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Evaluation of the cytoprotective effects of bovine lactoferrin against intestinal toxins using cellular model systems

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Abstract Lactoferrin is an iron-binding glycoprotein that exhibits a range of health benefits including immune regulation and disease prevention derived from its structural properties. The present study employed immune cell models and a colon epithelial cell model to investigate the protective effects of bovine lactoferrin (BLf) on both immune cells and colon epithelium cells. BLf caused significant reduction of faecal genotoxin-induced DNA damage in HT29 cells, and down-regulation of lipopolysaccharide (LPS)-induced macrophage cell stress and endotoxic response, in an infection status.

Keywords Bovine lactoferrin · Cellular model system · DNA damage · Lipopolysaccharide · Faecal water

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Introduction

Lactoferrin is a multifunctional glycoprotein which may have beneficial preventive and therapeutic effects on infection, inflammation, and cancer as well as enhancing the iron status and growth of vulnerable groups (Lonnerdal 2009).

Lactoferrin has demonstrated an endotoxin-neutralizing capability in vitro and in vivo (Zhang et al. 1999). In addition, the preventive effects of BLf on colon carcinogenesis have been observed in animal models (Sekine et al. 1997; Tsuda et al. 2002). However, there are still many gaps in our knowledge of the detailed molecular mechanisms involved, including the binding interaction of Lf to intestinal toxins such as genotoxins and endotoxins, and on the locations of receptors associated with the immune response.

In light of these questions on the mechanisms of lactoferrin biological activity, the human and animal cellular model approach was considered appropriate to study the immunomodulatory and antitumor activities of BLf, with specific focus on the cellular level reduction of DNA damage and immune cell stress.

Materials and methods

Bovine lactoferrin (BLf)

BLf (NZMP7100) was kindly supplied by Fonterra Co-operative Group, New Zealand. In this product

the lactoferrin content is >90% of protein, and iron saturation is 10–20%. The pH value (5% solution at 20°C) is 5.5–6.5. Bacterial analysis is negative.

Immune cell models

Murine macrophage RAW 264.7 cell line was provided by Bioactives Research New Zealand (Auckland, New Zealand). Cells were incubated with 1 μg/ml of LPS with and without BLf for 24 h at 37°C in a 5% CO_2 humidified atmosphere (Cell culture CO_2 incubator: *SANYO*, Japan). This model was used to evaluate any relationships between inflammatory mediators and cell growth responses in the presence of LPS (from *E.coli* O127:B8, Sigma, USA) and BLf.

Human acute monocytic leukemia THP-1 cell line was provided by Plant and Food Research Ltd. (Auckland, New Zealand). Cells in concentration of 1 × 10^6 cells/ml were differentiated using 10 nM Phorbol myristrate acetate (PMA) (Sigma, USA) for 72 h. The differentiated THP-1 cells were then stimulated with 100 ng/ml of LPS at 37°C in a 5% CO_2 humidified atmosphere for 4 h. This model was used to test the inflammation responses of LPS during incubation with and without BLf. The TNF-α production was determined using Enzyme-linked immunosorbent assay (ELISA) (Park et al. 2007).

Colon carcinogenesis model

The human colon carcinoma cell line HT29 was obtained from the School of Medical Sciences (The University of Auckland, Auckland, New Zealand). HT29 cells were harvested and exposed to a faecal water sample in the presence of BLf. The Comet assay (Glei et al. 2006; Venturi et al. 1997) was performed to measure the antigenotoxic activity of lactoferrin on faecal water-induced DNA damage in HT29 cells.

Human faecal water preparation

Faecal samples were obtained from a healthy non-vegetarian, non-smoking female with no history of gastrointestinal disease, consuming a mixed diet. Faecal water was prepared as described elsewhere (Klinder et al. 2004).

Statistical analysis

Data were processed on an Excel spreadsheet. Results were expressed as means ± SD, the significance of differences was determined using ANOVA.

Results and discussion

Protective effects of bovine lactoferrin on LPS-induced murine macrophage cell stress

Cell proliferation and nitric oxide production were determined using MTT and Griess assays in the RAW 264.7 cell model. The results (Fig. 1) showed that bovine lactoferrin alone (1.25 mg/ml) did not influence murine macrophage cell stress or production of nitric oxide. In contrast, exposure of RAW 264.7 cells to LPS (1 μg/ml) at 37°C for 24 h significantly increased nitric oxide production and reduced cell proliferation (Fig. 1a). However, BLf at the concentration of 0.02–1.25 mg/ml significantly ($P < 0.05$) down-regulated nitric oxide production, or protected against LPS-induced immune cell stress, in a dose response manner (Fig. 1b).

