Inhibitory effect of plant extract on tumor necrosis factor-α formation from carboxymethyllysylsine stimulated macrophages

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Abstract

Objective: Proteins modified with \(N^\epsilon\)-carboxymethyllysylsine (CML), one of the advanced glycation end products (AGEs), activate macrophages and increase tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)) production. In terms of preventive medicine, it is meaningful to suppress the production of TNF\(\alpha\) which is involved in various lifestyle-related diseases. In this study, we examined the inhibitory effect of a plant extract on AGE-stimulated TNF\(\alpha\) production of CML-stimulated cultured macrophages.

Methods: The mouse macrophage cell line RAW264.7 and, as a stimulator, human serum albumin (HSA) with CML modification (CML-HSA) was used. Test samples were extracts from the following 5 plants and also a mixture of the latter 4 plants: water chestnut (Trapa bispinosas), banabá (Lagerstroemia speciosa), Chinese blackberry (Tien-cha) (Rubus suavissimus), persimmon leaves (Diospyros kaki), and kuma bamboo (Kumazasa) (Sasa veitchii). TNF\(\alpha\) concentration in the culture medium was measured by ELISA after CML-HSA stimulation with or without the pre-treatment of these test samples.

Results: TNF\(\alpha\) production from RAW264.7 cells was significantly elevated 3 hours after adding CML-HSA to the medium (p < 0.001). Pretreatment with extracts of water chestnut, banabá, Chinese blackberry, persimmon leaf, and mixed plant extract inhibited the TNF\(\alpha\) production by 14.6%-22.7%. Cell viability was not affected by the pre-treatment with these extracts.

Conclusion: The data that CML-HSA-stimulated TNF\(\alpha\) production from macrophages was significantly reduced by pre-treatment with extracts of water chestnut, banabá, Chinese blackberry, persimmon leaf, or kuma bamboo, indicated that the components contained in these plants may have a favorable action against the glycate stress-induced inflammation.

KEY WORDS: glycative stress, advanced glycation end products (AGEs), inflammation, receptor for AGE (RAGE), \(N^\epsilon\)-carboxymethyllysylsine (CML)
cultured macrophages were stimulated by human serum albumin (HSA) modified with CML (CML-HSA), and assessed, using this model, the actions of 5 kinds of plant extracts (water chestnut, banaba, Chinese blackberry, persimmon leaf, and kuma bamboo), that are known to show a high activity preventing AGE formation in the in vitro reaction between glucose and albumin.

**Methods**

**Plant extract preparation**

Materials were provided from the following companies: water chestnut extract from Hayashikane Sangyo (Yamaguchi, Japan) and banaba, Chinese blackberry, persimmon leaf, kuma bamboo, and a mixture of these 4 leaves from Hikawa (Shimane, Japan). Each leaf 1.0 g was immersed in 40 mL distilled water at 80°C for 1 hour, followed by centrifugation, and then extracts were prepared. Powered water chestnut extract 0.6 g was dissolved in 40 mL distilled water.

**Cell culture**

Cultured macrophage, RAW264.7, was provided from Prof. Yukiko Minamiyama, Kyoto Prefectural University. As previously reported [12], RAW264.7 was cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) added to 10% fetal bovine serum (FBS) (Nichirei Biosciences, Tokyo, Japan) containing penicillin 10,000 units/mL, streptomycin 10 mg/mL and amphotericin B 25 μg/mL at 37°C under the condition of 5% CO2.

For the evaluation of CML-HSA-induced inflammation, cells, adjusted as 5 × 10^5 cells/mL, were cultured for 24 hours in 24-well plate dishes, followed by stimulation by 0.5~1.0 μg/mL CML-HSA (CircuLex, Nagano, Japan). For the assessment of plant extracts (water chestnut, banaba, Chinese blackberry, persimmon leaf, and kuma bamboo), each sample, diluted with DMEM to the final concentration of 1.0~10 μg/mL, was applied and incubated 24 hours before CML-HSA (0.5 μg/mL) stimulation. The medium supernatant after CML-HSA stimulation was used for measurement of TNFα by enzyme-linked immunosorbent assay (ELISA).

