN, SPR analyses were performed. As shown in Fig. 5A, the injection of hVN (2000 nM) onto the hLF sensorchips produced clear response curves, but no response was obtained for hTF. Fig. 5B shows the concentration-dependent response curves of the sensorgrams after injection of different concentrations of hVN (125, 500, and 2000 nM) onto the hLF-immobilization chip. From these analyses, the K_d value for their binding was calculated to be 370 nM ($k_a = 3.8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, $K_d = 1.4 \times 10^{-3} \text{ s}^{-1}$).

To further evaluate that the observed interaction between hLF and hFN occurs through the RGD motif, an inhibition assay was performed by using hLF-binding phage clones (Fig.

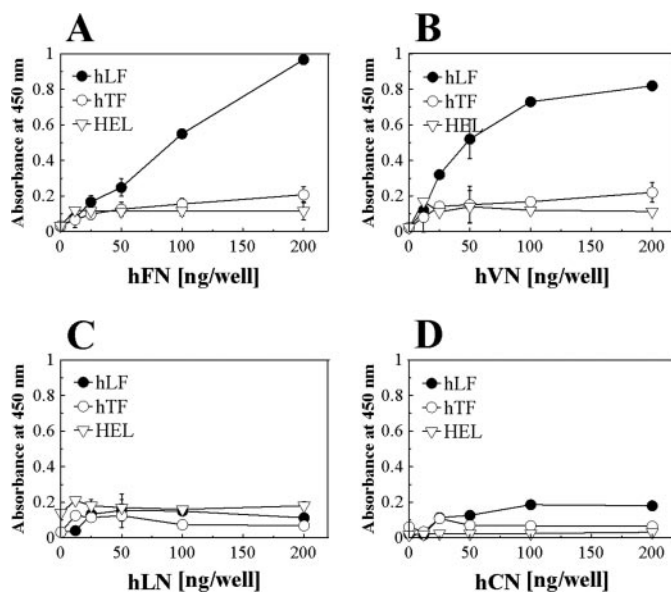


FIGURE 4. Specific binding activity of hLF to hFN and hVN in ELISA. The binding of hLF, hTF, and HEL was examined in ELISA plate coated with hFN (A), hVN (B), hLN (C), and hCN (D).

6A), LFpep-1 peptide, and its Ala-substituted peptides (Fig. 6B). The hLF-binding phage clones LF-1 and LF-4 effectively inhibited the hLF/hFN interaction in a titer-dependent manner. However, LF-7 phage clone, which did not possess the RGD motif, showed modest inhibitory effect despite its relatively high hLF-binding activity (Fig. 2), implicating that the LF-7 phage recognizes the different surface of hLF molecule from LF-1 and LF-4 phages. The biotinylated LFpep-1-forming tetrameric complex with SA also disturbed the interaction between hLF and hFN (Fig. 6B). All the Ala-substituted peptides (LFpep-1A, -3A, -4A, and -5A) did not show inhibitory activities, which is in good agreement with their binding abilities to hLF in ELISA (Fig. 3A).

Inhibition of Cell Adhesion by hLF—The cellular receptor for the extracellular matrix proteins (hFN and hVN) is the $\alpha\beta$ -heterodimeric transmembrane glycoprotein integrin ($\alpha 5\beta 1$, $\alpha V\beta 1$, $\alpha \text{IIb}\beta 3$, and $\alpha V\beta 3$), and they interact with each other through the RGD motif. Therefore, we examined whether LF could inhibit adhesion of three typical adherent cells (CHO, RAW, and HeLa cells) to hFN-coated plate (Fig. 7). Human LF preincubated with the hFN-coated well apparently inhibited cell-adhesion of CHO, RAW, and HeLa cells to the hFN-coated plate, while no changes in cell adhesion were occurred by the addition of control proteins (hTF and HEL). This specific inhibitory effect of hLF on cell adhesion was not observed in the case where the cells were first treated with hLF, and the mixture was added to the wells coated with hFN (data not shown), indicating that the inhibitory effect of hLF was exerted by specifically binding hFN.

