Oral Administration of Lactoferrin Inhibits Inflammation and Nociception in Rat Adjuvant-Induced Arthritis

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ABSTRACT. Lactoferrin (LF) is a ubiquitous protein which exists in milk, plasma, synovial fluids, cerebrospinal fluid and other biological fluids. LF is also well known as a natural immunomodulator. Recently, we found that bovine milk-derived LF (BLF) produced µ-opioid receptor-mediated analgesia. In this study, we examined whether oral administration of BLF causes anti-nociceptive and anti-inflammatory effects, and also whether it modulates LPS-induced TNF-α and IL-10 production in rat model of rheumatoid arthritis (RA), rat adjuvant arthritis. BLF was administrated once daily, starting 3 hr before (preventive experiment) or 19 days after (therapeutic experiment) adjuvant injection. In both experiments, BLF suppressed the development of arthritis and the hyperalgesia in the adjuvant-injected paw. The single-administered BLF produced a dose-dependent analgesia, which was reversed by naloxone, in the adjuvant arthritis rats. Both repeated and single administration of BLF suppressed TNF-α production and increased IL-10 production in the LPS-stimulated adjuvant arthritis rats. These results suggest that orally administered BLF has both preventive and therapeutic effects on the development of adjuvant-induced arthritis and pain. Moreover, the immunomodulatory properties of BLF, such as down-regulation of TNF-α and up-regulation of IL-10, could be beneficial in the treatment of RA. Thus, we concluded that LF can be safely used as a natural drug for RA patients suffering from joint pain.

KEY WORDS: adjuvant arthritis, IL-10, lactoferrin, pain, TNF-α.

Rat adjuvant arthritis is often used as an animal model of rheumatoid arthritis (RA) in the evaluation of anti-rheumatic drugs. Recent cumulative evidence suggests that proinflammatory cytokine, TNF-α, increases in the synovial tissue and synovial fluid in RA, and plays a pivotal role in the pathology of RA. On the other hand, the anti-inflammatory activities of IL-10, which suppresses TNF-α production, are well known. It is reported that systemic IL-10 treatment suppresses the development of collagen-induced arthritis in rats [17]. Thus, down-regulation of TNF-α and up-regulation of IL-10 may be a rational strategy for the treatment of RA. In addition, it is well known that the major complaint of RA patients is joint pain. Appropriate treatments for pain in RA patients are thus also required.

Based on these evidences, we were interested in lactoferrin (LF), a natural iron-binding protein with a wide spectrum of biological activities. Lactoferrin is a ubiquitous protein which exists in milk, plasma, synovial fluids, cerebrospinal fluid (CSF), and other biological fluids [3, 14–16, 21]. Under inflammatory conditions, LF production is increased in the periphery by neutrophils [2, 14] and in the central nervous system (CNS) by microglia [8]. LF is well known as a natural immunomodulator. LF suppresses TNF-α and IL-1β productions in human mononuclear cells in vitro [5]. Intravenous or intraperitoneal administration of LF reduces the production of TNF-α and IL-6 in vivo [13, 26]. Oral administration of bovine milk-derived LF (BLF) also decreases TNF-α and IL-6 production [25] and increases IL-10 production [24] in vivo. In the mouse collagen-induced and septic arthritis models, periarticular injection of human LF reduces inflammation [9]. From these evidences, an anti-inflammatory effect of orally administered LF on arthritis is expected. Recently, we found that BLF produced µ-opioid receptor-mediated analgesia in the thermal, visceral, and formalin-evoked nociceptions in rats following oral, intraperitoneal, and intrathecal administration [11]. Thus, we hypothesize that the oral administration of BLF would be effective in treating arthritis with its anti-inflammatory and anti-nociceptive activity.

In this study, we examined whether oral administration of BLF has the anti-nociceptive and anti-inflammatory effects (preventive and therapeutic experiments) in the rat adjuvant arthritis. In order to clarify the immunomodulatory activity of BLF, we also examined whether BLF modulates LPS-induced TNF-α and IL-10 production in the adjuvant arthritis rats.