**Murine TNFα measurement**

TNFα concentration in the cell culture medium was measured by ELISA as previously reported [12]. Briefly, 0.8 μg/mL mouse TNF-alpha antibody (R&D Systems, Minneapolis, MN, USA), diluted with 50 mM carbonate buffer (pH 9.6), was added to 96-well plates (Thermo Scientific, Waltham, MA, USA) and converted into solid phase. After 2-hour blocking of the reaction by 0.3% skim milk (Difco Laboratories, Detroit, MI, USA), each well was incubated for 1 hour with medium supernatant diluted with DMEM. Then wells were reacted with 0.1 μg/mL mouse TNFα biotinylated antibody (R&D Systems) for 2 hours, followed by 30-minute standing with application of Pierce Streptavidin Poly-HRP Conjugate (Thermo). After 15-minute coloring reaction with TMB One Component HRP Microwell Substrate (Surmodics, Eden Prairie, MN, USA), the reaction was stopped by H 2 SO 4 (Wako Pure Chemical Industries, Osaka, Japan), and absorbance at 450 nm was measured by SpectraMax Paradigm Multi-Mode Detection Platform (Molecular-Devices Japan). The TNFα concentration was calculated by the standard curve prepared by recombinant mouse TNF-alpha aa80-235 (R&D Systems) with known concentrations (15.6 pg/mL~1,000 pg/mL).

**Cell viability assessment**

When CML-HSA and plant extracts were applied to the Kit-8 (CCK-8; Dojindo, Kumamoto, Japan), in which viable cell numbers can be counted by colorimetry determination of water-soluble formazan reduced by intracellular dehydrogenase. This assessment was conducted parallel with TNFα measurement.

Cells, adjusted to 5 × 10^5 cells/mL, were inoculated into 96-well plates followed by application of 100 μL each test sample in the same manner as in the anti-inflammation evaluation. After 3 hours of incubation with CML-HSA, cells were reacted with 10 μL CCK-8 for 1 further hour. Absorbance at 490 nm was measured by SpectraMax Paradigm Multi-Mode Detection Platform (Molecular-Devices Japan). The wells were prepared as follows: test sample (S), no-reagent-applied well as the negative control (C), and no-cell-inoculated well as the blank (B). Viability was calculated using the following equation

\[
\text{Cell viability (\%)} = \frac{(\text{Abs. (S)} - \text{Abs. (B)})}{(\text{Abs. (C)} - \text{Abs. (B)})} \times 100
\]

**Anti-oxidative activity measurement**

Anti-oxidative activity was evaluated by using i-Pack Oxystress Test [13] (ARKRAY, Kyoto Japan). Results of anti-oxidative activity was expressed as ascorbic acid equivalent (mg/L) using ascorbic acid as the standard reagent.

**Statistical analysis**

Data were expressed as mean ± standard deviation (SD). All statistical analyses were performed using SPSS II Statistics 22 (IBM Japan, Tokyo, Japan). Dunnett’s test was used for intergroup comparison in the TNFα inhibition study and cell viability assessment. Differences were considered significant at a significance level of 5%.

