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Comparison of Proliferative Effect of Human Lactoferrin and Its Proteolytic Peptide on Normal and Transformed Epithelial Cells

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Abstract Human lactoferrin (hLF) is an iron-binding glycoprotein with a variety of functions. hLF undergoes proteolytic cleavage to smaller peptides in the stomach following ingestion. In the present study, we evaluated the effects of hLF and its proteolytic product, human lactoferrin peptide (hLFP), on the proliferation of two epithelial cells, HEK293 normal cells and KATO III gastric carcinoma cells, using an MTT assay and expression of proliferative nuclear cell antigen (PCNA), a notable proliferation marker. When the two epithelial cells were stimulated with hLF and hLFP in the presence of fetal bovine serum (FBS), hLFP stimulated proliferation of both cell types at lower concentrations than hLF by two orders of magnitude. The cancer cells exhibited proliferative responses to both hLF and hLFP at lower concentrations by 2~3 orders of magnitude than the normal cells. Either hLF or hLFP alone did not support appreciable proliferation of these cell lines in the absence or low concentrations of FBS. Bovine serum albumin or its proteolytic product failed to promote cellular proliferation even in the presence of 10 % FBS, indicating the specificity of the proliferative activity of hLF and hLFP. These data highlight feasibility of hLF and its peptide for adjuvants for tissue culture medium.

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Keywords Epithelial cells · HEK293 cells · KATO III cells · Lactoferrin · Lactoferrin-derived peptide · PCNA · Proliferation

Abbreviations

BSA	Bovine serum albumin
BSAP	Pepsin-lysate of BSA
FBS	Fetal bovine serum
LF	Lactoferrin
LFP	Lactoferrin-derived peptide
hLF	human lactoferrin
hLFP	Pepsin-lysate of human lactoferrin
PCNA	Proliferating cell nuclear antigen

Introduction

Human lactoferrin (hLF) is a cationic, multifunctional glycoprotein in the transferrin family with a molecular weight of 78 kDa that is composed of 691 amino acids. Lactoferrin (LF) exhibits a bi-lobal structure with four domains for the reversible binding of two Fe^{+} ions [1]. LF is isolated mainly from human milk in concentrations ranging from 1 g/l in mature milk to 7–8 g/l in the colostrum [2]. This protein is also found in mucosal secretions, including plasma, saliva, tears, vaginal fluids, semen, nasal, gastrointestinal fluids, and urine [3].

Multiple functions of hLF were reported [4]. hLF plays a role in the regulation of iron absorption and immune responses. hLF also plays a role as an innate host defense system against infection from a variety of bacteria, fungi, viruses, and even some parasites. Additionally, hLF has antioxidant and anticancer activities [3]. The antimicrobial and antiviral activities of lactoferrin-derived peptide (LFP) were also reported [1, 4–6].

A cell growth-promoting activity of LF was also reported in a wide variety of cell types [7–15]. The growth-promoting effect was thought to be due to the ability of LF to transport iron into the cell. However, there are many reports that LF functions as a growth factor for small intestine epithelial cells [16]. Additionally, LF itself is an effective growth factor that stimulates the growth of endometrium stroma cells in culture in the absence of any other cytokines or growth factors [9]. Another report suggested that the cell growth promotion was due to the DNA-synthesis-stimulating activity of LF, which was proven by the incorporation of ^{3}H-thymidine into the DNA of rat enterocytes and mouse Balb/c 3T3 fibroblasts [17, 18]. In contrast, there are many reports that LF does not have a growth-stimulating effect on cellular proliferation [19]. Moreover, LF showed a growth-inhibitory effect on some cell types [20–23]. Therefore, the inconsistent effects of LF on cell growth presumably depend on cell types, the doses used, and possibly the presence of co-growth-promoting or co-growth-inhibiting factors or signals [11, 24].

There are many reports that LFP, the pepsin-hydrolysate of LF, also has the same antibacterial, antiviral, and anticarcinoma activities [3]. These activities of LFP are mainly due to lactoferricin, the major peptide that is composed of 11 to 34 amino acids [25]. Additionally, LFP showed cell proliferation effects on some cell types such as a rat intestinal epithelial cell line [16] and oral cancer cells [26]. LFP also inhibited the growth or induction of apoptosis in

certain cell types [27–29]. The effect on the cell proliferation was inconsistent, as evidenced by the controversial effect depending on cell type and dose similar to LF. The mechanism of this effect is elusive.

This study examined and compared the effects of hLF and human lactoferrin peptide (hLFP) on the proliferation of normal human epithelial cells and gastric carcinoma cells with an emphasis of feasibility of hLFP as a growth-stimulating ingredient for tissue culture. We report that the peptides exhibited a growth proliferation effect, and hLFP was more effective than hLF. The growth proliferation includes the upregulation of proliferative nuclear cell antigen (PCNA).

