ANTI-INFLAMMATORY AND ANTI-TUMORAL ACTIVITY OF HUMAN RECOMBINANT LACTOFERRIN

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(Received October 19, 2012)

Lactoferrin (Lf) is an iron-binding glycoprotein which exhibits a variety of properties, including anti-inflammatory and anti-tumoral activity. The recombinant Lf (rLf) retains the activities of the native protein, being in many cases even more active. Given their biological effects, Lf and rLf are potential useful compounds for pharmaceutical and food-related applications. Due to the difficulty in obtaining high amounts of human milk Lf (hLf), different methods have been developed to obtain human recombinant Lf.

In a previous study, the capacity of hLf to inhibit the LPS-induced IL-6 secretion in a human monocytic cell line THP-1 has been reported. Our aim was to extend this investigation by evaluating the anti-inflammatory activity of recombinant hLf (rhLf, Ventria, Bioscience) in this experimental model of inflammation and to compare its effect with those obtained with native protein. We found that hrLf inhibited the secretion of LPS-induced pro-inflammatory cytokines TNF-α, IL-6 and IL-8. The level of inhibition was similar to that obtained in the presence of native hLf, regardless whether rhLf was added to the cells before or after the stimulation with LPS. The iron content of protein did not affect its anti-inflammatory capacity.

We have also investigated the effect of iron-free hrLf (Apo hrLf) on the growth, morphology and cell cycle of melanoma B16-F10 cell line, used as an *in vitro* model of metastasis in skin cancer. Apo hrLf induced changes in the cell morphology and a decrease in the S (DNA synthesis) phase of the cells from 14% to 1%, suggesting that B16-F10 DNA synthesis was affected in the sample treated with recombinant protein.

The results are encouraging for a possible future application of hrLf as anti-inflammatory and anti-tumoral agents.

Key words: lactoferrin, anti-inflammatory, anti-tumoral.

INTRODUCTION

Lactoferrin (Lf) is an iron-binding glycoprotein situated in the frontline of host defense and may be considered a marker for inflammation due to the fact that its

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level is increasing during inflammation and in some pathology (1-4). A variety of properties including anti-inflammatory (5), and anti-tumoral activity, protection against metastasis (6), involvement in iron homeostasis (7), DNA binding and transcriptional activation (8, 9), and anti-bacterial activity (10) have been described so far.

ANTI-INFLAMMATORY ACTIVITY

In some inflammation processes, the bacterial endotoxin lipopolysaccharide (LPS) stimulation of macrophages activates signaling pathways. LPS interacts with specific receptors which trigger activation of MAPKinases and NF-κB transcription factor. All these events lead to pro-inflammatory cytokines production (11).

Lf was reported to modulate the inflammatory response by its capacity to bind bacterial LPS and LPS binding protein (12, 13), leading to down-regulating effects on both the activation and recruitment of immune cells in inflamed tissues. Previous studies have reported the capacity of Lf to greatly reduce LPS-induced cytokines production in monocytes from blood and THP-1 promonocytic cell line, through different mechanisms suggesting Lf binding at the cell surface (14), internalization (15), nuclear localization and interference with the nuclear factor NF-κB and/or MAPK pathway activation (16-18).

ANTI-TUMORAL

A number of studies have reported the anti-tumoral activity of Lf, a property mediated by a variety of mechanisms such as inhibitory effects on tumor cell growth, NK cell activation, inhibition of VEGF-mediated angiogenesis and enhancement of apoptosis (3, 4, 19, 20).

RECOMBINANT LF

Recent studies have shown the benefit of using recombinant Lf (rLf) in fibroblast proliferation and wound repair (21), bacterial infections (22-24), iron deficiency and diarrhea (25) and tumours (26). Given its biological effects, rLf is a potential useful compound for pharmaceutical and food-related applications (23, 27). Due to the difficulty in obtaining high amounts of human milk Lf (hLf), different methods have been developed to obtain human recombinant Lf (hrLf) (28-33).

Our aim was to extend this investigation by evaluating the *in vitro* anti-inflammatory and anti-tumoral activity of hrLf. The effect was compared to that obtained with native protein.

We found that hrLf inhibited the secretion of LPS-induced pro-inflammatory cytokines TNF-α, IL-6 and IL-8 in THP-1 promonocytic cell line. Similar with hLf treatment, the inhibitory effect was observed regardless whether hrLf was added to

the cells, before or after the stimulation with LPS. Notably, the iron content of protein does not affect its anti-inflammatory capacity.

Using murine melanoma cells as an *in vitro* model of metastasis in skin cancer, we have demonstrated the antitumoral effect of Apo hrLf on the growth and morphology of melanoma B16-F10 cell line. FACS analysis of cell cycle progression showed that the DNA synthesis was dramatically affected in the sample treated with Apo hrLf.