It is well known that extracellular matrix proteins like hLN and hCN also participate in cell adhesion functioning as ligands of integrin family proteins. These two proteins, however, utilize the different binding interactions other than RGD motifs in the integrin binding. Therefore, we tested the effect of hLF on the RGD-independent cell adhesion using

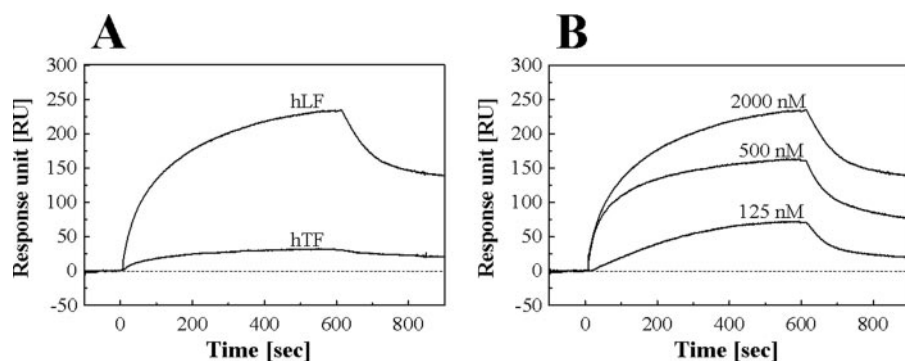


FIGURE 5. **SPR analysis of hLF binding to hVN.** The binding of hVN to hLF was examined using a BIAcore 2000 biosensor. *A*, sensorgram of the binding of hVN (2000 nM) to immobilized hLF on sensorchip. Human TF was used as a control protein. *B*, sensorgrams in concentration-dependent analysis of hVN (125, 500, and 2000 nM) to immobilized hLF. At time = 600 s, the injection phase was stopped and the sensorchips were washed with HBS buffer.

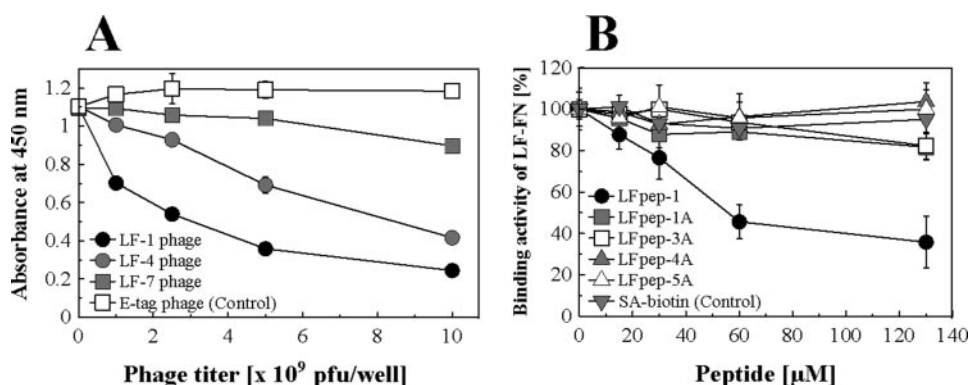


FIGURE 6. **Inhibition of the LF/FN interaction by phage clones (A) or the LFpеп-1 peptide and Ala substituted peptides (B).** Human LF was reacted with immuno plate coated with hFN in the presence of indicated phages or peptides. All peptides were biotinylated and supplied for analysis after forming tetrameric complex with SA. Data represent the mean $\pm$ S.D. of duplicate measurements.

hLN and hCN-coated plate. The inhibitory effect of hLF for cell adhesion to hLN and hCN-coated plate was not observed in all the three cell lines (data not shown). This result indicated that hLF blocked the specific interaction between hFN and integrin through RGD motif in cell adhesion. The IC_{50} of hLF in the inhibition of cell adhesion was approximately estimated to be 34–44 nM, which was far lower than that of cyclic RGD peptide, a typical inhibitor of cell adhesion (1.49 μ M) (13).

