Goat milk CSN1S2 is able to decrease the severity scoring, TNF-α, and RAGE expression in complete Freund’s adjuvant-induced rheumatoid arthritis model of rats

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Abstract This study aimed to elucidate whether CSN1S2 protein of goat’s milk was able to inhibit pro-inflammatory cytokine [interleukin (IL)-17 and tumor necrosis factor (TNF)-α], RAGE, and caspase-3 expression in rheumatoid arthritis (RA) rats. A total of 24 Wistar female rats, were randomly assigned into four groups: control group (C), CSN1S2 protein of goat’s milk group (CM), CFA-induced RA-rats group (RA), and the RA group treated by CSN1S2 protein of goat’s milk (RAM). The severity of erythema and swelling in lower extremities were counted by scoring. IL-17, TNF-α, RAGE, and caspase-3 expression in synovial membranes were analyzed by confocal laser scanning microscopy (CLSM) and western blotting. Erythema and swelling in the RA group was significantly attenuated by goat’s milk CSN1S2 (p < 0.05), but did not reach the level in the control group (p < 0.05). The use of CLSM, CSN1S2 protein of goat’s milk could decrease the TNF-α, caspase-3, and the number of hyperplasia cells in comparison with the RA group (p < 0.05), to reach the level in the control group (p > 0.05). Western blotting analysis showed that the expression of IL-17, RAGE, TNF-α, and caspase-3 were higher in the RA group compared with the control group. CSN1S2 protein of goat’s milk decreased RAGE and TNF-α expression, but increased the IL-17 and caspase-3 expression. In conclusion, CSN1S2 protein of goat’s milk decreased erythema, swelling, and in inflammation in lower extremities. The CSN1S2 protein of goat’s milk also decreased TNF-α and RAGE expression in the synovial membrane of ankle joints. Unfortunately, CSN1S2 protein of goat’s milk may induce the production of IL-17. Therefore CSN1S2 protein of goat’s milk

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Introduction

One of the autoimmune diseases that is characterized by chronic inflammation due to various risk factors is rheumatoid arthritis (RA). The disease prognosis and development are related to synovial joint inflammation, cartilage destruction, and bone erosion. Proinflammatory cytokines that may affect joint damage in RA is interleukin (IL)-17 which is produced by T-helper (Th)17. Previous studies showed that IL-17 causes joint inflammation in mice through intra-articular injection. Otherwise, blocking of IL-17/IL-17R signaling may control the RA symptoms and prevent the joint degradations. This fact indicates that every compound that inhibits IL-17 production would be beneficial in rheumatoid arthritis.

Rheumatoid arthritis patients have a high level of advanced glycation end products (AGEs) in plasma and synovial fluid. AGEs react with their receptors (RAGEs) to induce oxidative stress. This reaction activates nuclear factor kappa beta (NF-κB) which triggers the over-expression of genes for cytokines, growth factors, and adhesive molecules. Intracellular signal transduction pathways, such as extracellular signal-regulated protein kinase (ERK), the p38 mitogen-activated protein kinases (p38MAPK), the c-Jun N-terminal kinase (JNK kinase), transcription of cytokine gene, growth factor gene, including tumor necrosis factor (TNF)-α or for apoptosis pathway, and the NF-κB (nuclear factor-κB) pathway activated by AGE-RAGE interaction. This finding also indicates that every substance that inhibits AGE-RAGE will have beneficial effects to RA progression. In addition, previous studies indicate that the modulation of synovial fibroblast apoptosis (marked using caspase-3) may contribute to the synovial hyperplasia and have beneficial effects for rheumatoid arthritis.

Bioactive peptides from food proteins represent a source of health enhancing components that may be incorporated into functional foods and/or used as nutraceuticals. Milk protein-derived bioactive peptides are health-enhancing components that can be used to reduce the risk of disease or to enhance certain physiological functions. Casein protein and electrophoretic casein fractions have been shown to exhibit different biological activities, such as immunomodulation. Our previous study reported that we found specifically the alpha-S2 casein (CSN1S2) protein has molecular weight (MW) 36 kDa only in goat milk which has no band in bovine milk; we also determined this 36 kDa contains eight peptides analyzed by matrix assisted laser desorption ionization time-of-flight. Previous in vitro study concluded that CSN1S2 protein of goat’s milk (0.100 mg/L) inhibits the decrease of viability due to increases in the proliferation of MC3T3E1 preosteoblast cells due to methyl glyoxal exposure. As far as we known, there is no study investigating the effect of CSN1S2 protein of goat’s milk in rheumatoid arthritis. Therefore, this study aimed to investigate whether CSN1S2 protein of goat’s milk is able to inhibit clinical severity and modulate the IL-17, RAGE, TNF-α, and caspase-3 expression of rheumatoid arthritis rats.

