

## Protective Effects of Lactoferrin against Intestinal Mucosal Damage Induced by Lipopolysaccharide in Human Intestinal Caco-2 Cells

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Indirect evidence suggests that lactoferrin (Lf), a major iron-binding protein in human milk, induces enterocyte growth and proliferation, depending on its concentration and affects the function and permeability of the intestinal mucosa. The bacterial endotoxin (lipopolysaccharide, LPS) is known to cause mucosal hyperpermeability *in vivo*. However, protective effects of Lf against LPS-mediated intestinal mucosal damage and barrier function in epithelial cells are not yet fully clarified. The aim of this study was to investigate whether Lf can reduce the cellular injury and alter epithelial hyperpermeability caused by LPS in human intestinal Caco-2 cells. When cell viability was measured by a WST-1 assay (tetrazolium salt-based assay), the protective effects against LPS-induced damage to Caco-2 cells were observed at doses of 800 and 1000  $\mu\text{g}/\text{ml}$  Lf. The barrier function of Caco-2 monolayer tight junctions was assessed by measuring transepithelial electrical resistance (TEER) and permeability of FITC-labeled dextran 4000 (FD-4). The treatment of Caco-2 cells with Lf at doses of 400 and 1000  $\mu\text{g}/\text{ml}$  significantly increased TEER as compared to treatment with LPS alone for 2 h ( $p < 0.05$ ). Further, at doses of 400 and 1000  $\mu\text{g}/\text{ml}$ , Lf inhibited the enhancement of LPS-mediated permeability in Caco-2 cell monolayer. The results of this study suggest that Lf may have protective effects against LPS-mediated intestinal mucosal damage and impairment of barrier function in intestinal epithelial cells.

**Key words**—lactoferrin; lipopolysaccharide; epithelial cell; cellular injury; barrier function

### INTRODUCTION

Lactoferrin (Lf), present in large amounts in breast milk, is found to bind to enterocytes and brush-border membranes in the infant intestine.<sup>1,2</sup> Several biological functions are attributed to Lf. In addition to its iron-binding capacity, regulation of inflammation and first-line defense against bacterial infections are the known effects of Lf.<sup>3</sup> When disruption in intestinal mucosal barrier function occurs, there is a leakage of water and plasma protein into the lumen and translocation of intestinal bacteria into the systemic circulation; these factors contribute to the development of systemic septicemia.<sup>4,5</sup> Bacterial endotoxins such as lipopolysaccharide (LPS) are known to cause mucosal hyperpermeability *in vivo*.<sup>6,7</sup> LPS was found to promote gut-barrier dysfunction through an oxidative mechanism, and incubation of Caco-2 enterocytic monolayers with a mixture containing interferon- $\gamma$ , IL-1 $\beta$ , and tumor necro-

sis factor- $\alpha$  increased epithelial permeability.<sup>8,9</sup>

Recently, it has been reported that treatment with many substances could ameliorate considerable structural and functional damage to the intestinal mucosa.<sup>10,11</sup> Despite these promising findings, the use of many substances as therapeutic agents may be limited by factors such as poor stability. However, Lf could be a potential therapeutic agent for the intestinal epithelial damage caused by LPS.<sup>3</sup>

In the present study, we examined the protective effects of Lf against intestinal epithelial damage and tight junction (TJ) barrier function impairment by using a filter-grown Caco-2 intestinal epithelial monolayer. The Caco-2 cells grown on permeable inserts form a TJ and attain many morphological and functional characteristics of the small intestinal enterocytes<sup>12</sup> and are shown to possess Lf receptors<sup>13</sup> that allows its internalization and delivery into the nucleus of the cells.<sup>14</sup> This cellular model system can be used to assess the effects of Lf on paracellular permeability.

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## MATERIALS AND METHODS

**Materials** Dulbecco's modified Eagle's medium (DMEM), penicillin, and streptomycin were purchased from Sigma-Aldrich, Inc. (MO, USA). Hank's balanced salt solution (HBSS) was purchased from GIBCO Laboratories (NY, USA). Caco-2 cells and fetal bovine serum (FBS) were purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). Millicell-PCF permeable filters (pore size: 3.0  $\mu\text{m}$ ; diameter: 12 mm) were purchased from Millipore Corp. (MA, USA). A Cell Counting Kit was purchased from DOJINDO Laboratories (Kumamoto, Japan). Lf from human milk and LPS from *Escherichia coli* serotype 055: B5 were purchased from Sigma-Aldrich, Inc. (MO, USA). All the other chemicals were of reagent grade.