Inhibition Activity of the LFpеп-1 Peptide on Cell Internalization of hLF—The internalization of hLF into a human monocytic cell line THP-1 was previously described (20), although the uptake mechanism remain to be known. To examine the biological activities of the LFpеп-1 peptide, we examined the effect of the addition of the LFpеп-1 peptide on the uptake of hLF in THP-1 cells. THP-1 cells were incubated with biotinylated hLF in the presence of LFpеп-1 peptide or hLF in the medium containing 10% fetal bovine serum, and the incorporated hLF was detected by SA-fluorescein isothiocyanate on a fluorescence microscopy. In the absence of the LFpеп-1 peptide, internalized biotinylated hLF was seen as positive staining (Fig. 8A), whereas no staining could be observed in the cells treated in the presence of the LFpеп-1 peptide (Fig. 8B) or hLF itself (Fig. 8C). This result indicates that the LFpеп-1 peptide inhibits the uptake

of hLF into THP-1 cells, probably by disturbing the interaction between hLF and hFN through the RGD motif.

DISCUSSION

In the present study, we isolated hLF-binding peptides from a T7 phage-displayed random hexameric peptide library. The obtained four peptides were all specific to hLF (Fig. 1), which was also confirmed by kinetic affinity experiments using SPR analysis. The apparent affinities of the isolated T7 phage clones seemed to be very strong since of the dissociation rate was very slow ($K_d < 10^{-4}$ s $^{-1}$) (Fig. 2). However, a synthetic peptide LFpеп-1, derived from the LF-1 phage clone, had very weak affinity to hLF (Fig. 3). This discrepancy in affinity is thought to be occurred because of the avidity effect on the multivalent interactions between peptides on T7 phage and the hLF immobilized on the SPR sensorchip. Despite the low affinity of the peptide, it is significant that the RGD motif was found in the hLF-binding peptides among the major clones isolated (LF-1 and -4). The RGD motif is observed in many acceptors for integrins as a

common recognition moiety (Table 2). The interaction between integrins and their acceptors through RGD motif is involved in various bioactivities, including cell adhesion, blood coagulation, angiogenesis, migration of lymphocytes, inflammation, and tumor metastasis. In this study, we demonstrated that hLF bound to the extracellular matrix proteins hFN and hVN which possess RGD motif and inhibited the hFN-mediated cell adhesion by blocking the interaction between the cellular surface integrin and RGD motif of hFN. Such blocking ability for cell adhesion may give a plausible interpretation for multiple functions of hLF.

We scanned the important residues responsible for hLF binding in LF-1 sequence by using Ala-substituted peptides (Fig. 3A). As the result, we found not only Arg¹ and Asp³, which form RGD motif, but also Asp⁴ and Trp⁵ located after the RGD motif play an important role in binding to hLF. These results suggest that the RGD region is necessary for binding to the specific site on hLF and that the Asp⁴ and Trp⁵ are involved in enhancement of affinity and/or specificity of LF-1 sequence to hLF. In natural RGD-containing proteins, the sequence after RGD motif is not conserved as summarized in Table 2. A study regarding the binding motifs recognized by integrin suggests that there are no favorable common features in amino acid sequence after the RGD motif (14). Therefore, it is thought that