Materials and Methods

Preparation of hLF and hLFP

Human colostrum collected from lactating donors was stored at −20 °C until needed. For the purification of hLF, 20 ml of human colostrum was centrifuged at 1000×*g* for 1 h at 4 °C. The supernatant was applied to a CM-Sepharose column (2.5 × 20 cm) equilibrated with a 0.02 M sodium phosphate buffer (pH 7.2) at a flow rate of 20 ml/h. After washing the column with the same buffer (flow rate 100 ml/h), hLF was eluted using 0.8 M NaCl at a flow rate of 20 ml/h. The fractions were collected, and the protein concentration was determined. After analyzing by SDS-PAGE, the purified hLF was lyophilized and stored at −20 °C until necessary. For the preparation of hLFP, treatment of purified hLF with pepsin was performed as described elsewhere [30] with minor modifications. Briefly, 10 mg of lyophilized hLF was dissolved in 1 ml of sterile water. The pH was adjusted to 3.0 with 1 N HCl. Porcine pepsin (Sigma Co., St. Louis, MO, USA) was added to a final concentration of 3 % (*w*/*w*). The reaction mixture was incubated for 4 h at 37 °C with agitation. The reaction was terminated by the inactivation of the pepsin via incubation at 80 °C for 15 min. The reaction mixture was rapidly cooled on ice followed by adjusting the pH to 7.0 with 1 N NaOH. After centrifugation of the reaction mixture at 13,000×*g* for 30 min, the supernatant was passed through an ultrafilter (5000 MW cutoff) to remove the pepsin. Finally, approximately 4.2 mg of hLFP was obtained. This hLFP was used for cellular proliferation.

Analysis of hLFP by HPLC

HPLC analysis of hLFP was performed on an Agilent 1260 HPLC system (Agilent technologies, Waldborn, Germany) with a YMC-Triart C18 column (150 × 4.6 mm, Agilent Zorbax XDB). The flow rate was 1 ml/min and the injection volume was 50 μl. hLFP was eluted with a linear gradient of solvent B (acetonitrile with 0.1 % trifluoroacetate) in A (water with 0.1 % trifluoroacetate) going from 0 to 60 % over 60 min. The elution was monitored at the absorbance of 214 nm.

Measurement of Antibacterial Activity

Antibacterial activities of hLF and hLFP were conducted as described elsewhere [31] with minor modifications. The indicated concentrations of purified hLF and hLFP were incubated

with *Staphylococcus aureus* (approximately 2.4×10^7 cells/ml) for 3 h at 4 °C. The bacteria were spread on agar plates containing 10 g peptone/l and further incubated at 37 °C overnight. Colonies that appeared on plates were examined.

Cell Culture for KATO III Cells and HEK293 Cells

KATO III human gastric carcinoma cells and HEK293 human embryonic kidney cells were purchased from ATCC (Manassas, VA, USA). KATO III and HEK293 cells were cultured in Iscove's Modified Dulbecco's Medium (Welgene, Daejeon, Korea) and Dulbecco's Modified Eagle Medium, respectively. These media were supplemented with 10 % fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml), HEPES (25 mM), and sodium pyruvate (1 mM). MTT [3-(4, 5)-dimethylthiazol-z-yl-2, 5-diphenyl-tetrazolium bromide] was purchased from Sigma (St. Louis, MO, USA).

Measurement of Cell Proliferation

KATO III and HEK293 cells were seeded at 1.5×10^4 cells/well on 96-well plates and treated with varying concentrations of hLF and hLFP for 48 and 72 h. After incubation, cell proliferation was assessed using MTT assays. A total of 10 μl of MTT (5 mg/ml) was added to each well. After an additional 4 h, 100 μl of the culture supernatant was removed, and 100 μl of isopropanol with 0.04 N HCl was added to solubilize the blue crystals. After dissolving the crystals, plates were read on a microplate reader (Biotek, Winooski, VT, USA) at a wavelength of 595 nm. The OD of cells that were cultured with medium alone was set as "1." Cell proliferation following hLF or hLFP was expressed as a fold increase relative to that of cells cultured with medium alone. Data are shown as means ± SEM of two to four independent experiments performed in triplicate ($*p < 0.01$ and $**p < 0.05$ compared to cells that were cultured in media alone).

Western Blot Analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl, 0.1 % NaN_3, 1 % Nonidet P-40, 0.25 % sodium deoxycholate, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitor mixture) supplemented with 0.4 and 0.1 M NaCl for nuclear and cytosolic fractions, respectively. The fractions were resolved using SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5 % non-fat dry milk and probed with anti-PCNA (PC10: Santa Cruz Biotechnology, Santa Cruz, CA, USA). Anti-GAPDH (Santa Cruz Biotechnology) was used as a loading control. Immunostained proteins were visualized using an ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical Analysis

We performed two or more independent sets of the experiments in triplicate, and each experiment was run at least three times. All data were analyzed using the SPSS program for

independent t tests. Differences with a p value <0.05 were considered to be statistically significant. Results are expressed as means ± SEM.

