Human rheumatoid arthritis is an inflammatory disease that affects about 1% of the adult population worldwide (1). It is characterized by systemic and local inflammation resulting in destruction of cartilage and bone (1). The disease-modifying anti-rheumatic drugs (DMARDs) used for most commonly prescribed medication of rheumatoid arthritis treatment work by improving the immunological abnormalities (2, 3). However, the therapeutic effects of DMARDs are also not always satisfactory, because of their efficacy and side effects. On the other hand, non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to have therapeutic effects on rheumatoid arthritis in spite of their gastric toxicity (4). Although NSAIDs have beneficial effects on pain and edema, they have no effect on the basic process in the joint lesions (5).

In a previous study, it has been reported that some food components such as glucosamine (6) and a-linked galactooligosaccharide (7) were effective on the joint lesions in rat adjuvant arthritis or mouse collagen-induced arthritis. In addition, it was recently demonstrated that casein hydrolysate prepared from Aspergillus oryzae protease was also effective for the treatment of rat adjuvant arthritis (8). It was explained that an oral administration of this substance had efficacy on the arthritis through mediation of the anti-inflammatory and/or immunomodulatory action (8). The results disclosed evidence that the substance may have efficacy as a functional food against rheumatoid arthritis in humans.

Flavangenol (FG), one trade name (registered trademark) of extract of French maritime pine bark (Pinus maritima), is a rich source of water-soluble polyphenols (oligomeric proanthocyanidins, OPCs) (9). OPCs are a class of phenolic compounds, which acquire the form of oligomers or polymers of polyhydroxy flavan-3-ol units, such as (+)-catechin and (−)-epicatechin (9). OPCs, predominant food sources of which are red wine, tea, chocolate and fruits like apples, grapes, pears, and cranberries, are present in plants in a complex mixture of polymers (10). OPCs have powerful antioxidative activity (11) and have been shown to prevent or attenuate atherosclerosis (12), diabetes (13, 14), and hypertension (15). Furthermore, it has been shown that another French maritime pine bark extract had anti-inflammatory effects on carrageenan-induced paw edema (16) and UV radiation (17) in animals and osteoarthritis (18) in humans.

Based on these findings, we hypothesized that FG might have a therapeutic effect on inflammatory disorders in the joints of rheumatoid arthritis models. In the present study, we fed an FG diet to the rats for 4 wk, and evaluated the effects on the progression of inflammation.

Effects of Flavangenol, an Extract of French Maritime Pine Bark on Collagen-Induced Arthritis in Rats

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Summary Flavangenol (FG), an extract of French maritime pine bark (Pinus maritima) mainly contains proanthocyanidin in oligomers. It has many physiological effects, including antioxidant and anti-atherosclerosis. In this study, we evaluated the effects of FG on rat collagen-induced arthritis, a model of human rheumatoid arthritis. The rats were fed with the diet of control, 0.3% FG, or 1% FG for 4 wk after the induction of arthritis. The FG diets, compared with the control diet, suppressed the increase in articular score and swelling of the paws in a dose-dependent manner. Histopathological examination revealed evidence that the 1% FG diet suppressed acute and chronic articular lesions in the rats. In addition, the FG diets (0.3% and 1%) suppressed the production of nitric oxide in the plasma of the rats. These results suggest that dietary FG has beneficial effects on collagen-induced arthritis in rats by inhibiting the acute and chronic inflammatory reactions.

Key Words Flavangenol, pine bark extract, collagen-induced arthritis, nitric oxide, anti-inflammation

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MATERIALS AND METHODS

Animals. Female DA rats (7 wk of age) were purchased from Japan SLC, Inc. (Shizuoka, Japan), and were used. The animals were given a period of 1 wk to adjust to the new environment (room temperature, 24°C ± 3°C; relative humidity, 55% ± 15%; 12 h light/12 h dark illumination cycle). The rats were kept in specific pathogen free (SPF) conditions. The rats received standard powder feed (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water ad libitum. All procedures using the animals were in accordance with the Guidelines for the Care and Use of Experimental Animals of the Japanese Association for Laboratory Animal Science and were approved by the Animal Use and Care Committee of Mercian Cleantec Corporation (Experimental No. 08R309).