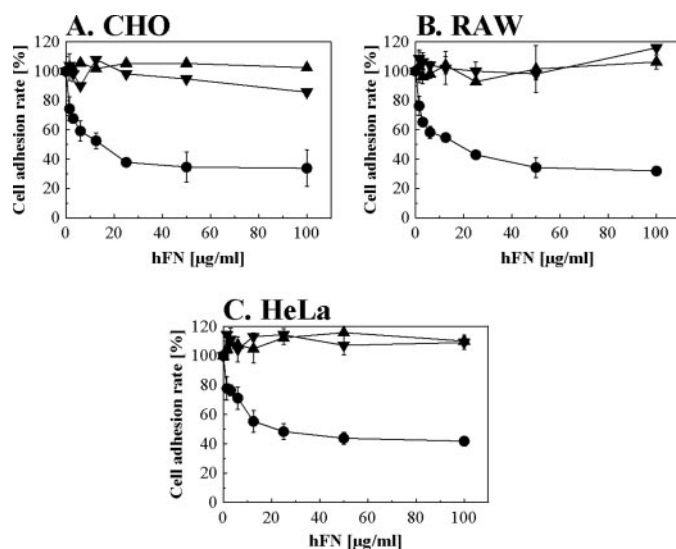


FIGURE 7. Specific inhibition of hFN-mediated cell adhesion by hLF. The cell adhesion was observed in the wells which was coated with hFN and subsequently treated with hLF (closed circles), hFN (closed triangles), or hFN + hLF (inverted closed triangles). Three types of adherent cells, CHO (A), RAW (B), and HeLa (C) (1×10^5 cells/well), were employed for the experiments. The data represent the mean $\pm$ S.D. of duplicate measurements. The detailed protocols of the adhesion assay were described under "Experimental Procedures."

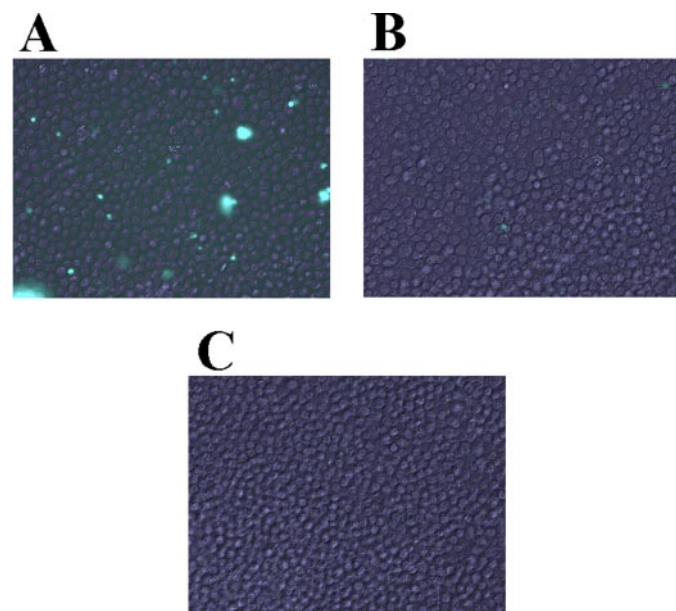


FIGURE 8. Inhibition of hLF internalization into cells by LFpep-1 peptide. Biotinylated hLF (600 μ M) was added to THP-1 cells in the absence (A), presence of 250 μ M LFpep-1 peptide (B), or 6 μ M hLF (C).

the amino acids responsible for hFN and hVN to bind to hLF are essentially limited to the RGD motif.

It has been reported that the RGD motif is involved in the binding of TF to its receptor (15, 16). On the basis of the tertiary structure of the complex, as analyzed by cryo-electron microscopy (17), the first Arg⁶⁴⁶ residue of the RGD motif on the receptor forms a salt bridge with the side chain of Asp³⁵⁶ on the C-terminal lobe of TF. The corresponding site of Asp³⁵⁶ of TF is replaced by Asn in LF, leading to an absence of the electrostatic interaction. In fact, it has been reported that LF barely binds to the TF receptor (18). Therefore, the binding mode or site of RGD motif is probably different in hLF and hTF.

TABLE 2

Amino acid sequence similarities between hLF-binding peptides and representative RGD-harboring proteins

The bold letters indicate the region of the common RGD sequence.