Materials and methods

Animal

Twenty four, 12-week-old, male Wistar rats, weighing 250–300 g, were obtained from the Laboratory of Experimental Animal, UPT LPPT, Gadjah Mada University, Yogyakarta, Indonesia. The animals were acclimatized for 1 week in Biosains Laboratory, Brawijaya University, Malang, Indonesia conditions prior to experimental procedure. They were exposed to a 12-hour light and 12-hour dark cycles at room temperature (24°C) and had free access to standard laboratory chow or drinking water ad libitum. After that, the animals were randomly divided into four groups (n = 6 each): control (untreated) group (C), CSN1S2 protein of goat’s milk group (CM), rheumatoid arthritis group (RA), and rheumatoid arthritis group treated by CSN1S2 protein of goat’s milk (RAM). The treatment with the CSN1S2 protein of goat’s milk was performed for 60 days.

Rat adjuvant-induced arthritis model

Rat adjuvant-induced arthritis model was performed according to previous studies with modification. Adjvant-induced arthritis in rats were established by a single subcutaneous injection of 100 μL of Complete Freund’s Adjuvant (CFA; Sigma–Aldrich Inc., Saint Louis, Missouri, USA). Fourteen days later, intradermal injection of 50 μL of FCA (Sigma–Aldrich Inc., Saint Louis, Missouri, USA) were performed into the lower extremity. The treatment with the CSN1S2 protein of goat’s milk was conducted 24 hours after the last injection.

Preparation and administration of casein

Isolation of CSN1S2 protein was performed on fresh goat’s milk. A 250-mL quantity of milk was heated to 40°C, glacial acetic acid was added and then stirred until a precipitate formed. The precipitate was separated by meshing with nylon. The protein concentration was measured by Nano Drop and stored at −20°C if not immediately used. CSN1S2 protein was isolated from 36 kDa protein sequence from SDS PAGE gel. CSN1S2 protein isolated from goat’s milk was orally administrated by gavage at a dose of 2 mg/kg body weight per day for 60 days.
weight according to a previous study with dose modification.\(^1^6\)

**Clinical sign of arthritis**

The sign of arthritis was assessed by evaluation of erythema and swelling in lower extremities. This assessment was conducted by three independent evaluators. The severity of arthritis in each of the two paws was graded in a scale of 0–4 according to established protocol and ranked as follows: 0 = no evidence of erythema and swelling (normal); 1 = erythema and mild swelling confined to the tarsals or ankle joint; 2 = erythema and mild swelling extending from the ankle to the tarsals; 3 = erythema and moderate swelling extending from ankle to metatarsal joints; and 4 = erythema and severe swelling encompass the ankle, foot and digits, or ankylosis of the limb.\(^1^7\)

**Histopathological analysis**

The rats were sacrificed at the end of the experiment, their synovial membrane of the ankle joint were isolated and fixed in 4% PFA. Afterwards, tissues were dehydrated with ethanol (96%), then cleared using xylene, and infiltrated using liquid paraffin at 56–57°C for 3 hours, the last embedded with paraffin and sectioned with 4 µm thickness. Histopathological profile of the ankle synovial membrane was stained by hematoxylin–eosin (HE). Deparaffinization using xylene, dehydration with alcohol series (absolute ethanol, 90 until 70%), then slides were washed with phosphate buffered saline (PBS) for 15 minutes. Thereafter slides were first stained with hematoxylin then washed with running water. Afterwards slides were stained with eosin and washed with running water. Slides were dehydrated again with 70% and 90% ethanol, each for 2 minutes, followed by absolute ethanol. Then the slides were immersed in xylene for 10 minutes, and then dried and covered with Entellan R (Merck, Darmstadt, Germany) then covered by cover glasses. These slides were observed using Olympus BX53 microscope (Olympus Corporation, Tokyo, Japan) with CellsSens Standart software version 1.5 (Olympus Corporation, Tokyo, Japan) and evaluated by a pathology expert.