Results

Preparation of hLFP and Its Biological Activity

To prepare the hLFP by pepsin digestion of hLF, human colostral hLF was purified using CM-Sepharose column chromatography. Figure 1a shows the SDS-PAGE of the purified hLF. A single hLF band was obtained in the fraction numbers 5 to 10 . After collection and concentration of the fractions, a total of 9.25 mg of hLF was obtained from the 20 ml of human colostrum. The purified hLF was lyophilized and stored at −20 °C until necessary. To prepare hLFP, 10 mg/ml of the lyophilized hLF was incubated with pepsin as described in the Materials and Methods. After reaction, the pepsin was inactivated and removed from the reaction mixture. Finally, 4.2 mg of hLFP was obtained. Figure 1b shows the profile of the hLFP analyzed by HPLC. Lots of small peptides derived from hLF are shown. To confirm whether the hLF and hLFP preparations were biologically active, antibacterial activity was measured using the *S. aureus* as an indicator strain. Table 1 shows that both hLF and hLFP peptides exhibited strong antibacterial activities as evidenced by the decreased number of bacteria as the increase in the concentration. When target bacteria were incubated with 0.5 and 1.0 mg/ml of hLF for 3 h at 4 °C, the growth was inhibited by 49.6 and 84.2 %, respectively, while hLFP inhibited 72.5 and 94.7 %, respectively. These results indicated

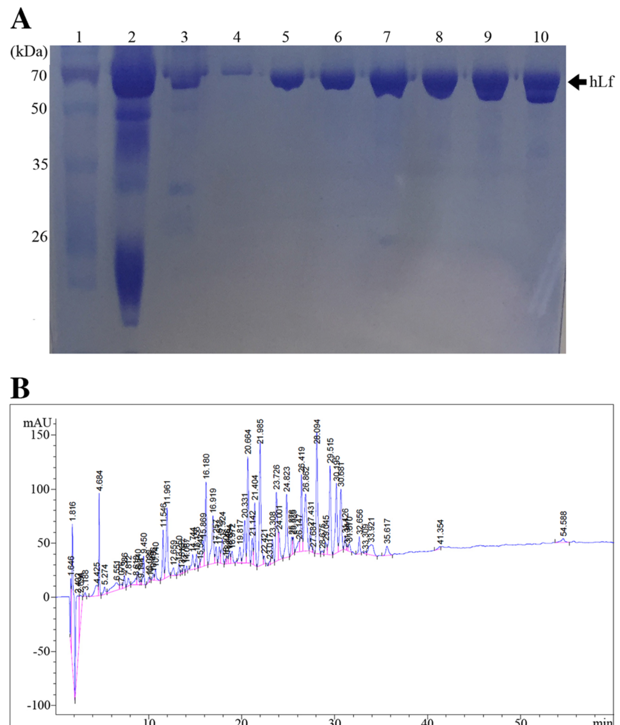


Fig. 1 Purification of hLF and HPLC pattern of hLFP prepared by pepsin digestion. hLF was purified to homogeneity from colostrum through CM-Sepharose column chromatography and then subjected to pepsin digestion for preparation of hLFP. **a** SDS-PAGE of purified hLF. For the separation of proteins, Laemmli-SDS-PAGE was performed with 12 % separation gel in the Tris-glycine buffer. *Lane 1* protein size marker, *lane 2* crude colostrum, *lane 3* flow-through, *lane 4* washout, *lanes 5~10* eluted fractions. **b** HPLC profile of hLFP prepared by digestion of the purified hLF with pepsin (see details in the text). Various sizes of peptides are shown

Table 1 Antibacterial activity of hLF and hLFP by colony counting assay

	Concentration (mg/ml)	Number of cells (CFU × 10^6)[a]	Antibacterial activity (%)[b]
S. aureus	–	24.4 ± 0.8	–
S. aureus + hLF	0.5	12.3 ± 1.0	49.6
	1.0	3.8 ± 0.3	84.2
S. aureus + hLFP	0.5	6.7 ± 0.4	72.5
	1.0	1.3 ± 0.3	94.7

[a] Target cells (*S. aureus*, 2.4 × 10^7 cells/ml) were incubated with the indicated concentration of hLF or hLFP at 4 °C for 3 h followed by spreading on the agar medium containing 10 g peptone/l. The total number of cells that appeared on the plate after overnight incubation was counted. All the values are the means ± SEM of the three separate experiments

[b] Antibacterial activity (%) is defined as follows: 100 − (number of cells survived / number of cells of *S. aureus* on the control plate) × 100

that the hLF and hLFP prepared in our study are biologically active. The hLF and hLFP preparations were subsequently used to examine their effects on the proliferation of epithelial cells.