**Results**

**CML-HSA-stimulated TNFα formation in RAW264.7 cells**

CML-HSA (0.5~1.0 μg/mL) was applied onto RAW264.7 for 1~6 hours and the release of TNFα was measured by ELISA. After 3- and 6-hour stimulation, TNFα (p<0.001, 0.5 μg/mL and p<0.001, 1.0 μg/mL) was significantly increased compared with control (Fig. 1).
Inhibition by plant extracts on CML-HSA-induced TNFα formation

The following extracts were reacted with RAW264.7 for 24 hours and diluted with DMEM, medium: water chestnuts (1.0 μg/mL), banabá and Chinese blackberry (10 μg/mL), and persimmon leaf and kuma bamboo (5.0 μg/mL). The TNFα concentration was measured after 3-hour stimulation with CML-HSA (0.5 μg/mL). TNFα production was significantly reduced by the pretreatment with water chestnut (22.7%, p < 0.001), banabá (17.2%, p < 0.001), Chinese blackberry (17.0%, p < 0.001), persimmon leaf (14.6%, p = 0.001), and mixed extract (17.2%, p < 0.001), when compared with CML-HSA-stimulation without pretreatment (Fig. 2). The mixed extract (Mix) consisted of banabá, Chinese blackberry, persimmon leaf, and kuma bamboo.

Cell viability assessment

The influence on cell viability by CML-HSA and plant extracts was evaluated. The number of viable cells was significantly increased by CML-HSA stimulation compared with the control (p = 0.018). There was no significant change in viable cell numbers in the plant extract-pretreatment group.
Effect of Plant Extract on INFα Formation from CML Stimulated Macrophage

compared with the CML-HSA-stimulated group without pretreatment (Fig. 3).

Anti-oxidative activity measurement

Anti-oxidative activity of 4 test samples were as follows: banabá 2,243 mg/L, Chinese blackberry 1,882 mg/L, persimmon leaf 604 mg/L and kuma bamboo 200 mg/L. Each result is expressed as ascorbic acid equivalent.

Discussion

In this study, using cultured macrophage cells, RAW264.7, stimulated by CML-HSA as a glycative stress, TNFα formation was measured and the anti-inflammatory actions of plant extracts were assessed. The following receptors are known to bind to AGEs as a ligand on the cell surface in addition to RAGE: macrophage type-I and type-II class A scavenger receptors (MSR-A), CD36・SR-BI and LOX-1 (lectin-like oxidized low density lipoprotein receptor-1) which belong to the MSR-B family, galectin-3 complex, FEEL1・2 (fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1・2), megalin, and toll-like receptor 4 (TLR4) 14. These receptors play roles in inflammation reaction, degeneration and removal of AGES, or cell proliferation. RAGE participates in inflammation, recognizes proteins with CML modification or glyceraldehyde-derived AGES (toxic AGES; TAGE) which are known to be cytotoxic 10. Mediated by NF-κ B activation, RAGE induces inflammatory cytokines (i.e. TNFα, interleukin [IL]-β, IL-6), matrix metalloproteinase (MMP), vascular endothelial growth factor (VEGF) and adhesion factors (i.e. intercellular adhesion molecule-1 [ICAM-1], vascular cell adhesion molecule-1 [VCAM-1]) 16. The CML-HSA-stimulated TNFα formation observed in this study (Fig. 1) may be mainly due to the RAGE actions. Precise analysis is necessary for further investigations to determine the specific contributions of each receptor.

CML accumulation induces chronic inflammation in tissue. Released inflammatory cytokines are reported to play a pathogenetic role in Alzheimer’s disease 15, age-related macular degeneration 16, cancer 17, and bronchial asthma 18, and also to inhibit activation of Sir1 (Sirtuin 1) 21 which is known as a longevity gene.