**Cell Cultures** Caco-2 cells (passage 55–60) from a human colonic carcinoma were grown in a culture medium composed of DMEM with 4.5 g/l glucose, 50 U/ml penicillin, 50  $\mu\text{g}/\text{ml}$  streptomycin, 1% (v/v) nonessential amino acids, and 10% (v/v) heat-inactivated FBS in 25-cm<sup>2</sup> tissue culture flasks (Becton-Dickinson Ind., NJ, USA). The cells were maintained at 37°C in an atmosphere of 5% CO<sub>2</sub>/95% air in a CO<sub>2</sub> incubator until they reached 80–90% confluence, which usually took 5–7 days. The cells were subcultured by partial digestion with 0.25% trypsin and 1 mmol/l EDTA in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free phosphate-buffered saline (PBS) solution. The Caco-2 cells were then detached from the stock cultures by trypsin digestion, washed once by centrifugation, resuspended, and subcultured in 6 ml of the medium in culture flasks at a concentration of  $1 \times 10^5$  cells/ml. The cultures were observed on a regular basis under an inverted light microscope to monitor growth and contamination. For growth on filters, high-density Caco-2 cells ( $2.0 \times 10^5$  cells/ml) were plated on nitrocellulose-based Millicell-PCF filters. The Caco-2 cells were fed with culture medium every alternate day for 6 days and then daily for 18 or 25 days by replacing 0.4 ml of the medium in the apical chamber and 0.6 ml of the medium in the basolateral chamber. They were monitored regularly for confluence by measuring transepithelial electrical resistance (TEER).

**Cell-viability Experiment** The viability of Caco-2 cells was determined by a cell proliferation assay using WST-1 reagent—a water-soluble sulfonated tetrazolium salt that is cleaved by cellular succinate

dehydrogenases in living cells, yielding dark blue formazan. Damaged or dead cells exhibit reduced dehydrogenase activity. Briefly, Caco-2 cells ( $5 \times 10^3$  cells/well) were plated on a 96-well multiplate (Becton-Dickinson Ind., NJ, USA). On day 3, the medium was replaced by medium without FBS and cells were preincubated for 1 h with Lf. Then, the cells were treated with LPS for 2 h. Lf and LPS was solved by HBSS. WST-1 solution/culture medium (final concentration: 5 mmol/l) was added to each well. Following 3-h incubation at 37°C, absorbance at 450 nm (reference at 630 nm) was measured by a Multiskan JX microplate reader (Thermo LabSystems, MA, USA). The percentage cell viability was calculated in terms of absorbency in cells treated with Lf relative to that in cells exposed to culture medium alone.

**Determination of Epithelial Monolayer Resistance and Permeability** The permeabilities of fluorescein isothiocyanate-labeled dextran 4000 (FD-4) (Sigma-Aldrich, Inc., MO, USA; loading dose of 10  $\mu\text{M}$ ) through Caco-2 cell monolayers were determined in the apical-to-basolateral (A→B) directions at pH 7.4. Before conducting the permeability experiments, the cell monolayers were washed twice with PBS (pH 7.4). After washing, the cells were allowed to reach equilibrium in HBSS for 1 h. Next, we observed in the cells preincubated with Lf to order to evaluate the protective effects of Lf in both sides of Caco-2 cell monolayers in the present study. Fresh medium containing 1000  $\mu\text{g}/\text{ml}$  Lf was added to both sides of the cell monolayers and incubated further for 1 h. Subsequently, LPS was added to the basolateral side at various concentrations. The medium on the basolateral side of Caco-2 cell monolayers was replaced with (1) 600  $\mu\text{L}$  HBSS, (2) HBSS with Lf, or (3) HBSS with 200, 400, or 1000  $\mu\text{g}/\text{ml}$  LPS and/or Lf. TEER was measured using a Millicell-ERS voltohmmeter (Millipore Corp., MA, USA). Cell monolayers with TEER values below 300  $\Omega \cdot \text{cm}^2$  were not used. Samples were obtained after 2 h by moving the cell monolayer to a new receiver well containing fresh HBSS. The samples were diluted with 500  $\mu\text{l}$  HBSS, and fluorescence was determined at 485/528 nm (excitation/emission) using a fluorometer (Powerscan HT fluorescent plate reader, Dainippon Sumitomo Pharm Co., Ltd., Osaka, Japan). All the permeability experiments were performed in triplicate. The TEER values were measured after each ex-

periment.