Effects of hLF and hLFP on the Epithelial Cells

The effect of hLF and hLFP on the growth proliferation of two human epithelial cells, HEK293 normal epithelial cells and KATO III gastric carcinoma cells, was measured. The two cells were cultured with a wide range of concentrations of hLF or hLFP in the presence of 10 % FBS for 48 and 72 h, and cell proliferation was determined using the MTT assay. HEK293 cells began to proliferate in response to as low as 1 μg/ml hLF and 0.01 μg/ml hLFP, and the proliferation rates increased linearly with 1~100 and 0.01~100 μg/ml of hLF and hLFP, respectively (Fig. 2a). When KATO III cells were cultured with hLF and hLFP, they had proliferative responses linearly to 0.01~100 and 0.0001~0.1 μg/ml for hLF and hLFP, respectively (Fig. 2b). Therefore, hLFP promoted the proliferation of both cell types at lower concentrations by a factor of approximately 2 compared to hLF. Additionally, the carcinoma cells responded to approximately 100-fold lower concentrations of hLF and hLFP for proliferative responses than the normal epithelial cells. Notably, HEK293 cells continued to proliferate through 72 h in response to hLF and to a lesser extent hLFP, whereas the numbers of KATO III cells was decreased at 72 h, compared to 48 h. The decrease in the growth yield of KATO III cells with the longer culture period appeared reasonable because the cancer cells (KATO III cells) generally had more rapid growth kinetics than normal cells (HEK293 cells) and therefore experience earlier depletion of essential nutrients.

Characterization of Cell Growth Proliferation Effect by hLF and hLFP

Next, we examined whether the proliferative effects of hLF and hLFP were consistent with the expression of a proliferation marker. HEK293 or KATO III cells were cultured with a broad range of concentrations of hLF or hLFP in the presence of 10 % FBS for 48 h, and the expression of PCNA, a universal proliferation marker, was determined. PCNA expression of HEK293 cells was increased linearly at concentrations of 10~1000 and 0.1~100 μg/ml for hLF and hLFP, respectively (Fig. 3a). A similar pattern was observed in KATO III cells. Upon

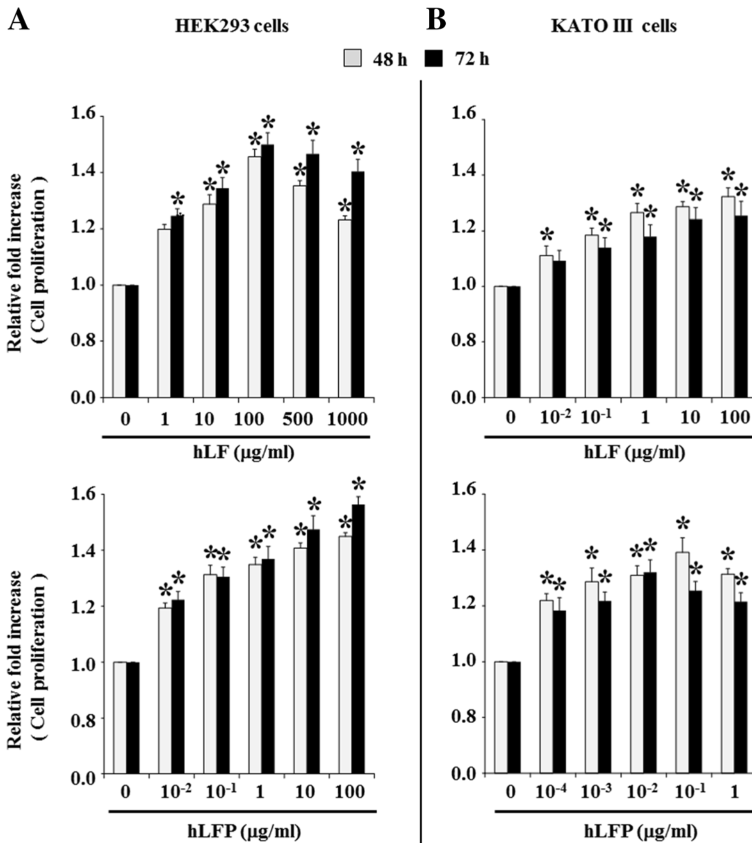


Fig. 2 Effects of hLF and hLFP on the proliferation of epithelial cell lines. HEK293 cells (**a**) and KATO III cells (**b**) were seeded at 1.5×10^4 cells/well on a 96-well plate and treated with varying concentrations of hLF and hLFP in the presence of 10 % FBS for 48 and 72 h. Cell proliferation was determined using the MTT assay, as described in the Materials and Methods. Data are shown as the mean ± SEM of four independent experiments performed in triplicate and expressed as a fold increase (*$p < 0.01$; **$p < 0.05$ compared to cells that were cultured in medium alone)