In addition to these disorders, "locomotive syndrome," which has recently attracted attention in preventive medicine, has been suggested in relation to glycate stress, and glycate stress may reduce the bone quality by forming pathological cross-links between bone collagen fibers 22. Inflammatory cells infiltrated into the synovium induce synovitis, which plays a significant role in the pathogenesis of rheumatoid arthritis and knee osteoarthritis 23. The TNFα produced in the synovium induces the expression of the transcription factor CCAAT/ enhancer-binding protein (C/EBP) β, through the induction of inflammatory mediators (i.e. prostaglandins) and vascular endothelial growth factor (VEGF), and enhances inflammation and causes articular swelling. Furthermore, TNFα activates MMP-3 and MMP-13, cartilage matrix degrading enzymes, and finally causes the degeneration and destruction of cartilage 24. In addition, TNFα induces the expression of receptor activator of nuclear factor kappa-B ligand (RANKL), essential for the differentiation of osteoclasts, and accelerates bone resorption, thus increasing the risk of osteoporosis developing 25. On the other hand, since the half-life of cartilage collagen is long, CML-modified proteins are abundant in tissue 25. It has been suggested that AGE deposits in the joint cartilage tissue may participate in the pathogenesis and progress of osteoarthrosis of the knee 26. It is possible that the CML-modified protein-induced inflammatory substance in the cartilage may be involved in the pathogenesis of locomotive syndrome and in the hypofunction of locomotive organs in a similar manner as in the synovium.

The AGES-RAGE interaction is related to various lifestyle-related diseases. Therefore it is important to prevent TNFα production, which is produced in the early phase of
inflammation and influences the inflammatory response and injury. The common component in the anti-inflammation action of the 5 plant extracts in this study has not yet been reported. The following actions are indicated by the polyphenol included in each extract. Tannin is included in water chestnut peel extract which inhibits rat hind limb edema. In a randomized controlled trial using banabá extract, production of the adhesion molecules ICAM-1 and VCAM-1, which are related to inflammation, was inhibited in patients with type 2 diabetes. The main polyphenol of Chinese blackberry (Tiencha) is galloyl-oxygen-diphenyl (GOD) type ellagitannin; it is reported that Chinese blackberry extract prevents the release of leukotriene B4 from the rat peritoneal macrophage thus manifesting an anti-inflammation action. The persimmon leaf flavonoid is effective for inflammatory disturbance in the nervous system in cases of ischemia reperfusion injury. There is also a report that proanthocyanidin inhibits RAGE manifestation. In addition to its anti-inflammatory action, persimmon leaf proanthocyanidin improves hyperglycemia and dyslipidemia in the type 2 diabetes model mouse. Ellagitannin in banabá extract possesses an insulin-like action and activates the glucose transport in adipocytes. Therefore these components contribute to the improvement of glucose metabolism. Although the precise identification of the responsible ingredients was not undertaken in the present study, the results that these polyphenols prevent TNFα production and support disease prevention are considered to be worthy findings.

It's confirmed that the plant extracts used in this study indicate the inhibitory actions of in vitro AGE formation which is stronger than that of aminoguanidine (Fig. 2). It is not likely that each plant extract specifically inhibits the CML-HSA-stimulated TNFα formation. However, we can add the new action of AGE-induced inflammatory response prevention in addition to AGEs generation inhibition.

The anti-oxidative activity of the 4 extracts in the mixed plant extract, as measured by i-Pack Oxystress Test, showed an ascorbic acid equivalent of banabá 2,243 mg/L, Chinese blackberry 1,882 mg/L, persimmon leaf 604 mg/L and kuma bamboo 200 mg/L. According to "Standard Dietary Intake in Japan (2010)" guidelines announced by the Ministry of Health, Labour and Welfare, the daily recommendation value of ascorbic acid is 100 mg/day for adults. The ascorbic acid equivalent of the mixed plant extract is markedly large compared with the recommended value. Since the oxidative stress is known to promote the glycative reaction and increase the AGE production in the skin or other tissues, it is also important to control the oxidative reaction. These plant extracts may contribute to reduce glycative stress by acting as antioxidants.

Conclusion

In the present study model, employing TNFα formation from CML-HSA-stimulated macrophages as the index, some plant extracts inhibited TNFα formation, thus reducing inflammatory reaction. These actions of plant extracts may be useful since inflammation induced by glycative stress occurs in the body. As the next step, we plan to study the correlation between CML and TNFα in the body and also the inhibitory effect of these extracts on CML and TNFα in a clinical study.

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Conflict of interest statement

The authors have no conflict of interest related to this study to declare.

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