MATERIALS AND METHODS

Drugs

Bovine serum albumin (BSA, Sigma, Tokyo, Japan) and bovine lactoferrin (BLF, Tatua, Morrinsville, New Zealand) were dissolved in distilled water (100 mg/ml) and then diluted with a 0.5% carboxymethyl cellulose sodium (CMC, Wako, Osaka, Japan) solution for oral administration (5 ml/kg). Dexamethasone (DEX, Wako) was dissolved in ethanol (10 mg/ml) and then diluted with 0.5% CMC solution.
Naloxone hydrochloride (naloxone, Sigma), and morphine hydrochloride (morphine, Sankyo, Tokyo, Japan) were dissolved in saline for subcutaneous injection (1 ml/kg).

Animals and the induction of adjuvant-induced inflammation

Male Wistar rats (6 weeks old; body weight, 130–170 g) were used. All animals were maintained at a controlled temperature (22 ± 2°C), and a regular light/dark cycle (7:00 to 19:00 hr, light), and all animals had free access to food (CE-2, CLEA, Tokyo, Japan) and water. The induction of adjuvant-induced inflammation was performed according to the method described in a previous report [22]. On the first day of the experiment (day 0), the rats received a subplantar injection of 100 µl of Freund’s complete adjuvant (Sigma, Tokyo, Japan) in right hind paw under brief ether anesthesia following pre-value measurements of paw volume and pain score (described below). Behavior tests were conducted during the light period. All experiments were conducted in accordance with the guidelines of the Physiological Society of Japan regarding the care of experimental animals.

Measurements of paw volume and pain score

The method for measuring pain score (joint hyperalgesia) followed that used in the previous report with modifications [4]. The number of pain-related responses (vocalizations) was recorded during ten flexions of the tarsotibial joints of the adjuvant-injected paw. Immediately after the hyperalgesia testing, the hind paw volume was determined by water displacement plethysmometry. The results are expressed as the mean number of vocalizations and the mean paw volume in the adjuvant-injected paw ± S.E.

Experimental procedure

Experiment 1: Preventive effects of orally administered BLF on adjuvant-induced inflammation and hyperalgesia

Rats were divided into four groups: 1) bovine serum albumin (BSA, 100 mg/kg)-treated group (n=7), 2) BLF (30 mg/kg)-treated group (n=7), 3) BLF (100 mg/kg)-treated group (n=7), and 4) DEX (0.1 mg/kg)-treated group (n=5). Each drug was administered once a day, starting 3 hr before the adjuvant injection (day 0) and continuing to day 18 (preventive administration). Measurements of paw volume and pain score were performed each day 3 hr after the drug administration. The use of this time point for the measurements was determined on the basis of the preliminary experiment in which the plasma concentration of BLF reached its maximum level approximately 3 hr after oral administration.

Experiment 2: Therapeutic effect of orally administered BLF on inflammation and hyperalgesia in adjuvant arthritis rats

Rats were divided into three groups: 1) BSA (100 mg/kg)-treated group (control group for both the daily and single-administered groups), 2) BLF (100 mg/kg) single-administered group, and 3) DEX single-administered group. In both the daily and single-administered groups, the drugs were given 3 hr prior to the intraperitoneal injection of 100 µg/kg of lipopolysaccharide (LPS, Escherichia coli 0111:B4, Sigma), which was dissolved in saline. This dose of LPS was determined from the preliminary experiment. Blood samples were obtained at 3 hr after LPS injection. Plasma was then stored at –80°C until the cytokine assay. The concentration of TNF-α and IL-10 in plasma was measured with ELISA kits using the manufacturer’s instructions (Pierce Endogen, Rockford, IL, U.S.A.).

Statistics

Data are expressed as mean ± S.E. Differences between treatment groups were assessed by the un-paired t-test or, when appropriate, ANOVA followed by Dunnett’s post-hoc test for multiple comparisons. In all cases, a probability (P) value of <0.05 was considered to indicate statistical significance.