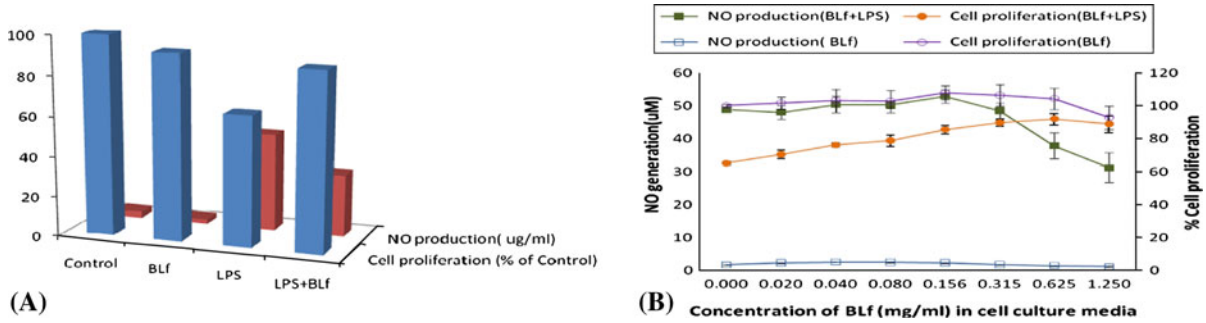

Fig. 1 **a** The effects of BLf or LPS and their combination on macrophage cell proliferation and nitric oxide production. **b** Dose responses of BLf on reduction of LPS-induced nitric oxide production and cell proliferation in macrophages

Effects of bovine lactoferrin on TNF-α production in LPS-induced THP-1 cells

To evaluate the endotoxin-neutralizing capability of lactoferrin by regulating production of pro-inflammatory cytokine TNF-α, BLf or bovine serum albumin (BSA), at concentrations of 0.125–2 mg/ml, were added to LPS-challenged (100 ng/ml) differentiated THP-1 cells. BLf, but not BSA, significantly reduced TNF-α release from LPS-activated THP-1 cells dose-dependently (Fig. 2a). Furthermore, to judge whether BLf blocks LPS receptors on THP-1 cells or detoxifies LPS by binding it directly, pre-treated THP-1 cells that were incubated with BLf for 1 h prior to challenge with LPS for 4 h were compared with BLf and LPS co-treatment for 4 h at 37°C in a 5% CO_2 humidified incubator. Figure 2b shows that a significant reduction in TNF-α production occurred during BLf plus LPS co-treatment rather than during BLf pre-treatment. These results confirm that the key reaction mediated by BLf was not directly on the THP-1 receptors; rather, the anti-inflammation actions were mediated by neutralization of endotoxin LPS.

Antigenotoxic effects of bovine lactoferrin on faecal water-induced colon epethelium DNA damage

The possibility of lactoferrin having antigentoxic properties on faecal water-induced DNA damage in HT29 cells was determined by measuring the DNA tail moment in the Comet assay. BLf (0.02–20 mg/ml) was incubated with faecal water at 37°C for 30 min prior to exposure to HT29 cells. The Comet images (Fig. 4) showed that lactoferrin, at 20 mg/ml and 10 mg/ml, significantly reduced faecal genotoxins-

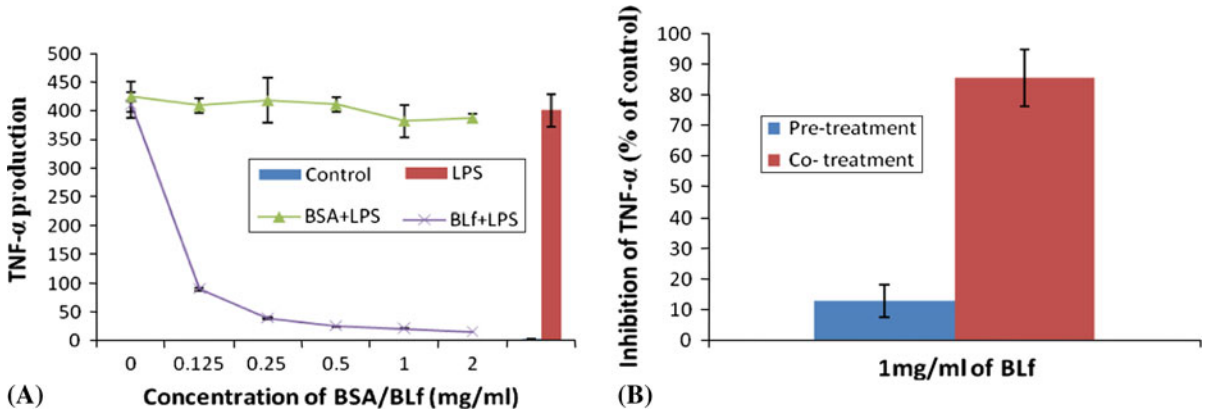

Fig. 2 **a** The effects of BLf and BSA on LPS-induced TNF-α production in THP-1 cell supernatant, tested using the ELISA method. **b** The comparison of TNF-α reduction in THP-1 cells during BLf plus LPS co-treatment and during BLf pre-treatment