All these data suggest the possibility of using hrLf in anti-inflammatory and anti-tumoral therapeutic strategies.

MATERIAL AND METHODS

REAGENTS

Human native lactoferrin (hnLf) was purchased from (Sigma) and recombinant human lactoferrins Apo – iron free –, Holo – fully saturated – and Assi – 50% iron saturated- (hrLf) were kindly provided by Ventria Bioscience. The purity of protein was checked by SDS-PAGE and was higher than 90%. Contaminating endotoxin (lipopolysaccharide, LPS) was removed using Detoxi-gel (Pierce Chemical, Co). The endotoxin content of Lf preparations was less than 3 EU/ml (250 ng LPS/mg protein) as measured by the Limulus Amoebocyte Lysate (LAL) assay (Sigma Chemicals, St Louis, MO, USA).

CELLS

Human promonocytic cells THP-1 (ATCC TIB 202) and murine melanoma cells, B16-F10 (ECACC, Porton Down, UK) were routinely cultured in RPMI-1640 medium (EuroClone) supplemented with 5% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Biochrom). Cells were used for experiments while in the log phase of growth.

CELL PROLIFERATION AND VIABILITY

Cells were seeded on 96-well plates at a density of 8 x 10^3 cells/well in a RPMI medium and a non-radioactive proliferation assay (CellTiter96 AQueous Non-Radioactive cell proliferation assay, MTS, Promega) was performed for cell viability. Briefly, cells were incubated with either medium or different concentrations of hrLf or hnLf or (0-1000 μg protein/ml) for 24 h. Then 20 μL of MTS were added to the wells and the plate incubated for 1 h at 37°C. The amount

of formazan product, measured as the absorbance at 450 nm, is directly proportional to the number of living cells in culture. All experiments were run in triplicate. The inhibitory concentration (IC_{50}) was determined and expressed as the concentration of compound which inhibited cell proliferation of 50% of the viable cells. Cell viability was assessed in parallel by the Trypan Blue method after cell dispersion with trypsin.

CYTOKINE ASSAY

The levels of TNF-α, IL-6 and IL-8 released by macrophage-differentiated cells THP-1 stimulated with LPS (*E. coli* serotype 055:B5, 100 ng/ml) as described previously (18) untreated or treated with hnLfs or hrLf samples were analyzed by capture Duo-ELISA using antibodies pairs and protocols recommended by R&D system.

CELL MORPHOLOGY

The morphology of $5x10^4$ B16-F10 cells untreated or treated with 500 µg/ml Apo hrLf was examined by light microscopy with a Nikon Eclipse TS100 microscope. Images (x 20) were captured with a NIS Elements software controlled digital camera.

CELL CYCLE ANALYSIS

Cells ($2x10^4$ cells/well in 24 well plates) were incubated for 24 h at 37^0C with Apo hrLf at a concentration of protein corresponding to the IC_{50} (500 µg/mL). After 24 h, cells were washed, fixed with 100% ethanol for 2 h at 4^0C, washed again and than labeled with propidium iodide (PI, 50 µg/mL). Cell cycle distribution was determined by flow cytometry using a FACS Calibur (BD, Bioscience). Data were recorded on a logarithmic scale and a minimum of 10 000 events was collected for each sample. The gating strategy excluded dead cells in FSC/SSC dot plot and the percentage of cells in G0/G1, S and G2/M cell cycle phases was determined statistically. Analysis of data was performed with CellQuest Pro Software program (BD Biosciences).

RESULTS AND DISCUSSION

Given the beneficial effects of Lf, in the past few years there have been a broad interest in exogenous sources of Lf for therapeutic and nutrition approaches. Several transgenic approaches to produce Lf have been developed: hLf can now be

obtained from yeast, transgenic cows, and rice. The rLf has to meet all the important criteria: structural similarity, high yield, and ease of protein isolation. However, the activity of heterologous Lf cannot be assumed to identically mimic that of the homologous protein. Human protein from *Aspergillus awamori* has been mainly directed to therapeutic approaches with advanced phases of clinical trials currently being in progress. In contrast, hLf produced in transgenic cows and rice has brought the important advantage of origins compatible with use in foods.

Previous studies have shown that native hLf possesses anti-inflammatory capacities by reducing the amount of pro-inflammatory cytokines secretion in human monocytic cells (16, 18). Therefore, we wanted to study the anti-inflammatory activity of hrLf.