RESULTS

Experiment 1: Preventive effects of orally administered BLF on adjuvant-induced inflammation and hyperalgesia

The adjuvant-injected paw volume in BSA-treated rats (control rats) showed its first increase (2.73 ± 0.11 ml) at day 1, and declined (2.46 ± 0.08 ml) at day 4 (Fig. 1A). The injected paw volume showed a second increase from day 14 (2.85 ± 0.12 ml). From these observations, we defined the inflammation in the injected paw as “an acute inflammation (day 1)” and “a chronic inflammation (after day 14)” in this study. To evaluate the preventive effects of drugs on the development of adjuvant-induced arthritis, BLF (30, 100 mg/kg/day) and DEX (0.1 mg/kg/day) were orally applied once a day, starting from 3 hr before the adjuvant injection and continuing to day 18. DEX suppressed the development
of the acute and chronic inflammation in the injected paw in comparison with the control. BLF also significantly suppressed the development of acute and chronic inflammation compared to the control rats during the experiment.

As shown in Fig. 1B, none of the rats showed the pain score (hyperalgesia) before adjuvant injection (day 0). After adjuvant injection, the control rats showed clear hyperalgesia in the injected paw during the experiment. BLF and DEX markedly inhibited hyperalgesia in the injected paw during the experiment.

Experiment 2: Therapeutic effect of orally administered BLF on inflammation and hyperalgesia in adjuvant arthritis rats

The control rats (BSA-treated rats) showed gradual development of arthritis (increase of paw volume) during the experiment, from 2.8 ± 0.05 (day 18) to 3.25 ± 0.05 mL (day 25) in the adjuvant-injected paw (Fig. 2A). BLF-treated rats did not show the apparent development of arthritis but kept almost the same level during the experiment. A significant difference in the paw volume between the control and BLF-treated rats was observed at day 25. On the other hand, DEX markedly inhibited the development of arthritis. The significant difference in the paw volume between the control and DEX-treated rats was observed from day 19.

In correlated with the development of arthritis, the hyperalgesia in the injected paw also gradually increased from 5.9 ± 0.9 (day 18) to 8.4 ± 0.6 (day 25) in the control rats (Fig. 2B). BLF showed a significant analgesia in the injected paw on the first day of treatment (day 19) without significant changes of paw volume, and thereafter kept the same potency during the experiment. DEX showed significant analgesia in the injected paw from day 21.

Experiment 3: Effect of single oral administration of BLF on joint hyperalgesia in adjuvant arthritis rats

BSA (100 mg/kg, control) did not affect the hyperalgesia in the adjuvant-injected paw (Fig. 3). BLF (10, 100 mg/kg) showed a dose-dependent analgesia in the adjuvant-injected paw, and a significant difference compared to the control was observed at a dose of 100 mg/kg of BLF. Subcutaneous administration of an opioid antagonist, naloxone (3 mg/kg), per se did not affect the hyperalgesia, but completely reversed BLF-induced analgesia. In this experiment, subcutaneous administration of a μ-opioid agonist, morphine (3 mg/kg), showed a marked analgesia in the adjuvant-injected paw.
Experiment 4: Effect of single or daily oral administration of BLF on LPS-induced TNF-α and IL-10 production in adjuvant arthritis rats

To assess the immunomodulatory feature of BLF, we measured LPS-induced TNF-α and IL-10 production in adjuvant arthritis rats following daily or single oral administration of BLF (10, 100 mg/kg), DEX (0.1 mg/kg), and BSA (100 mg/kg, control). Daily administration of BLF dose-dependently suppressed TNF-α production, and increased IL-10 production in the LPS-stimulated adjuvant arthritis rats (Fig. 4). In both cases, a significant difference between the control and BLF-treated rats was observed at a BLF dose of 100 mg/kg. Moreover, single administration of BLF (100 mg/kg) also suppressed TNF-α production and increased IL-10 production with almost the same potency as daily administration of the same dose. Both single and daily administration of DEX also significantly inhibited TNF-α production in the LPS-stimulated adjuvant arthritis rats. However, DEX did not increase the IL-10 production in either administration regime. Rather, both single and daily administration of DEX tended to suppress IL-10 production.