Preparation of diets. FG used in this study was a commercial product, and produced by Toyo Shinyaku Co., Ltd. (Saga, Japan). The FG contains 72.5% polyphenols (determined by the Folin-Denis method) including 5% proanthocyanidin B1, 2.98% catechin and 0.23% epicatechin. Ibuprofen, one of the NSAIDs was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and was employed as a positive control drug. After a 1-wk adjustment period, the rats were randomly separated into a non-induction group (normal) and an induction group. The latter was further separated into four diet groups: control group, 0.3% FG group, 1% FG group, and 0.05% ibuprofen group. Bovine type II collagen was dissolved and emulsified with an equal volume of Freund’s incomplete adjuvant. Each rat was injected intradermally with the emulsion in four sites on the back. The day of induction was designated as day 0. Treatment with the diet of FG or ibuprofen was started at day 0 and continued for 28 d. Each point represents the mean±SE of eight animals in an experimental group. *p<0.05, **p<0.01 and ***p<0.001 vs. the control.

Fig. 1. Effects of Flavangenol (FG) and ibuprofen on the body weight changes in collagen-induced arthritic rats. After a 1-wk adjustment period, the rats were randomly separated into a non-induction group (normal) and an induction group. The latter was further separated into four diet groups: control group, 0.3% FG group, 1% FG group, and 0.05% ibuprofen group. Bovine type II collagen was dissolved and emulsified with an equal volume of Freund’s incomplete adjuvant. Each rat was injected intradermally with the emulsion in four sites on the back. The day of induction was designated as day 0. Treatment with the diet of FG or ibuprofen was started at day 0 and continued for 28 d. Each point represents the mean±SE of eight animals in an experimental group. *p<0.05, **p<0.01 and ***p<0.001 vs. the control.

Fig. 2. Effects of Flavangenol (FG) and ibuprofen on the arthritic score in collagen-induced arthritic rats. See legend to Fig. 1. Arthritis of forepaws (A) and hind paws (B) was clinically evaluated and scored by grading each paw from 0 to 4 points based on erythema and swelling of the joint. Each point represents the mean±SE of eight animals in an experimental group. *p<0.05, **p<0.01 and ***p<0.001 vs. the control.

Fig. 1:

- **Normal**
- **Control**
- **FG 0.3%**
- **FG 1%**
- **Ibuprofen 0.05%**

Fig. 2:

- **Normal**
- **Control**
- **FG 0.3%**
- **FG 1%**
- **Ibuprofen 0.05%**

Induction of arthritis. Bovine type II collagen was purchased from Cosmo-Bio Co., Ltd. (Tokyo, Japan) and was dissolved in 0.1% acetic acid at a concentration of 2 mg/mL with constant mixing overnight at 4°C. Then the solution was emulsified with an equal volume (at a 1:1 ratio) of Freund’s incomplete adjuvant (Difco Labs., Detroit, MI, USA), and each rat was injected intradermally with 200 μL of the emulsion containing 200 μg of collagen in four sites on the back under light ether anesthesia. The day of induction was designated...
as day 0.

Clinical evaluation of arthritis. The rats were clinically observed for characteristic signs and symptoms. The rats were scored by grading each paw, from 0 to 4 based on the erythema, swelling and rigidity of the joint (0 = no erythema or swelling; 1 = erythema or swelling of one toe; 2 = erythema or swelling of two or more of the toes; 3 = erythema and swelling of the entire paw; 4 = complete erythema and swelling of the entire paw and incapacity to bend the ankle). All four legs were scored and the maximum possible score reached 16 (4 points for each paw). The volume of swelling was also measured in both hind paws with a foot volume meter (TK-105, Muromachi Kikai Co., Ltd., Tokyo, Japan) and the average of the volume in each rat was calculated.

Histopathological evaluation of arthritis and the plasma analysis. Blood samples were taken from the abdominal aorta under ether anesthesia on day 28 after the induction of arthritis. The heparin-anticoagulated blood was centrifuged at 2,200×g for 10 min at 4˚C, and then the supernatant was stored at 80˚C as blood plasma.