**Double-labeling immunofluorescence staining**

Immunofluorescence staining was conducted to determine the IL-17, RAGE, TNF-α, and caspase-3 protein expression on synovial membrane according to previous studies with little modification.\(^1^8\) Slides were incubated with first primary anti IL-17 rabbit (1:1000 dilution; Santa Cruz Biotechnology Inc., USA) and first of secondary mouse antirabbit IgG-FITC (1:1500; Santa Cruz Biotechnology Inc., USA) for 1 hour each in dark conditions. Slides were stained secondly with of primary antiRAGE mouse monoclonal (1:1500 dilution; Santa Cruz Biotechnology Inc., USA) antibodies and secondly with secondary goat-anti mouse IgG-R (1:1500; Santa Cruz Biotechnology Inc., USA) antibodies each for 1 hour in dark conditions. By using a similar technique, we also performed immunofluorescence staining for TNF-α and caspase-3 expression. To visualize and measure the IL-17, RAGE, TNF-α, and caspase-3 expressions were analyzed using Olympus confocal laser scanning microscopy (CLSM; Olympus Corporation, Tokyo, Japan). Slides were captured on three fields. IL-17, RAGE, TNF-α, and caspase-3 expression were quantified with the Olympus FluoView software version 1.7a (Olympus Corporation, Tokyo, Japan).

**Immunoblotting of IL-17, RAGE, TNF-α, and caspase-3**

Protein (10 mg/mL) of synovial membrane was separated in 12.5% SDS–polyacrylamide gels electrophoresis and blotted into a nitrocellulose membrane. Nonspecific protein-binding sites were blocked by overnight incubation of 5% skim milk in PBS. The membrane was subsequently incubated overnight at 4°C with rabbit anti-IL17, mouse antiRAGE, rabbit antiTNF-α, and mouse anticaspase-3 (Santa Cruz Biotechnology). The membranes were washed and incubated for 1–2 hours at room temperature with peroxides-labeled polyclonal antirabbit IgG and antimouse IgG (KPL, Gaithersburg, Maryland, USA). The antigen–antibody complexes were visualized with Western Blue Stabilized Substrate (KPL, Gaithersburg, Maryland, USA). The reaction was stopped by the addition of sterile aquadest. Membrane-labeled was visualized by ChemiDoc Imaging (Bio-Rad Laboratories Inc., Hercules, USA) and the intensity of specific protein bands were counted by Quantity One software. (Bio-Rad Laboratories Inc., Hercules, USA).

**Ethics**

This research has been approved by research ethics committee Faculty of Brawijaya University, Malang, East Java, Indonesia (Registration number, KEP-90-UB).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Body weight, daily intake, clinical scoring, and hyperplasia in each group.</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>372.49 ± 30.12</td>
</tr>
<tr>
<td>Daily intake (g)</td>
<td>23.61 ± 2.26</td>
</tr>
<tr>
<td>Scoring (unit)</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Hyperplasia (%)</td>
<td>6.67 ± 0.58</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD.

*\(p < 0.05\) in comparison with control group.

**\(p < 0.05\) in comparison with CM group.

***\(p < 0.05\) in comparison with RA group.

%= percentage; CM = CSN1S2 protein of goat’s milk group; g = gram; RA = rheumatoid arthritis group; RAM = rheumatoid arthritis group treated by CSN1S2 protein of goat’s milk.
Statistical analysis

Data are presented as mean ± SD and differences among groups were analyzed using analysis of variance and Honestly Significant Difference test using SPSS 16.0 statistical package (SPSS Inc., Chicago, Illinois, USA). A p value < 0.05 was considered statistically significant.