exposure of the cancer cells to hLF and hLFP, PCNA expression increased at 0.1 and 0.001 μg/ml for hLF and hLFP, respectively, and reached a plateau at approximately 10 and 1 μg/ml for hLF and hLFP, respectively (Fig. 3b). PCNA expression nearly perfectly matched the cell proliferation in response to hLF and hLFP; the upregulation of PCNA by hLFP was observed at lower concentrations by approximately two orders of magnitude compared to hLF in normal and cancer cell lines. These data also showed that KATO III cells exhibited the proliferative response to lower concentrations of hLF and hLFP than HEK293 cells.

Effects of FBS on the Cell Growth Proliferation by hLF and hLFP

We examined whether hLF or hLFP alone promoted cell proliferation or served auxiliary functions under a strong growth-stimulating signal, such as FBS. When HEK293 cells

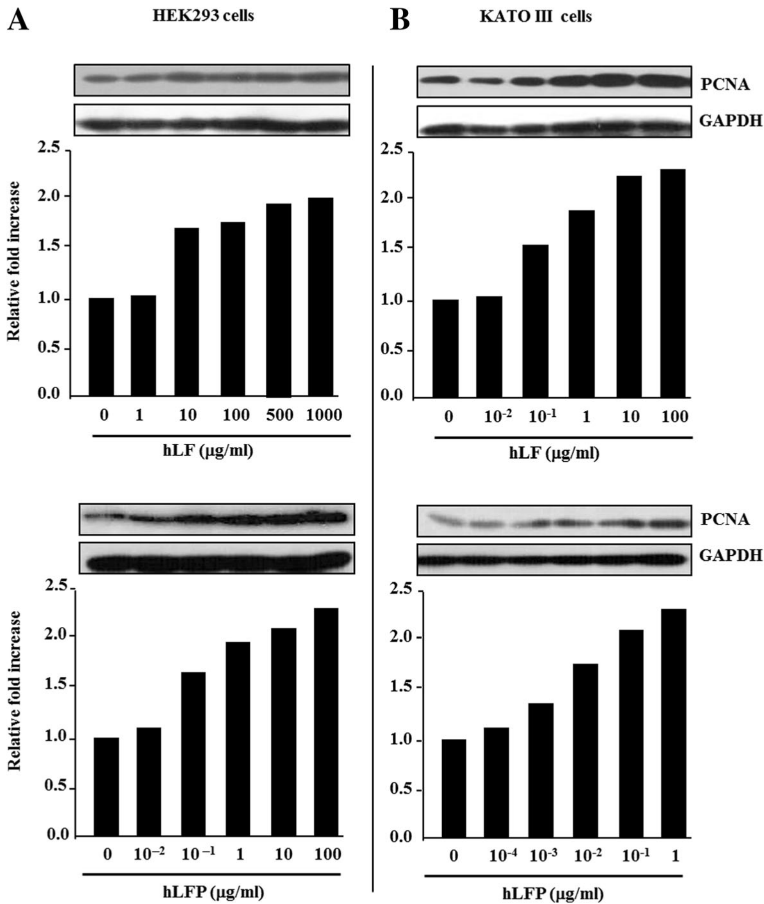
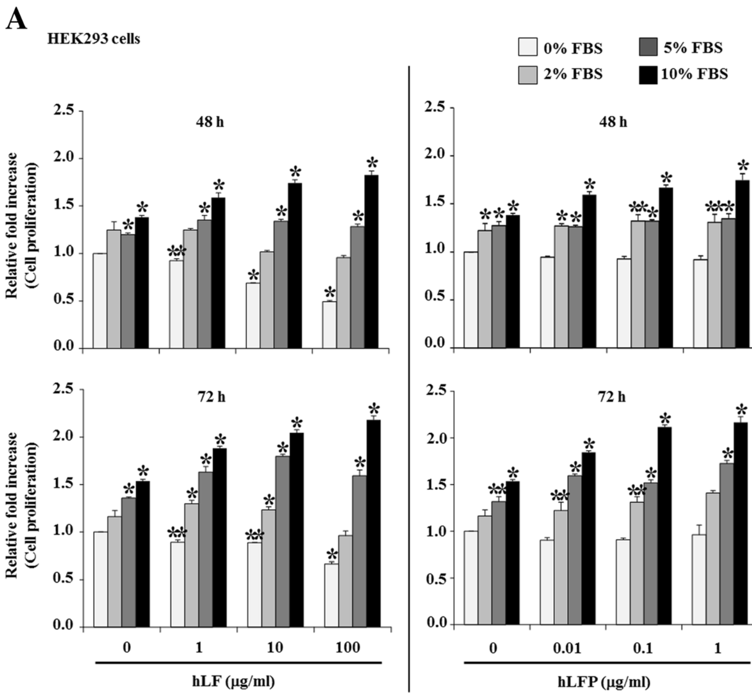
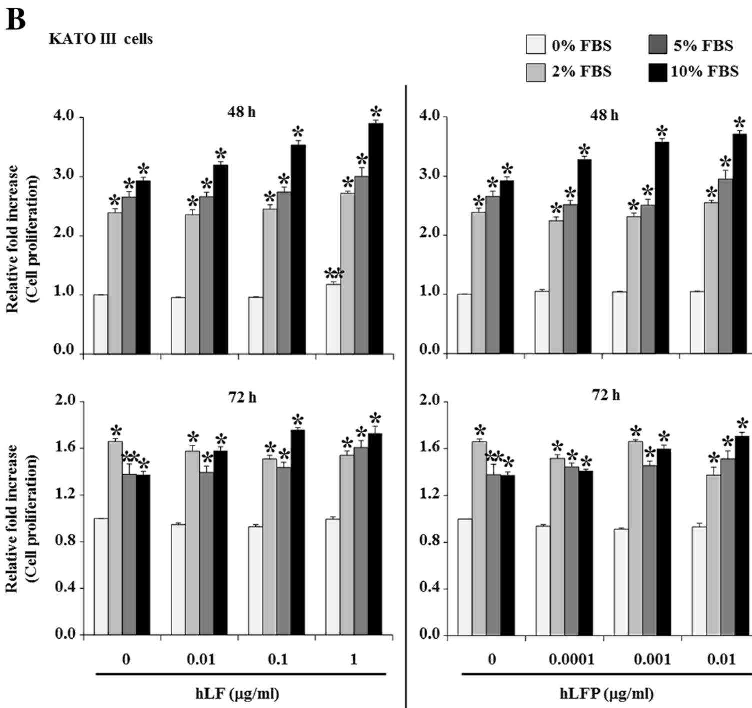