The limbs were then dissected and fixed in 10% neutral buffered formalin. After decalcification with saturated ethylene diamine tetracetic acid (EDTA), the joints were longitudinally sectioned, and tissue sections (10 μm) were mounted on glass slides and stained with hematoxylin and eosin. Articular lesions were observed under an optical microscope, histopathological findings being graded into four levels (− = intact; + = mild; ++ = moderate; +++ = severe). Multiplication of the synovial lining cells was evaluated as “intact,” three layers or less; “mild,” four or five layers; “moderate,” six to eight layers; and “severe,” nine layers or more of the cells.

Plasma samples were used for measuring the NO level. Total NO (nitrite and nitrate) was measured by the Griess method, using a nitrite/nitrate CII colorimetric assay kit (Dojin Chemical Labs., Kumamoto, Japan).

Statistical analysis. All data are expressed as the mean ± standard error (mean ± SE). The results in each group were compared by one-way analysis of variance (ANOVA) followed by Dunnett’s test or Steel’s test, as appropriate. A p value of less than 0.05 was considered to be statistically significant.

RESULTS

Body weight change with collagen-induced arthritis in the rats

The body weight gains in all the groups treated with

| Number of examined rats |
|-------------------------|
| Normal | Control | FG 1% | Ibuprofen 0.05% |
| − | + | ++ | +++ | − | + | ++ | +++ | − | + | ++ | +++ |

Multiplication of synovial lining cell layer

8 0 0 0 0 5 3 0 7 1 0 0 4 2 2 0

Subsynovial soft tissue edema

8 0 0 0 0 1 2 5 7 1 0 0 4 0 1 3

Fibrin exudation

8 0 0 0 0 1 2 5 4 3 1 0 3 1 1 3

Fibroblast proliferation

8 0 0 0 0 1 2 5 4 3 1 0 3 1 1 3

Lymphocyte infiltration

8 0 0 0 0 3 0 5 1 3 2 2 3 0 2 3

Cartilage degeneration

6 2 0 0 0 3 1 4 4 4 0 0 1 7 0 0

Bone/cartilage replaced by connective tissue

8 0 0 0 0 2 0 6 7 0 0 1 4 1 0 3

Pannus formation

8 0 0 0 0 2 0 6 5 1 0 2 2 3 1 2

New bone formation

8 0 0 0 0 4 4 0 0 7 1 0 0 7 1 0 0

Periostitis

8 0 0 0 0 1 1 1 5 6 0 1 1 5 3 0 0 7

The histopathological evaluation was carried out on the joints of the forepaws. The histopathological findings were graded into four levels (−, intact; +, mild; ++, moderate; ++++, severe). *p<0.05 vs. Control rats; **p<0.01 vs. Control rats.
Fig. 4. Histopathological changes in collagen-induced arthritic rats treated with Flavangenol (FG) and ibuprofen. At day 28, the limbs were dissected from each rat and fixed in 10% neutral buffered formalin. After decalcification, the joints were sectioned longitudinally, and tissue sections were mounted on glass slides and stained with hematoxylin and eosin. Articular lesions were observed under an optical microscope. (A-1, A-2) The forepaw obtained from a non-induction rat (normal rat) (×20). (B-1) The non-treated control rats demonstrated severe articular changes. Synovial fibroblast proliferation (F) and multiplication of the synovial lining cells (M) are observed (×10). (B-2) The non-treated control rats demonstrated severe articular changes. Pannus formation (P) is observed (×20). (C-1) The articular changes were almost normal in those which had received treatment with 1% FG (×40). (C-2) The articular changes were slight in those which had received treatment with 1% FG. Pannus formation (P) is slightly observed (×20). (D-1) The articular changes were moderate in those which had received treatment with 0.05% ibuprofen. Synovial fibroblast proliferation (F) and bone/cartilage replaced by connective tissue (CT) are observed (×10). (D-2) The articular changes were slight in those which had received treatment with 0.05% ibuprofen. Synovial fibroblast proliferation (F) is observed (×20).
collagen was almost the same as that in normal group, which had not been immunized with collagen, but the body weight in the groups decreased on day 18 (Fig. 1). No significant differences were observed between the groups of control and 0.3% FG, and 1% FG throughout the experiment period. However, in the group of 0.05% ibuprofen, the decrease in body weight was suppressed, and significant differences were observed between the groups of control and 0.05% ibuprofen on days 18, 21, 25 and 27 (p<0.05 or 0.01, Fig. 1).