Results

Clinical sign of arthritis

Clinical severity of lower extremities in control and experimental groups was measured by scoring, as given in Table 1. The level of scoring was higher significantly in the

| Table 2 | Expression of IL-17, TNF-α, RAGE, and caspase-3 in all experimental groups. |
|---------|------------------|----------------|----------------|
|          | Control | CM       | RA             | RAM             |
| IL-17 (int/μm) | 535.41 ± 359.82 | 603.58 ± 245.36 | 1196.09 ± 354.89 | 724.33 ± 813.24 |
| TNF-α (int/μm) | 144.27 ± 21.07  | 161.54 ± 23.72 | 720.62 ± 80.30*** | 243.73 ± 32.86*** |
| RAGE (int/μm)  | 543.77 ± 306.32 | 600.88 ± 415.39 | 1163.95 ± 441.26 | 643.64 ± 554.32 |
| Caspase-3 (int/μm) | 213.85 ± 30.95 | 251.22 ± 53.81 | 543.18 ± 85.83*** | 307.96 ± 52.71*** |

Values are presented as mean ± SD.
*p < 0.05 in comparison with control group.
**p < 0.05 in comparison with CM group.
***p < 0.05 in comparison with RA group.
CM = CSN1S2 protein of goat’s milk group; IL-17 = interleukin 17; int/μm = intensity/micrometer; RA = rheumatoid arthritis group; RAGE = receptor for advanced glycation end products; RAM = rheumatoid arthritis group treated by CSN1S2 protein of goat’s milk; TNF-α = tumor necrosis factor-α.

Figure 1 Micrograph illustrating the expression of RAGE and IL-17 in the synovial membrane of each group. Immunofluorescence of RAGE was labeled with FITC staining (green), IL-17 was labeled with Rhodamine (red) (Magnification ×400; bar is 30 μm). C = control group; CM = CSN1S2 protein of goat’s milk group; HE = hematoxylin eosin; IL-17 = interleukin-17; RA = rheumatoid arthritis group; RAGE = receptor for advanced glycation end products; RAM = rheumatoid arthritis group treated by CSN1S2 protein of goat’s milk.
RA group than the control group \((p > 0.05)\). CSN1S2 protein of goat’s milk significantly attenuated the erythema and swelling degree compared with the RA group \((p < 0.05)\), but can not reach the level in the control group \((p < 0.05)\).

**Body weight and intake**

Table 1 also presented the body weight and level of intake in each group. The body weight was lower significantly in the RA group compared with the control group \((p < 0.05)\), but the daily quantity of intake was not significantly different between the groups \((p > 0.05)\).

**Hyperplasia cell**

The number of hyperplasia cells in synovial membranes was higher significantly in the RA group compared with the control group \((p > 0.05)\). Administration of CSN1S2 protein of goat’s milk was able to decrease the number of hyperplasia cells compared with the RA group \((p < 0.05)\), to reach the level in the control group \((p > 0.05)\), as seen in Table 1.

**IL-17, RAGE, TNF-\(\alpha\), and caspase-3 expression**

The expression of IL-17, RAGE, TNF-\(\alpha\), and caspase-3 in the control and experimental groups was presented in Table 2, and Figs. 1 and 2. Confocal micrographs show that the IL-17 and RAGE expression were not significant in all groups \((p > 0.05)\). A confocal micrograph of TNF-\(\alpha\) and caspase-3 was higher significantly in RA compared with control group \((p < 0.05)\). Indeed, CSN1S2 protein of goat’s milk reduces the level of TNF-\(\alpha\) and caspase-3 significantly than the RA group \((p < 0.05)\), to reach similar levels at a control group \((p > 0.05)\). Western blotting analysis showed that the expression of IL-17, RAGE, TNF-\(\alpha\), and caspase-3 were higher in the RA group compared with the control group. CSN1S2 protein of goat’s milk decrease RAGE and TNF-\(\alpha\) expression, but increase the IL-17 and caspase-3 expression, as seen in Fig. 3.

**Histopathological of synovial membrane**

Synovial membrane of lower extremities in each group are presented in Fig. 4. Pathological section from synovial membranes of control (untreated) rats show lipid cells proliferation with minimal connective tissue. The

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**Figure 2**  Micrograph illustrating the expression of TNF-\(\alpha\) and caspase-3 in synovial membrane of each group. Immunofluorescence of TNF-\(\alpha\) was labeled with FITC staining (green), caspase-3 was labeled with Rhodamine (red) (Magnification \(\times 400\); bar is 30 \(\mu\)m). C = control group; CM = CSN1S2 protein of goat’s milk group; CSP-3 = caspase-3; HE = hematoxylin eosin; RA = rheumatoid arthritis group; RAM = rheumatoid arthritis group treated by CSN1S2 protein of goat’s milk; TNF-\(\alpha\) = tumor necrosis factor-\(\alpha\).
Figure 3  (A) Immunoblotting; (B) density and intensity level of IL-17; (C) density and intensity level of TNF-α; (D) density and intensity level of RAGE; and (E) density and intensity level of caspase-3 in synovial membrane. C = control group; CM = CSN1S2 protein of goat’s milk group; IL-17 = interleukin-17; RA = rheumatoid arthritis group; RAGE = receptor for advanced glycation end products; RAM = rheumatoid arthritis group treated by CSN1S2 protein of goat’s milk; TNF-α = tumor necrosis factor-α.