**Data Analysis** Results have been expressed as mean ± S.D. Statistical analyses were performed using Student's unpaired *t*-test, and the differences were considered to be significant when  $p < 0.05$ .

**RESULTS**

**Effects of LPS and LPS Plus Lf on Cell Viability**

In a dose-dependent manner LPS reduced the cell viability compared with control medium. The percentage cell viability decreased to approximately 30% of control medium in 800 and 1000 µg/ml LPS, respectively (Fig. 1). LPS produced marked damage at higher concentrations. However, at similar concentrations of LPS, the cell viability rates (%) of the cells preincubated with Lf were significantly higher than those of untreated cells ( $p < 0.05$ ).

**Effects of Lf and LPS Plus Lf on the TJ Barrier of Caco-2 Cell Monolayers**

The effects of LPS and LPS plus Lf on TJ barrier function of Caco-2 cell monolayers were evaluated by measuring TEER. After the 2-h treatment, TEER was significantly decreased at 400 and 1000 µg/ml of LPS compared with control medium ( $p < 0.01$ ). At similar concentrations of LPS, TEER significantly increased at 400 and 1000 µg/ml of Lf as compared with LPS alone (respectively,  $p < 0.05$ ,  $p < 0.01$ ; Fig. 2).

**Effects of Lf and LPS Plus Lf on FD-4 Permeability of Caco-2 Cell Monolayers**

The permeability activity on the TJ barrier of Caco-2 cell monolayers was evaluated by measuring the paracellular penetration amount of FD-4 across Caco-2 cell monolayers. The FD-4 permeability of Caco-2 monolayers increased following incubation with LPS for 2 h (Fig.

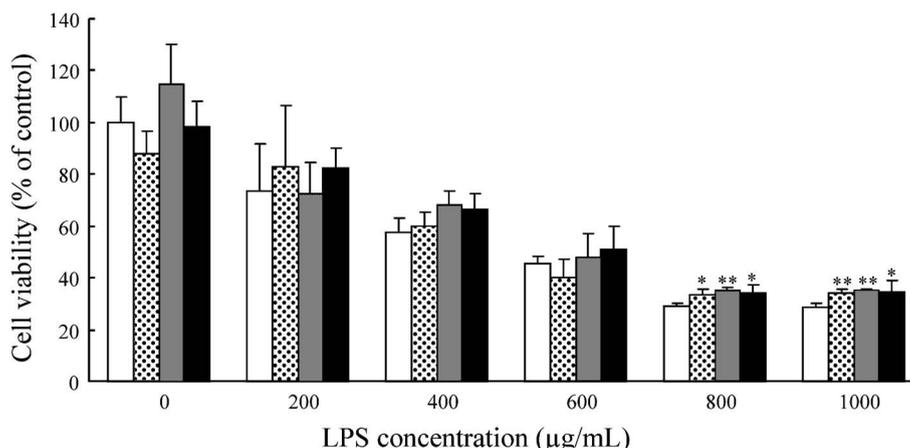


Fig. 1. Effects of Lf on LPS-induced Damage in Caco-2 Cells  
 Values are mean ± S.D. (n=3). Cell viability (% of control) was calculated as 100% in medium.  
 \* $p < 0.05$ , \*\* $p < 0.01$  compared with LPS alone. □; Lf 0 µg/ml, ▨; Lf 250 µg/ml, ▩; Lf 500 µg/ml, ■; Lf 1000 µg/ml.