Arthritic score with collagen-induced arthritis in the rats

In the control group, erythema and swelling of the forepaw joints started to appear on day 13, the arthritic score reaching the maximum level (4.9±0.5) on day 27. The 0.3% FG slightly suppressed the arthritic score in the forepaws, although not significantly. The 1% FG markedly suppressed the arthritic score on days 25 and 27 (p<0.05 compared with the control, Fig. 2A). The 0.05% ibuprofen also suppressed the arthritic score on days 25 and 27 (p<0.05 or 0.01 compared with the control, Fig. 2A).

On the other hand, in the control group, erythema and swelling of the hind paw joints also started to show on day 13, the arthritic score reaching the maximum level (6.9±0.4) on day 27 (Fig. 2B). The 0.3% FG had no effect, but the 1% FG slightly suppressed the arthritic score in the paws. In contrast, the 0.05% ibuprofen markedly suppressed the arthritic score on days 18, 21, 25 and 27 (p<0.05 or 0.01 compared with the control, Fig. 2B).

Footpad volume with collagen-induced arthritis in the rats

In the control group, the swelling of hind paws also started to show on day 13, the volume reaching the maximum level on day 21 (Fig. 3). The 0.3% FG had no effect on the swelling of hind paws. Although the 1% FG slightly but not significantly suppressed the swelling in the hind paws on days 18, 21 and 27, the 1% FG significantly suppressed it in the hind paws on day 25 (p<0.05 compared with the control, Fig. 3). The 0.05% ibuprofen significantly suppressed the swelling in hind paws on days 18, 21, 25 and 27 (p<0.05 or 0.01 compared with the control, Fig. 3).

Histopathological findings with adjuvant arthritis in the rats

The histological findings in the forepaws are shown in Table 1 and Fig. 4A, B, C and D. Although 0.3% FG failed to suppress the collagen-induced arthritis (data not shown), 1% FG suppressed subantivocal soft tissue edema, fibrin exudation, fibroblast proliferation, cartilage degeneration, bone/cartilage replaced by connective tissue, pannus formation and periostitis (p<0.05 or 0.01 compared with the control). In contrast, 0.05% ibuprofen suppressed cartilage degeneration and periostitis (p<0.01 compared with the control).

NO production with collagen-induced arthritis in the rats

The plasma NO level in the collagen-induced arthritic rats was 5 times higher than that in the non-induction rats (Fig. 5). The FG (0.3% and 1%) significantly reduced the plasma NO level in the collagen-induced arthritic rats (p<0.001 compared with the control). The 0.05% ibuprofen also reduced it in the rats (p<0.001 compared with the control).

DISCUSSION

Experimental animal models for arthritis are widely used for the evaluation of anti-rheumatic drugs. Adjuvant arthritis rats (6–8, 20), collagen-induced arthritis mice or rats (7, 20, 21), pristane-induced arthritis mice (22), and spontaneous polyarthritis mice (MRL/lpr strain) (22) have been well studied. In particular, it is well known that the collagen-induced arthritis rats are a widely useful model in testing potent therapeutic drugs for use in patients with rheumatoid arthritis, because the arthritic models share similarities with rheumatoid arthritis in human patients (23). In the present study, the FG (1%) and ibuprofen (0.05%) significantly suppressed the increase in arthritic score and swelling of the hind paws (Figs. 2A, B, and 3). The effect of FG (1%) was observed in the forepaws, but the effect of ibuprofen (0.05%) was observed in all four paws (Fig. 2A and B). Although FG (0.3% and 1%) had no effect on the body weight suppression caused by the progression of arthritis, ibuprofen (0.05%) had a suppressive effect (Fig. 1). These results suggest that the mode of the alleviative action of FG on arthritis is different from that of ibuprofen.

