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Reaction of Cathelicidin-2 Secreted from Goats Milk Leukocytes to Lipopolysaccharide

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ABSTRACT: Since mastitis causes a great economic loss for dairy farmers, its prevention and treatment are essential for milk production. *Escherichia coli (E.coli)* is one of the main bacteria causing mastitis, and its cell membrane component, lipopolysaccharide (LPS), induces mammary inflammation through the recognition by Toll-like receptors (TLR)-4. In human, there are some reports that Cathelicidin (Cath), one of the antimicrobial peptides, could prevent LPS-induced inflammation by neutralization of LPS. The objective of the present study was to examine whether the goat Cath-2 bound to and neutralized LPS. Leukocytes derived from goat milk were cultured for 0, 1, 3, 6, 12, 24 h. Then the concentration of Cath-2 in the medium was measured. The binding ability of synthetic Cath-2 (0~100 ng/ml) to LPS was analyzed. Mammary epithelial cells (MEC) isolated from milk were cultured with LPS and/or Cath-2. Then mRNA expression of cytokines (*IL-1 β, IL-6, IL-8 and TNF-α*) in the cells and concentration of lactoferrin (LF) in the medium were examined. The concentration of Cath-2 in the cultured medium increased significantly with the time of culture. It was confirmed that Cath-2 bound to LPS. The mRNA expression of IL-8 was increased significantly in MEC challenged with LPS. No significant effect of LPS on the expressions of other cytokines was found. The expressions of all cytokines were not changed by the addition of Cath-2 in MEC challenged with LPS. However, the expression of *IL-1 β, IL-6 and TNF-α* tended to increase in association with the increase of Cath-2 concentration in the absence of LPS. The addition of Cath-2 together with LPS did not cause significant change in LF concentration in the medium. These results suggest that goat Cath-2 was secreted from leukocytes in milk and it has an ability to bind to LPS.

Keywords: Mastitis, Cathelicidin, LPS, Neutralization

INTRODUCTION

Mastitis is inflammation of the mammary glands. Because mastitis may cause death of dairy cows and great economic losses, the innate immune system in the mammary gland, which works rapidly after bacterial invasions, is important for its prevention. The innate immune system is initiated by the recognition of bacterial components through Toll-like receptors (TLRs), that are expressed in the epithelial cells and leukocytes, followed by the production of some cytokines and antimicrobial peptides (Isobe *et al.*, 2009, 2011). Escherichia coli (E. coli), one of the Gram-negative bacteria, is a major pathogenic bacteria of mastitis (Blowey and Edmondson., 1995) and frequently causes acute and severe mastitis. Although antibiotics are used for its treatments, it destroys *E. coli*, resulting in the release of a large amount of lipopolysaccharide (LPS). LPS can reach the whole body through blood circulation and cause an inflammatory reaction, leading to endotoxin shock (Kirikae et al., 1997) and sepsis (Su *et al.,* 2010).

Cathelicidin (Cath) is one of the antimicrobial peptides that play roles in the innate immune system (Ramanathan *et al.,* 2002). In goat, Cath-2 was expressed in leukocytes and included in milk (Zhang *et al.,* 2014). It is reported that the human Cath (LL-37) can prevent severe inflammations by binding to LPS, which is called its neutralization ability (Scott *et al.,* 2011). However, there is no report showing the neutralization ability of Cath in cows and goats. To prevent endotoxin shock

and sepsis, such ability of Cath is important. Therefore, the objective of the present study is to examine whether the goat Cath-2 can bind to and neutralize LPS.

MATERIALS AND METHODS

Four crossbred female goats were kept in Hiroshima University farm under the Guideline for Animal Experimentation, Hiroshima University, Japan.

Leukocytes were isolated from goats milk by centrifuging at 1,800 ×g at 4°C for 10 min and resulting precipitate was used. Leukocytes were cultured for 0, 1, 3, 6, 12, 24 h at the concentration of 1.0×10^8 cell/ml. After cultivation, Cath-2 concentration in the medium was measured using ELISA as reported previously (Zhang *et al.,* 2014).

To localize Cath-2 in leukocytes, they were smeared on slide glass and immunostained using anti-Cath-2 antibody and HRP-anti-rabbit IgG antibody.

To determine whether Cath-2 can bind to LPS, synthetic Cath-2 (0 ~ 100 ng/ml) was added to the LPS-coated 96-well plate followed by additions of anti-Cath-2 antibody and HRP-labeled anti-rabbit IgG antibody. Then the absorbance of each well was measured.

To examine whether Cath-2 can neutralize LPS reaction, goat mammary epithelial cells (gMEC) was used. Goat milk was centrifuged and precipitated MEC were cultured at a concentration of 1.0×10^8 cell/ml for 24 h. Attached cells were cultured to be confluent. MEC was cultured in the medium supplemented with LPS (0 or 10 μg/ml) and/or Cath-2 (0-10 μg/ml) for 6 h. After cultivation, total RNA of MEC and cultured medium were collected to examine the mRNA expression of cytokines (*IL-1 β, IL-6, IL-8, TNF-α*) and the concentration of LF, respectively.

The significance of differences was analyzed by using Kruskal-Wallis multiple comparison test and Tukey multiple comparison test. A probability of $P < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

The concentration of Cath-2 in the cultured medium increased significantly at 3-24 h compared with that at 0 h. When leukocytes were cultured for different time and immunostained for Cath-2, positive immuno-reaction was observed in the cytoplasm of leukocytes cultured for 0-24 h, suggesting that Cath-2 was synthesized and secreted from leukocytes into milk.

When binding ability of Cath-2 to LPS was examined, the absorbance of the wells added with Cath-2 was significantly higher than that without Cath-2 in the LPS-coated plate. This result suggests that Cath-2 can bind to LPS.

The mRNA expression of IL-8 was increased significantly in MEC cultured with LPS compared with that without LPS. However, no significant differences in the expressions of IL-1 β, IL-6 and TNF-α were found in MEC cultured between with and without LPS. The expressions of all cytokines were not changed by the addition of Cath-2 in MEC challenged with, or without, LPS. The addition of Cath-2 and/or LPS did not cause significant change in LF concentration in the medium.

In conclusion, these results suggest that goat Cath-2 is synthesized by leukocytes and secreted into milk and it has an ability to bind to LPS. But we cannot say that Cath-2 has the ability to neutralize LPS.

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