Protein/Peptide	Motif sequence
LF-1	R G D D W A
LF-4	R G D D W E
Human fibronectin	R G D S P A
Human vitronectin	R G D V F T
Human fibrinogen	R G D S T F
Human TFR	R G D F F R

The anti-bacterial activity of LF originates from the N-terminal Arg-rich region in hLF (Arg², Arg³, Arg⁴, Arg⁵, and Arg^{28–31}), and this region is also involved in binding heparin, LPS, and lysozyme (2). We examined the anti-bacterial activity of hLF using *E. coli* BL21 in the presence of the LFpep-1 peptide (1–500 μ M). However, even the presence of 500 μ M LFpep-1 peptide did not affect the inhibitory activity of human LF against bacterial proliferation (data not shown), suggesting that the binding site of the LFpep-1 peptide is not the N-terminal Arg-rich region of LF.

The suppressive activity of LF against the inflammatory cytokine production induced by LPS has been explained by its competitive binding to LPS with LPS-binding receptor CD14 (19). However, it has recently been proposed that LF directly suppresses cytokine production by inhibition of NF κ B binding to the cytokine promoter region (20). In contrast, LF acts as an LPS-binding protein under the low concentrations of LPS, leading to the enhancement of inflammatory reactions (21). Namely, LF forms a complex with LPS that is incorporated by macrophages, and LPS released in the intracellular fraction activates NF κ B through possible TLR4 signaling. Although the uptake of LF is necessary for both cases, its mechanism remains unclear. Therefore, we tried to examine whether the RGD sequence is involved in the incorporation of LF into THP-1 cells, as it is reported that monocyte-derived THP-1 cells efficiently incorporate LF into the intracellular fraction by endocytosis (20). The uptake of hLF was observed in THP-1 cells and efficiently inhibited by the addition of LFpep-1 peptide (Fig. 8), implicating that the interaction of hLF and RGD-containing proteins may have a key role in the uptake of LF into cells. It is well known that hFN participates in the cellular uptake of the extracellular proteins or microorganisms which bind to hFN (22). We consider that the uptake of hLF occurs in accompanying with the incorporation of hFN, which was attached with hLF by endocytosis. Actually the colocalization of hLF and hFN in the granules of polynuclear lymphocytes was observed (23), supporting our idea.

Several reports have described the suppressive effect of LF against angiogenesis in tumor growth. Shimamura *et al.* (9) found that orally administrated LF in mice represses tumor angiogenesis. This effect was explained by the direct growth inhibition of the vascular endothelial cells with the absorbed LF (9), which leads to anti-tumor activity in accompanied with immune enhancement by interleukin-18 induced by the intestinal LF receptor (9). Another group showed that LF inhibited *in vivo* angiogenesis induced by vascular endothelial growth factor (24). During angiogenesis, an adherent receptor integrin, α v β 3, is selectively expressed, and its interaction with FN and

VN promotes the viability of the angioblast to facilitate angiogenesis. Our finding that hLF inhibits the interaction between the integrin and FN in the RGD-dependent manor can plausibly explain the suppressive activities against angiogenesis involving in tumor metastasis.

In the present study, we proposed the base for explaining the multifunctionality of LF. Namely, hLF interacts with hFN, hVN, and possibly other RGD-harboring proteins and blocks their interactions with counter receptors. Our findings enable us to explain various functions of LF, although the further investigation is necessary for correct understanding of the biological functions of LF.