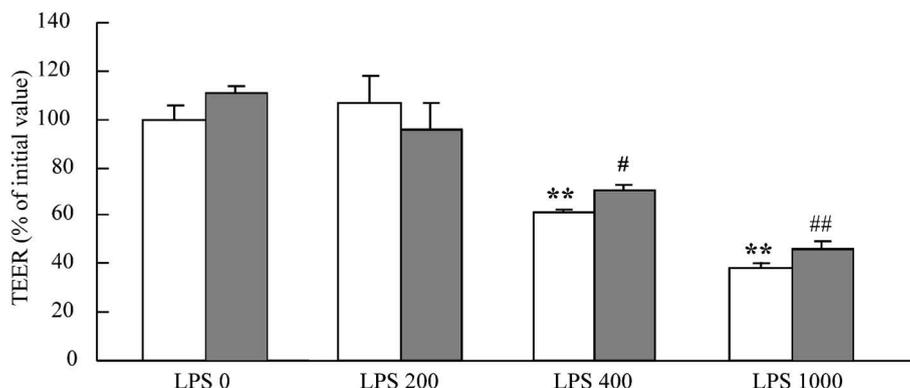


Fig. 2. Effects of Lf on Membrane Resistance (TEER) in Caco-2 Cell Monolayer  
 Values are mean ± S.D. (n=3). \*\* $p < 0.01$  compared with control (medium).  
 \* $p < 0.05$ , \*\* $p < 0.01$  compared with LPS alone. □; Lf 0 µg/ml, ■; Lf 1000 µg/ml.

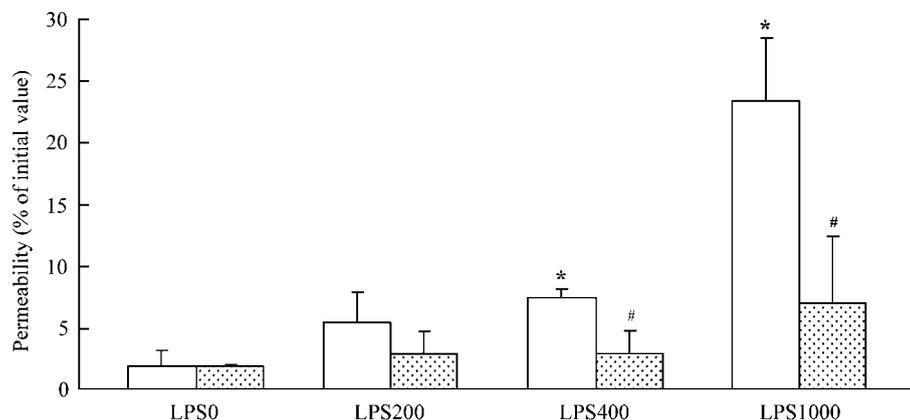


Fig. 3. Effects of LPS and LPS plus Lf on Permeability of FD-4 in Caco-2 Cell Monolayer

The permeability was calculated from permeation FD-4 amount after 2 h of incubation and 100% was the initial amount. Values are mean  $\pm$  S.D. ( $n=3$ ). \* $p < 0.05$  compared with control (medium). # $p < 0.05$  compared with LPS alone,  $\square$ ; LPS+Lf 0  $\mu\text{g/ml}$ ,  $\square$ ; LPS+Lf 1000  $\mu\text{g/ml}$ .

3). The elevation of permeability by LPS was concentration-dependent; it was statistically significant at 400 and 1000  $\mu\text{g/ml}$  of LPS (both,  $p < 0.05$ ). The addition of Lf to control medium, however, significantly prevented an increase in permeability to FD-4 induced by LPS (respectively,  $p < 0.01$ ,  $p < 0.05$ ).

## DISCUSSION

An important function of the intestinal epithelia is to protect against the mucosal penetration of toxic compounds, bacteria, and bacterial byproducts as well as dietetic additives present in the intestinal lumen. The TJ acts as a structural barrier against the paracellular permeation of luminal compounds.<sup>15,16</sup> A disruption of the TJ barrier causes increased epithelial penetration of toxic luminal substances that may promote mucosal injury. It is known that bacterial infection induces the impairment of intestinal mucosal barrier functions, although the mechanisms by which intestinal infection increases mucosal permeability have not yet been fully elucidated. Specific bacteria have been shown to damage the epithelium by releasing exotoxins that act on the tight junctions.<sup>17</sup> On the other hand, in our previous study, LPS was found to influence transcellular permeability; the expression of the intestinal epithelial transporter P-glycoprotein and its activity in rats.<sup>18</sup>