In our studies, THP-1 cells stimulated with 100 ng/mL LPS were used as experimental model of inflammation. We found that hrLf inhibited the secretion of LPS-induced pro-inflammatory cytokines TNF-α, IL-6 and IL-8 (Fig. 1 A-F). The level of inhibition of these cytokines by hrLf (~50%) was similar to the one resulted from treatment with native hLf (~40%). As for native protein, the inhibitory effect was observed regardless whether hrLf was added to the cells, before or after the stimulation with LPS. However, in the case of TNF-α inhibition, a slight more potentiating effect was observed when Holo hrLf or As-si hrLf was added before LPS stimulation (25% and 30% respectively) (Fig. 1F). The iron content of protein does not greatly affect its anti-inflammatory capacity, since the level of inhibition of cytokine release obtained with Apo hrLf resembles the level obtained with Holo hrLf or As-si hrLf.

Our results are in agreement with previous studies performed by us and others with hLf on inflammation model in monocytic cells (17, 18).

An increase in number of studies shows the benefit of hnLf and hrLf treatment in tumours *in vitro* and on animal model *in vivo* (25, 26, 31, 34, 35).

We have investigated the effect of Apo hrLf on the growth and morphology of murine melanoma B16-F10 cell line, used as an *in vitro* model of metastasis in skin cancer. The cytotoxicity experiments performed with different concentrations of Apo hrLf (0-1000 μg/mL) revealed a decreased of B16-F10 melanoma cells proliferation by ~ 50% when cells were incubated for 24 h at 37 °C in the presence of 500 μg/mL Apo hrLf (data not shown).

We further investigated the effect of Apo hrLf on B16-F10 melanoma cells morphology and cell cycle progression. Our experiments on light microscopy revealed that Apo hrLf induced dramatic morphological changes. As opposed to tumor cells that did not receive the treatment and exhibit normal morphology features, the cells incubated with Apo hrLf became round and floating, often agglutinated, suggesting that apoptosis was induced (Fig. 2).