Figure 4  Micrograph illustrating the histopathology of synovial membrane of lower extremity in each group; (A) synovial membrane of control (untreated) rats show lipid cells proliferation (red arrow) with minimal connective tissue (blue arrow); (B) CSN1S2 protein of goat’s milk in control rats induces the proliferation of lipid cells in larger size (red arrow) accompanied by growth of fibrous connective tissue (blue arrow) in separated morphology; (C) in the RA group, we found the dominancy of connective tissue (blue arrow), minimal lipid cells (red arrow) and massive inflammatory cells (green arrow); and (D) CSN1S2 protein of goat’s milk induces the connective tissue hardening and minimal inflammation/granulation and lytic process in connective tissue in RA group. Hematoxylin eosin staining (Magnification ×40; bar is 2 μm).
treatment of CSN1S2 protein of goat’s milk in control rats induces the proliferation of lipid cells of a larger size accompanied by growth of fibrous connective tissue in separated morphology. In the RA group we found the dominancy of connective tissue, minimal lipid cells, and massive inflammatory cells. CSN1S2 protein of goat’s milk induces the connective tissue hardening and minimal inflammation/granulation and lytic process in connective tissue in the RA group.

Discussion

The chronic soft tissue inflammation will determine the disease progression of RA. In this study we used Freund’s complete adjuvant to induce the arthritis process. The inactivated mycobacterium is the main active substance in Freund’s complete adjuvant to induce the arthritis process. The goat’s milk is able to act as an anti-inflammatory to inhibit (RAGE, TNF-α, caspase-3). Western blotting data showed that the expression of IL-17, TNF-α, and caspase-3 were higher in the RA group compared with the control group (p < 0.05) probably because of overproduction of TNF-α and IL-1. The nutritional status of RA patients was compromised despite adequate intake. The increased production of inflammatory cytokines affects the energy metabolism and protein metabolism, leading to a loss of body cell mass, namely as rheumatoid cachexia.

This study showed that in the RA group, we found the dominancy of connective tissue with minimal lipid cells. Conversely, synovial membrane of control (untreated) rats show lipid cell proliferation with minimal connective tissue. Our finding suggested that the CFA model established in this study was successful and stable. However, in RAM groups, we found the connective tissue hardest and minimal inflammation/granulation and lytic process in connective tissue, which indicated that the CSN1S2 protein of goat’s milk may induce the remodelling and decrease of inflammation in the synovial membrane. We found the level of scoring was higher significantly in the RA group than that of the control group (p < 0.05), and CSN1S2 protein of goat’s milk significantly reduced both parameters compared with the RA group (p < 0.05). This finding indicates that the CSN1S2 protein of goat’s milk is able to act as an anti-inflammatory to inhibit the erythema and swelling in the lower extremity.

Our confocal data show that the expression of IL-17 and RAGE were insignificant between groups (p > 0.05). Western blotting data showed that the expression of IL-17, RAGE, TNF-α, and caspase-3 were higher in the RA group compared with the control group. Previous arthritis mouse models that were induced by collagen showed that there were two stages; the early stage was dependent on the level of TNF stage and the advanced stage disease was mostly performed by IL-17. We propose that our model is established symptom in RA, in this study we found the level of body weight was significantly decreased in the RA group compared with the control group (p < 0.05) probably because of overproduction of TNF-α and IL-1. The increased production of inflammatory cytokines affects the energy metabolism and protein metabolism, leading to a loss of body cell mass, namely as rheumatoid cachexia.

In conclusion, CSN1S2 protein of goat’s milk decrease erythema, swelling, and inflammation in lower extremities. CSN1S2 protein of goat’s milk also decreased TNF-α and RAGE expression in synovial membranes of ankle joints. Unfortunately, CSN1S2 protein of goat’s milk may induce the production of IL-17.

Conflicts of interest

All authors declare that there is no conflict of interests regarding the publication of this article.

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