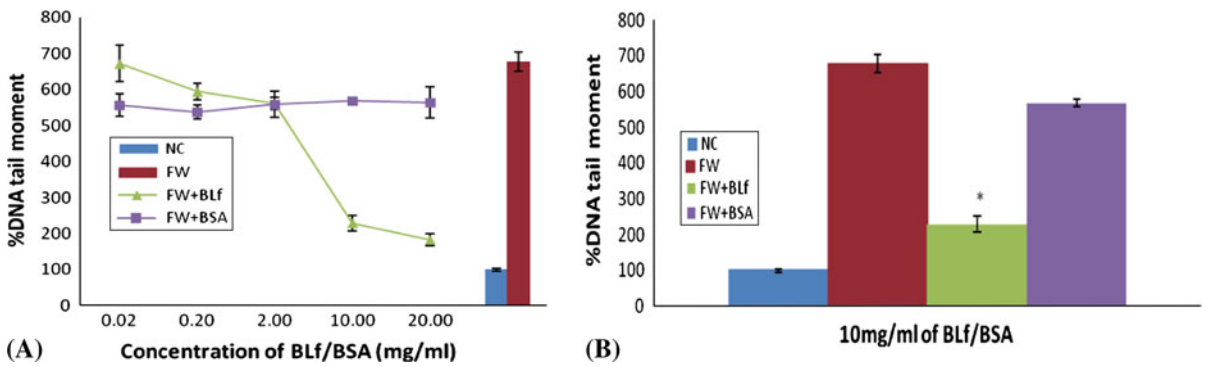

Fig. 3 **a** The dose responses of BLf and BSA on faecal water-induced HT29 cell DNA damage. **b** The reductive effect of BLf on faecal water-induced HT29 cell DNA damage ($P < 0.05$)

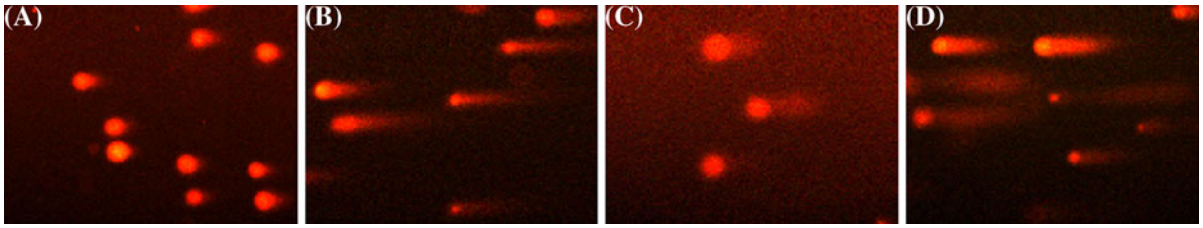

Fig. 4 Comet images are shown as negative control (NC, image **a**), positive control (faecal water, FW, image **b**) faecal water plus BLf (image **c**), faecal water plus BSA (image **d**)

induced DNA breakage (Fig. 3b). In contrast, bovine BSA, at same range of concentration as BLf, did not demonstrate any protective effects (Fig. 3a).

This study, using a cellular model approach, has demonstrated that BLf modulates the inflammatory process, mainly by preventing the release of cytokines and reactive nitro species from monocytes and macrophages, and by regulating the proliferation of immune cells via neutralization of endotoxin LPS. In terms of mechanism, these results not only confirm the effects of Lf on reduction of pro-inflammatory cytokines, but also partially explain the action of Lf involving a signaling pathway on immune receptors recognizing the pathogen-associated molecular pattern. Lf possibly modifies the necessary structures of LPS, which are able to be recognized by the CD14 and Toll-Like Receptors (TLRs) on immune cells. Taken as a whole, the present results and previous observations (Appelmelk et al. 1994) strongly suggest that lactoferrin is one of the key molecules which defend against pathogenesis and modulate the inflammatory response. These antibacterial and endotoxin-reduction activities are related to the ability of lactoferrin to bind lipopolysaccharides (LPS) with high affinity. Additionally, the results have shown that BLf is able to protect against epithelium DNA damage induced by genotoxic faecal water, and, thus, possibly reduce the risk of colon cancer. These results provide additional biological mechanism evidence to support the previous Lf anticancer activities in animal studies (Iigo et al. 2009; Tsuda et al. 2000).

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