DISCUSSION

In the present study, oral administration of BLF showed 1) the analgesia via an opioid receptor-mediated mechanism, 2) the inhibition of development of arthritis, and 3) the down-regulation of TNF-α and up-regulation of IL-10 in the well used animal model of RA, adjuvant arthritis rats. Recently, we found that BLF acts on the CNS and exerts μ-opioid receptor-mediated anti-nociceptive activity on the thermal, visceral, and formalin-evoked nociceptions in rats following oral, intraperitoneal, and intrathecal administration [11]. In the present study, we observed that orally administered BLF inhibited hyperalgesia in the various phases of adjuvant-induced inflammation (Figs 1B, 2B and 3). Naloxone completely reversed the single orally administered BLF-induced analgesia in the chronic state of arthritis (Fig. 3), suggesting that BLF produced an opioid receptor-mediated analgesia in the adjuvant arthritis rats. We used LF purified from bovine milk in the present study. The LF of humans, bovids, mice, and pigs share 70% of their overall amino acid sequence and showed 100% identity in several stretches of 10–15 amino acids at the C terminus [23]. We also confirmed that recombinant human LF possesses similar anti-nociceptive activity as BLF in the rat formalin test [11]. Thus, not only BLF but also LF from different species will produce analgesia.
that orally administered of BLF entered the CSF in piglets [10] and calves [21]. In the preliminary study, we also detected the presence of BLF in rat CSF following oral or intraperitoneal administration. Moreover, this macromolecular transcytosis may be enhanced in inflammatory conditions that induce pain, as suggested by a report that TNF-α increased the rate of transendothelial transport of BLF in a blood-brain barrier model [6]. It is also reported that the serum level of TNF-α increases in adjuvant arthritis rats [20]. From this evidence, it is likely that the transport of orally administered BLF to the CSF can be facilitated in adjuvant arthritis rats. Thus, oral administration of BLF might be more effective in reducing the nociception via the central opioidergic system in inflammatory pain, such as that caused by arthritis, than in non-inflammatory pain.

It is also reported that the level of LF increases in the synovial fluid from RA human patients [3]. Under inflammatory conditions, LF production is increased in the periphery by the neutrophils [2, 14]. However, the anti-nociceptive activity of LF in the periphery is still unclear. As exogenous and endogenous opioids produced both central and peripheral anti-nociception [18, 19], BLF and endogenous LF may cause analgesia not only in the CNS [11] but also in the periphery via the opioidergic system. Further investigation is required to clarify this point.

In addition to its anti-nociceptive effects, LF is well known as a natural immunomodulator. It is reported that the periartricular injection of human LF reduced inflammation in collagen-induced and septic arthritis in the mouse [9]. In the preventive experiment of the present study, the oral administration of BLF suppressed the development of the acute and chronic inflammation in the adjuvant-injected paw (Fig. 1A). In the therapeutic experiment (Fig. 2A), BLF also suppressed the development of arthritis in the adjuvant-injected paw. These results suggest that oral administration of BLF provides both preventive and therapeutic effects in the development of arthritis.

In order to evaluate the immunomodulatory feature of anti-rheumatic drugs, LPS-induced cytokines production in arthritic rats is often used. Indeed, LPS-induced cytokines production in adjuvant arthritis rats are augmented in comparison with normal rats [12]. In the present study, both daily and single administration of BLF suppressed TNF-α production and increased IL-10 production in the LPS-stimulated adjuvant arthritis rats (Fig. 4). It is reported that BLF causes the down-regulation of TNF-α and IL-1β and the up-regulation of IL-10 via inhibition of the nuclear factor-kB pathway, which is involved in the regulation of many inflammation-associated genes [24]. TNF-α increases in synovial tissue and synovial fluid [20] and plays a pivotal role in the pathology of RA. TNF-α is indeed at the apex of the inflammatory cascade that involves IL-1, IL-6, and IL-8 production in vivo and in vitro [1]. The anti-inflammatory activities of IL-10, which suppresses TNF-α production, are well known. Thus, the immunomodulatory properties of BLF, such as the down-regulation of TNF-α and up-regulation of IL-10, could be beneficial in the therapy for RA.

In summary, the present study demonstrates that oral administration of BLF produces the anti-nociceptive, anti-inflammatory, and immunomodulatory effects in rat adjuvant arthritis. It is already known that LF has many peripheral functions, such as inducing the primary defense against bacterial and viral infection and anti-tumor activity [2]. This wide range of LF activity will potentially be of great benefit to patients. The multifunctional milk-derived protein, LF, produces anti-nociceptive and anti-inflammatory effects, suggesting that LF can be a safely used natural drug for RA patients suffering from joint pain.

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REFERENCES