Fig. 3 Effects of hLF and hLFP on PCNA protein expression. HEK293 cells (**a**) and KATO III cells (**b**) were treated with varying concentrations of hLF or hLFP in the presence of 10 % FBS for 48 h and analyzed for PCNA protein expression using immunoblot analysis. GAPDH was used to normalize the levels of PCNA. Intensities were analyzed using densitometry. PCNA expression in cells cultured with hLF or hLFP was divided by GAPDH expression, and the values are expressed as a relative expression by comparing with cells that were cultured with medium alone. The blots shown are representatives of two independent experiments

were cultured with hLF in the presence of different concentrations of FBS for 48 h and 72 h, hLF had a pronounced effect on cell proliferative responses only in the presence of 10 % FBS and tended to have a modest effect in the presence of 5 % FBS (Fig. 4a). In contrast, hLF did not promote cell proliferation with 2 % FBS and decreased cell numbers at 0 %

Fig. 4 The effect of hLF and hLFP on the cell proliferation in the absence or presence of FBS. HEK293 cells (**a**) and KATO III cells (**b**) were seeded at 1.5×10^4 cells/well on a 96-well plate and treated with hLF or hLFP in the presence of 0, 2, 5, and 10 % FBS for 48 and 72 h. Proliferation of cells that were cultured with hLF or hLFP in the different FBS contents was compared with cells that were cultured with medium alone in the presence of 0 % FBS. Data are shown as the mean ± SEM of two independent experiments performed in triplicate and expressed as a relative fold increase (*$p < 0.01$; **$p < 0.05$ compared to cells that were cultured in medium alone)


A
HEK293 cells
0% FBS
2% FBS
5% FBS
10% FBS
48 h
72 h
Relative fold increase (Cell proliferation)
0
1
10
100
hLF (μg/ml)
0
0.01
0.1
1
hLFP (μg/ml)


B
KATO III cells
0% FBS
2% FBS
5% FBS
10% FBS
48 h
72 h
Relative fold increase (Cell proliferation)
0
0.01
0.1
1
hLF (μg/ml)
0
0.0001
0.001
0.01
hLFP (μg/ml)

FBS. A similar pattern was observed with hLFP. hLFP significantly promoted cell proliferation at 10 % FBS, and it did not significantly support proliferation at concentrations less than 5 % FBS (Fig. 4a). When KATO III cells were cultured with hLF or hLFP in varying concentrations of FBS for 48 h, their growth was linearly proportional to increasing doses of hLF or hLFP only in the presence of 10 % FBS (Fig. 4b). KATO III cells proliferated in response to these two agents but the dependency of this cell growth to hLF or hLFP decreased in the presence of 2 or 5 % FBS (Fig. 4b). Longer culture (72 h) of KATO III cells was detrimental to cell growth probably due to nutrient deficiency. These two agents did not support FBS-dependent cell growth without a clear coherence of their dose effect (Fig. 4b). This FBS-dependency was observed at their highest concentrations used (1 and 0.01 μg/ml for hLF and hLFP, respectively) (Fig. 4b).