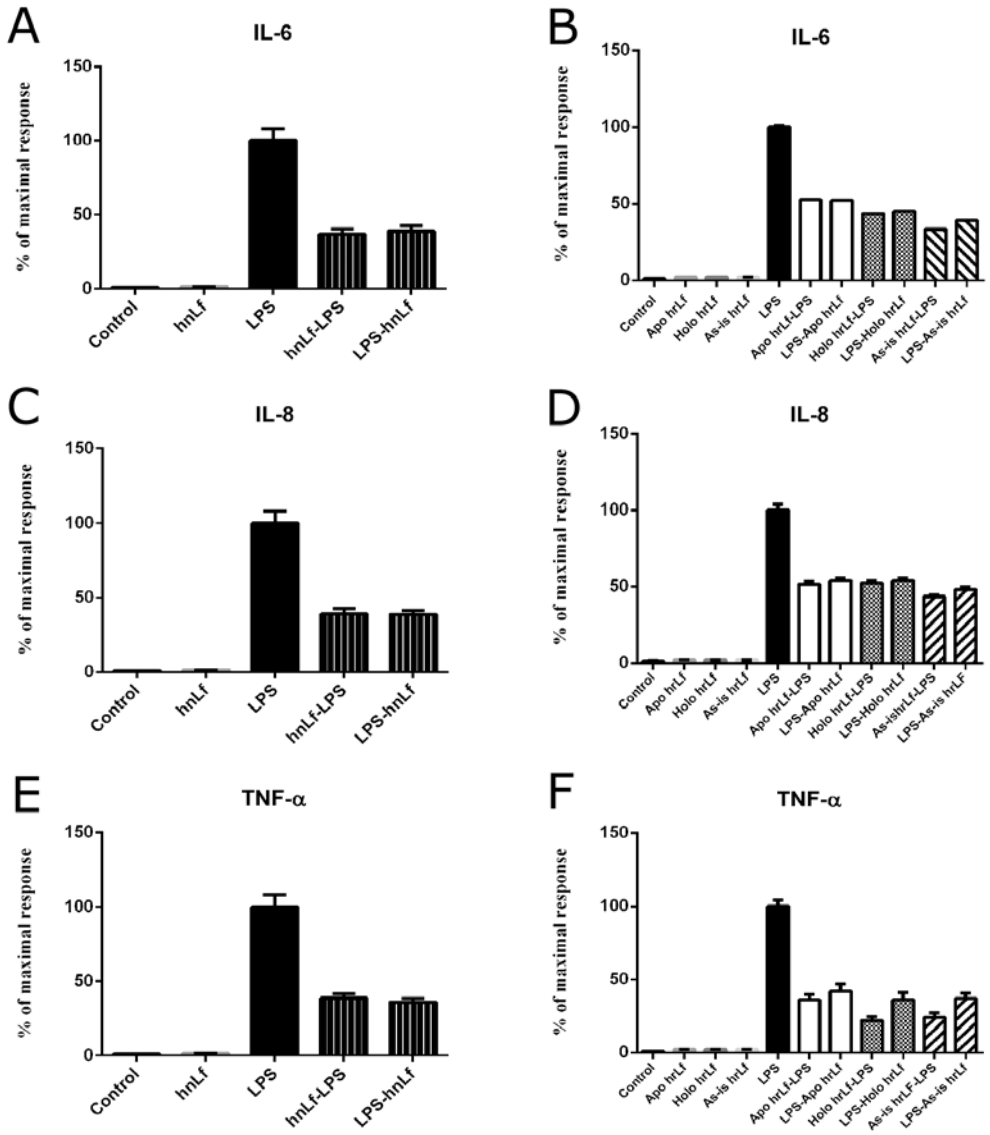


Fig. 1. – The effect of hnLf and hrLf on the LPS-induced pro-inflammatory cytokine secretion in THP-1 cells. Cells were treated with hnLf (A, C and E) or hrLf (B, D and F) (400 μg/mL) for 30 min before or after LPS stimulation (100 ng/ml) for 18 h. The IL-6, IL-8 and TNF-α secretion in the cell supernatant was assayed by ELISA. The results are expressed as percentage of maximal response (cells stimulated with LPS only). The data are mean of three independent experiments.

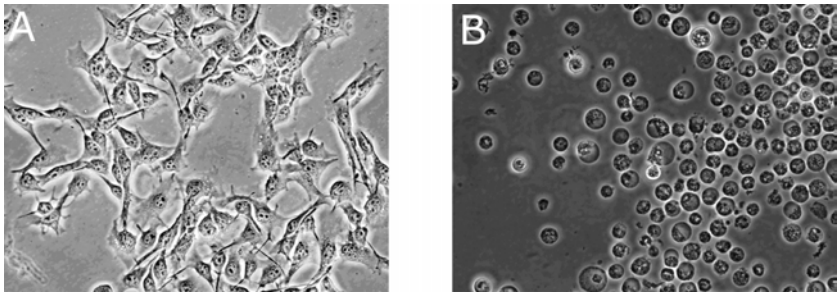


Fig. 2. – Morphological changes induced by Apo hrLf in B16-F10 melanoma cells. Cells attached on coverslips were cultured for 24 h in the absence *(A)* or the presence of Apo hrLf *(B)* (500 µg/mL). At the end of incubation time, cells were examined by light microscopy.

The regulation of cell growth by Lf has been intensely studied (3, 4, 9, 20, 36). Usually such activity may involve binding and internalization of Lf followed by activation of different signaling pathways.

To further investigate the effect of Apo hrLf on B16-F10 cell proliferation, cell cycle progression analysis was performed by flow cytometry. After melanoma cells were incubated for 24 h with Apo hrLf at a protein concentration corresponding to the IC_{50}, FACS analysis of cell cycle distribution revealed a decrease in the S (DNA synthesis) phase of the cells from 14% to 1%, suggesting that B16-F10 DNA synthesis was affected in the sample treated with Apo hrLf (Fig. 3).

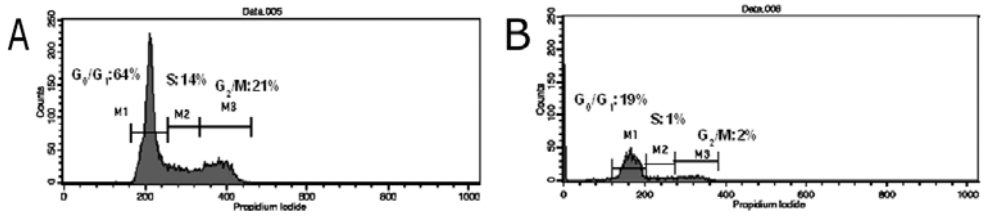


Fig. 3. – FACS analysis of cell cycle progression after Apo hrLf treatment of B16-F10 melanoma cells. Cells, both untreated *(A)* and treated for 24 h with 500 µg/mL Apo hrLf *(B)*, were stained with propidium iodide and then analyzed by flow cytometry. Numbers indicate the percentage of cells in the different phases of the cell cycle.

CONCLUSION

The recombinant protein decreased the pro-inflammatory cytokine response in an *in vitro* model of inflammation. Also, hrLf in its iron desaturated form inhibited cell proliferation and cell cycle progression in an *in vitro* model of metastatic melanoma.

The results are encouraging for a possible future application of rhLf as anti-inflammatory and anti-tumoral agents.

Acknowledgments. This work is part of IBAR project 4/ 2012. The authors would like to express their acknowledgment to Ventria Bioscience for providing the recombinant Lf for this study.

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