In the previous study, we examined the early articular lesions in four experimental animal models, including adjuvant arthritis in F344 rats, collagen-induced arthritis in DBA/1J mice, pristane-induced arthritis in DBA/1J mice, and spontaneous polyarthritis in MRL/lpr mice (24). We reported from histopathological findings in acute and chronic inflammatory lesions that there are differences in the quality of arthritis in the experimental animal models (24). In the present study, the histopathological findings were evaluated in the fore-
paws of the rats, because the changes in the hind paws were severe and we could not evaluate the finding in the case of severe score levels (score 3). The finding data showed that 0.3% FG failed to suppress the articular lesions, but 1% FG significantly suppressed the acute and chronic lesions, such as subsynovial soft tissue edema, fibrin exudation, fibroblast proliferation, cartilage degeneration, bone/cartilage replaced by connective tissue, pannus formation and periostitis (Table 1, Fig. 4B and C). These results suggest that FG suppresses the progression of collagen-induced arthritis by inhibiting the acute and chronic inflammatory lesions. In contrast, ibuprofen (0.05%) suppressed cartilage degeneration, bone/cartilage replaced by connective tissue, pannus formation and periostitis (Table 1, Fig. 4B and D). In our recent experiment to examine the effect of casein hydrolysate on rat adjuvant arthritis, ibuprofen as a positive control drug significantly suppressed the new bone formation (8). The differences between the two arthritic experiments might depend on the degree of articular lesions, because the score of new bone formation in control rats was slight in the present experiment (Table 1). Thus, histopathological examination of the arthritic joints revealed that FG (1%) more markedly suppressed the articular lesions than ibuprofen (0.05%).

NO is synthesized by the enzyme nitric oxide synthase (NOS) (25). NOS exists in three isoforms, neuronal (nNOS), inducible (iNOS) and endothelial nitric oxide synthase (eNOS), and the iNOS isoform is predominantly responsible for NO production in articular cartilage (25). It is well known that NO produced by cartilage and synovial cells is implicated in the pathogenesis of human osteoarthritis and rheumatoid arthritis (26, 27). NO has been also reported to play an important role in the pathogenesis of arthritis in animal models, including collagen-induced arthritis and adjuvant arthritis in rats (8). An inhibitor of iNOS has been reported to have a suppressive effect on arthritis in experimental animal models (28, 29). Furthermore, it was shown that procyanidins extracted from pine bark (Pinus maritime) show the scavenging activity of NO and the inhibitory activity of iNOS in activated murine RAW 264.7 macrophages (30). From these observations, in the present study we measured the plasma NO levels using the blood samples obtained from the rats. FG (0.3%, and 1%) and ibuprofen (0.05%) markedly reduced the plasma NO level in the rats, although FG (0.3%) failed to suppress the macroscopic and microscopic articular lesions. These results suggest that the reduction in plasma levels of NO caused by FG and ibuprofen may be partly associated with the attenuation of the inflammatory reaction in the rats.

FG is a unique mixture of phenols and polyphenols, containing more than 20 kinds of substances. FG is mainly divided into monomers (e.g. catechin and epicatechin), dimmers (e.g. proanthocyanidin B1), trimers, and oligomers up to 5–7 units. The maximum content analyzed in the FG mixture is proanthocyanidin B1, and it accounts for 5%. Therefore, in the present study we used the mixture as a sample instead of purified substances, in order to elucidate the whole action on the inflammation. It has been reported that procyanidin (proanthocyanidin) B1, catechin and epicatechin reduced the NO production, tumor necrosis factor α (TNFα) secretion, and nuclear factor (NF)κB-dependent gene expression in RAW 264.7 macrophages (31). Moreover, it has also been shown that procyanidin B1 inhibited the transactivation of NFκB driven genes and the increase of NFκB-DNA nuclear binding in jurkat T cells (32). On the other hand, anti-inflammatory effects of another pine bark extract were observed in animals with inflammatory bowel disease (33) and in patients with knee osteoarthritis (18). From these observations, it is possible that proanthocyanidin B1, catechin, epicatechin, and other substances in the FG mixture showed additive or synergistic effects on collagen-induced arthritis, probably mediated by these anti-inflammatory or immunomodulatory actions.

In conclusion, we revealed that FG suppresses the progression of collagen-induced arthritis, a model of rheumatoid arthritis, by inhibiting acute and chronic inflammatory lesions and the production of NO. Thus, FG can be anticipated as a novel and safe substance for treatment of human rheumatoid arthritis, although the precise mechanisms underlying the alleviative effect of FG on arthritis remain to be elucidated. We believe that these results might be helpful to understand the mode of action of FG and to encourage further related studies. The symptom-relieving effect of FG on rheumatoid arthritis should be comprehensively evaluated, and its underlying mechanism(s) remains to be elucidated in the future.

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