Bovine serum albumin (BSA) and their pepsin-digestive product, bovine serum albumin peptide (BSAP), were used as controls for hLF and hLFP, respectively, to verify the specificity of the cell growth-supplementing activities of hLF and hLFP. When two cell types were incubated with

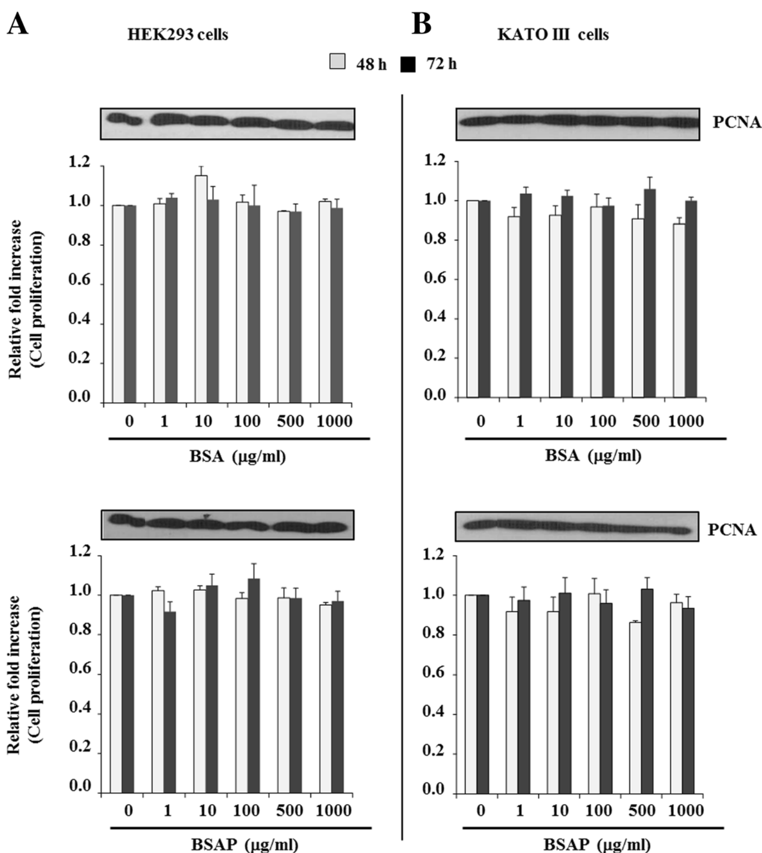


Fig. 5 Effects of BSA and BSAP on cell proliferation and PCNA expression. HEK293 cells (**a**) and KATO III cells (**b**) were seeded at 1.5×10^4 cells/well on a 96-well plate and treated with BSA or BSAP in the presence of 10 % FBS for 48 and 72 h. Proliferation of cells that were cultured with hLF or hLFP was compared with cells that were cultured with medium alone. Data are shown as the mean ± SEM of two independent experiments performed in triplicate. In parallel, western blot analysis was performed for PCNA expression, which is essentially not altered by varying concentrations of BSA or BSAP

varying concentrations of BSA or BSAP for 48 h, their proliferative responses remained unaltered to the concentrations examined (Fig. 5). In parallel, these two agents essentially had no effect on PCNA expression at the range of concentrations used for proliferation assay. These results reinforce the specificity of the cell growth-supplementing activities of hLF or its proteolytic product hLFP.

Discussion

The present study clearly demonstrates the promoting effect of hLF and its pepsin-lysate hLFP on the human normal epithelial cell line, HEK293, and the gastric cancer cell line KATO III, in culture. Consistent with our results, many previous studies revealed that hLF served as a growth factor to promote the proliferation of mammalian cells [7–16]. However, other studies showed that proliferation is not affected by hLF in some cells [19, 23, 32–34] or even that hLF has an inhibitory effect on cell proliferation in some cells [20–23]. Therefore, the effect of hLF on cellular proliferation is controversial. The reason for the conflicting effect of hLF is not clear, but the effect of hLF on the cell growth appeared to be dependent on the cell types, doses used to test, and both the endogenous and exogenous co-stimulating or co-inhibiting factors in the cells and culture medium [24]. Additionally, the preparation method and the status of hLF could be another possible reason [35].