Lf induces the concentration-dependent functional modulation of intestinal proliferation and differentiation.<sup>19</sup> Lf appears to have an important natural mechanism for regulating epithelial cell responses to pathogenic bacteria and in limiting cell damage and the spread of infections.<sup>20</sup> Lf-specific receptors have

been identified in the brush-border membrane of fetal and infant intestine,<sup>21</sup> and Lf receptor abundance has been reported to significantly increase with age.<sup>22</sup> Lf receptor is also identified in Caco-2 cell lines.<sup>13</sup> Asida et al. have reported that human Lf is internalized by Caco-2 cells from the apical side and localized to the nuclei.<sup>14</sup> These studies show that Caco-2 cell lines are useful models for assessing the effects of Lf in the intestine.

On the basis of these findings, we studied the capability of Lf in reducing the epithelial cell damage and tight junction opening in Caco-2 cells that received LPS. Lf showed an inhibitory effect on the decrease in cell viability by LPS (Fig. 1).

We then examined the effects of Lf on the barrier function of the monolayers of Caco-2 cells *in vitro*. Paracellular permeability has been correlated with a decrease in intestinal epithelial resistance.<sup>23</sup> The use of TEER as a measure of the integrity of membrane barriers has also been reported in studies evaluating the damage caused by drugs such as fenadine-HCl or indomethacin.<sup>24,25</sup> In addition, TEER measurements have been reported to indicate the permeability of cell monolayers and their barrier properties.<sup>26,27</sup> We therefore used TEER values to monitor the damage induced by LPS in Caco-2 cell monolayers. The confluent Caco-2 cell monolayers cultured for approximately 20 days and used in the experiments were subjected to 0, 200, 400, and 1000  $\mu\text{g/ml}$  LPS in HBSS from basolateral side for 2 h. Meanwhile, the observation that LPS affects the paracellular barrier function only when it interacts with the basolateral surface of intestinal epithelial cells would have a certain phys-

iological significance, because a huge amount of LPS exists in the animal alimentary canal even under physiological condition.<sup>28)</sup> Once bacterial translocation occurs locally under various pathological conditions, bacterial LPS gaining access to systemic circulation affects barrier function of TJ. When LPS was added to the basolateral side in our preliminary experiments, the TEER of Caco-2 cell monolayers in basolateral side declined more than the apical side. So, in this study LPS was added to the basolateral side of Caco-2 cell monolayers. A significant damage with a decrease in TEER was induced in these Caco-2 cell monolayers by treatment with 400 and 1000  $\mu\text{g/ml}$  LPS for 2 h (Fig. 2). However, Lf improved the reduction in TEER by LPS. As well, Lf concentrations used in this study is equal to the milk whey of breastfeeding mothers.<sup>29)</sup> An addition of Lf prevented increases in permeability to FD-4 induced by the proinflammatory bacterial product LPS (Fig. 3). In the present study, we demonstrated that high doses of LPS had direct cytotoxic effects on epithelial cells at the gastrointestinal surface, resulting in rupture of epithelial layers and formation of large open wounds on the epithelial surface. Thus, the LPS-induced increase in TJ permeability may be due to a notable change in the TJ barrier and may have been caused by cell death or the formation of large open wounds on the epithelial surface. As a one of the mechanism, interferon- $\gamma$  and tumor necrosis factor- $\alpha$  produced by LPS alter the expression and localization of two key TJ protein, ZO-1 and occludin.<sup>11)</sup> However, Caco-2 cell monolayer resistance and permeability changes induced by the LPS treatment were significantly attenuated by Lf supplementation. On the other hand, Shoji *et al.* demonstrated that Lf acts as an antioxidant in intestinal epithelial cells.<sup>30)</sup> And also, we found that LPS caused oxidative damage by decreasing superoxide dismutase activity in Caco-2 cells (data not shown). Additional studies are needed, but it is conceivable to hypothesize that Lf might play a role in reducing the oxidative stress induced by LPS.

In conclusion, the results of this study suggest that Lf may have protective effects against LPS-mediated intestinal mucosal damage and impairment of barrier function in intestinal epithelial cells.

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