REFERENCES

1. Caccavo, D., Pellegrino, N. M., Altamura, M., Rigon, A., Amati, L., Amoroso, A., and Jirillo, E. (2002) *J. Endotoxin Res.* **8**, 403–417
2. van Berkel, P. H., Geerts, M. E., van Veen, H. A., Mericskay, M., de Boer, H. A., and Nuijens, J. H. (1997) *Biochem. J.* **328**, 145–151
3. Legrand, D., van Berkel, P. H., Salmon, V., van Veen, H. A., Slomianny, M. C., Nuijens, J. H., and Spik, G. (1997) *Biochem. J.* **327**, 841–846
4. van der Strate, B. W., Beljaars, L., Molema, G., Harmsen, M. C., and Meijer, D. K. (2001) *Antiviral Res.* **52**, 225–239
5. Yi, M., Kaneko, S., Yu, D. Y., and Murakami, S. (1997) *J. Virol.* **71**, 5997–6002
6. Weng, T. Y., Chen, L. C., Shyu, H. W., Chen, S. H., Wang, J. R., Yu, C. K., Lei, H. Y., and Yeh, T. M. (2005) *Antiviral Res.* **67**, 31–37
7. Varadhachary, A., Wolf, J. S., Petrak, K., O'Malley, B. W., Jr., Spadaro, M., Curcio, C., Forni, G., and Pericle, F. (2004) *Int. J. Cancer* **111**, 398–403
8. Iigo, M., Shimamura, M., Matsuda, E., Fujita, K., Nomoto, H., Satoh, J., Kojima, S., Alexander, D. B., Moore, M. A., and Tsuda, H. (2004) *Cytokine* **25**, 36–44
9. Shimamura, M., Yamamoto, Y., Ashino, H., Oikawa, T., Hazato, T., Tsuda, H., and Iigo, M. (2004) *Int. J. Cancer* **111**, 111–116
10. Suzuki, Y. A., Shin, K., and Lonnerdal, B. (2001) *Biochemistry* **40**, 15771–15779
11. Legrand, D., Vigie, K., Said, E. A., Ellass, E., Masson, M., Slomianny, M. C., Carpentier, M., Briand, J. P., Mazurier, J., and Hovanessian, A. G. (2004) *Eur. J. Biochem.* **271**, 303–317
12. Zhao, T., and Newman, P. J. (2001) *J. Cell Biol.* **152**, 65–73
13. van Well, R. M., Overkleeft, H. S., van der Marel, G. A., Bruss, D., Thibault, G., de Groot, P. G., van Boom, J. H., and Overhand, M. (2003) *Bioorg. Med. Chem. Lett.* **13**, 331–334
14. Li, R., Hoess, R. H., Bennett, J. S., and DeGrado, W. F. (2003) *Protein Eng.* **16**, 65–72
15. Liu, R., Guan, J. Q., Zak, O., Aisen, P., and Chance, M. R. (2003) *Biochemistry* **42**, 12447–12454
16. Kawabata, H., Tong, X., Kawanami, T., Wano, Y., Hirose, Y., Sugai, S., and Koeffler, H. P. (2004) *Br. J. Haematol.* **127**, 464–473
17. Cheng, Y., Zak, O., Aisen, P., Harrison, S. C., and Walz, T. (2004) *Cell* **116**, 565–576
18. Kawabata, H., Yang, R., Hiramata, T., Vuong, P. T., Kawano, S., Gombart, A. F., and Koeffler, H. P. (1999) *J. Biol. Chem.* **274**, 20826–20832
19. Ellass-Rochard, E., Legrand, D., Salmon, V., Roseanu, A., Trif, M., Tobias, P. S., Mazurier, J., and Spik, G. (1998) *Infect. Immun.* **66**, 486–491
20. Haversen, L., Ohlsson, B. G., Hahn-Zoric, M., Hanson, L. A., and Mattsby-Baltzer, I. (2002) *Cell. Immunol.* **220**, 83–95
21. Na, Y. J., Han, S. B., Kang, J. S., Yoon, Y. D., Park, S. K., Kim, H. M., Yang, K. H., and Joe, C. O. (2004) *Int. Immunopharmacol.* **4**, 1187–1199
22. Jadoun, J., Ozeri, V., Burstein, E., Skutelsky, E., Hanski, E., and Sela, S. (1998) *J. Infect. Dis.* **178**, 147–158
23. Salcedo, R., Segura, C., Szekeley, L., de Mesquita, R., Biberfeld, P., and Patarroyo, M. (1997) *Exp. Cell Res.* **233**, 25–32
24. Norrby, K., Mattsby-Baltzer, I., Innocenti, M., and Tuneberg, S. (2001) *Int. J. Cancer* **91**, 236–240