Our results compared the proliferation-promoting effect of hLF versus hLFP and the effect on normal epithelial cells versus carcinoma cells. The activity of hLFP to promote proliferation was more robust than hLF in both cell types at low doses by a factor of 2. In addition, KATO III human gastrocarcinoma cells were much more sensitive to these two agents than HEK293 normal epithelial cells by two to three orders of magnitude. The effects of these two agents were prominent in the presence of FBS, as our results showed that higher FBS concentrations stimulated more growth-promoting effect. In contrast, the effects of hLF and hLFP were reduced severely at low concentrations of FBS, and these effects were minimal in the absence of FBS. In general, cell growth and proliferation can be activated by both intrinsic and extrinsic factors. The latter includes, most notably, extracellular growth factors that bind to and activate cell surface tyrosine kinase receptors. The tyrosine kinase receptors constitute the most principal axis for cell proliferation. FBS contains such growth factors, such as insulin-like growth factor, basic fibroblast growth factor, transforming growth factor, etc. [36], most of which strongly activate tyrosine kinase receptors. Either hLF or hLFP alone is unable to trigger or initiate cell proliferation in our cell models. It indicates that these two molecules cannot activate the tyrosine kinase receptors or functionally equivalent receptors that presumably initiate to drive cell proliferation. However, hLF has been shown to activate signaling molecules related to cell proliferation. For instance, the bovine LF can modulate the p44/p42 mitogen-activated protein kinase (MAPK) in fibroblast and epithelial cells derived from intestine [15] and mammalian gland [37]. Thus, the modulatory activity of MAPK by LF can contribute to its proliferation capacity, although it seems that the MAPK activation is necessary, but not sufficient, for cell proliferation. Alternatively, it is possible that the proliferative activity of hLF and hLFP can be attributable to the activation of proliferative factors or inactivation of inhibitory factors in FBS in both specific and nonspecific manners. Preincubation of hLF and hLFP with FBS helps address this possibility, although there is no available evidence that LF affects soluble proteins in FBS. The strict dependency of their proliferation-promoting effects on FBS concentrations suggests that hLF and hLFP themselves do not serve as an excellent trigger to drive cell proliferation. Rather, they serve as supplements to support the proliferation that is initiated by growth factors in FBS. The specificity of their

growth-supporting activity is corroborated because BSA and its pepsin-digestive product (BSAP), which were used as controls for hLF and hLFP, had no effect on cell proliferation and PCNA expression (Fig. 5), suggestive of hLF and hLFP as authentic growth supplements.

Two mechanisms were proposed to explain the growth proliferation activity of hLF. One mechanism is that hLF serves as an iron transferor that regulates the expression of genes involved in cell proliferation. This hypothesis is based on the fact that the holo-hLF shows a stronger growth promotion effect than apo-hLF. The ability of hLF to uptake iron and deliver it into cells might stimulate proliferation [38, 39]. However, our results showed that hLFP, which unlikely uptakes iron, also exerted strong growth-promoting activity, which implies that the ability of hLF to promote cell growth is not always associated with its binding and transferring activity of iron. These facts suggest the presence of different mechanisms to stimulate cell growth.

The other mechanism is receptor-associated transportation of hLF to the nucleus to interact with DNA [40, 41], which is based on the fact that both apo- and holo-LF stimulate the proliferation of cells [15]. Alternatively, hLF directly binds Toll-like receptor 4 (TLR4) and subsequently activates nuclear factor-κB (NF-κB) [42], which acts as the master regulator of cell proliferation and inflammation [43]. Although a molecular detail of the interaction of hLF with TLR4 remains to be established, it seems that intact hLF is required to bind TLR4. In contrast, the pepsin-digestive product hLFP is highly unlikely to bind its cognate receptor on the cell surface. Therefore, although the hLFP had positive effects of cell proliferation and PCNA expression, the mode of action of hLFP appears independent of hLF, if the effect of hLF is associated with receptor-binding. Therefore, the receptor model cannot explain the effect of hLFP. It would be interesting to see whether hLF exerts its function (cell proliferation) under conditions where TLR4 signaling is blocked.

hLF has been investigated extensively because of its antibacterial, antiviral, antifungal, and anticancer activities. In addition to these therapeutic activities, its growth-complementing or growth-promoting activity constitutes an important aspect for its commercial feasibility. For example, recombinant rice hLF is used as a culture ingredient to achieve high yield of hybridoma cells that produce monoclonal antibodies [39]. Our results strongly support the feasibility of hLF as a useful medium additive for the elevated growth of epithelial cells. Additionally, hLFP, the pepsin-hydrolysate of hLF, is a better growth stimulator for this cell type. We recently developed a recombinant hLF that is produced from *Pichia pastoris*. The recombinant hLF and its pepsin-hydrolysate have similar activities as native hLF (data not shown). Therefore, the use of hLF and hLFP could be a cost-effective way to use valuable therapeutic proteins by devising a mass scale culture system.

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Conflict of Interest The authors declare that they have no competing interests.

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