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Kaempferol, a Tea Flavonol, Effect on Interleukin-2 Signal Transduction of Human T Cell Leukemia

Kazumi ASAI*, Sawako MORIWAKI and Mari MAEDA-YAMAMOTO

Department of Physiology and Quality Science, National Institute of Vegetable and Tea Science (Kanaya, Shizuoka 428–8501, Japan)

Abstract

CCRF-CEM and Jurkat E6-1, human T cell leukemia, produced interleukin (IL)-2 stimulated with phorbol myristate acetate (PMA) and pokeweed mitogen (PWM). IL-2 cytokine production and mRNA expression of CCRF-CEM were increased by treatment with kaempferol, a tea (*Camellia sinensis* L.) flavonol, while those of Jurkat E6-1 were decreased by kaempferol treatment. We therefore investigated the nuclear factor expression of activated T cell (NFAT), a critical regulator of IL-2 gene transcription during T cell activation. In CCRF-CEM, NFAT induction of cytoplasmic extract and NFAT translocation to the nucleus were increased by kaempferol, demonstrating that kaempferol might affect the upstream cascade of T cell signaling. However, in Jurkat E6-1, NFAT induction of cytoplasmic extract was normal, but NFAT translocation to the nucleus was decreased by kaempferol treatment. In this report, it is demonstrated that kaempferol has different effects in the T cell signaling cascade.

Discipline: Tea industry / Animal industry
Additional key words: cytokine, tea (*Camellia sinensis* L.), NFAT (nuclear factor expression of activated T cell)

Introduction

It has been reported that tea has various bioregulatory activities and immuno-regulatory functions[2,15]. Flavonols, kaempferol, myricetin and quercetin are components of tea belonging to a group of polyphenolic compounds found in fruit, vegetables and tea[4]. Previous studies of the biological functions of kaempferol have focused on its anti-inflammatory effects and antioxidative activities in macrophages and neurons[9,17]. The immunoregulatory effects of kaempferol have weak inhibitory interleukin (IL)-5 bioactivity and inhibit histamine release in basophils and mast cells[1,12]. Recently, some reports showed that kaempferol suppressed interferon-γ and IL-2 production of T cells in *in vivo* experiments[11,16]. However, we have reported that IL-2 production in T cell leukemia CCRF-CEM was increased by kaempferol[3]. These results differ between *in vivo* and *in vitro*, and we therefore analyzed additional *in vitro* experiments using T cell leukemia, Jurkat E6-1. Human T cell leukemia, Jurkat, was used as a model to examine the requirements of T cell activation[8,18]. T cells, a type of lymphocyte, are very important in the immune system, and produce IL-2 when they are stimulated. IL-2 is an immune modulator and a major autocrine growth factor, and contributes to immune responses in part by promoting the rapid proliferation of activated T cells[14].

In order to clarify the effect of kaempferol on IL-2 production *in vitro*, we used two forms of T cell leukemia, CCRF-CEM and Jurkat E6-1. We analyzed IL-2 production, mRNA expression and transcriptional factor NFAT, to clarify the *in vitro* mechanism of kaempferol effect on IL-2 production.

Materials and methods

1. Cells and culture

CCRF-CEM (CCL-1199) and Jurkat E6-1 (TIB-152) were purchased from the American Type Culture Collection (ATCC). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum in the presence or the absence of 10 μg/mL of tea flavonols (kaempferol, myricetin) for 48 h, which time was the best among our testing culture times (4, 12, 24, 48, 72 or 96 h). The cells were washed and then stimulated with 5 μg/mL of pokeweed mitogen (PWM) and 10 ng/mL of phorbol myristate acetate (PMA) for 24 h. Kaempferol and

*Corresponding author: fax +81–547–46–2169; e-mail kazumia@affrc.go.jp
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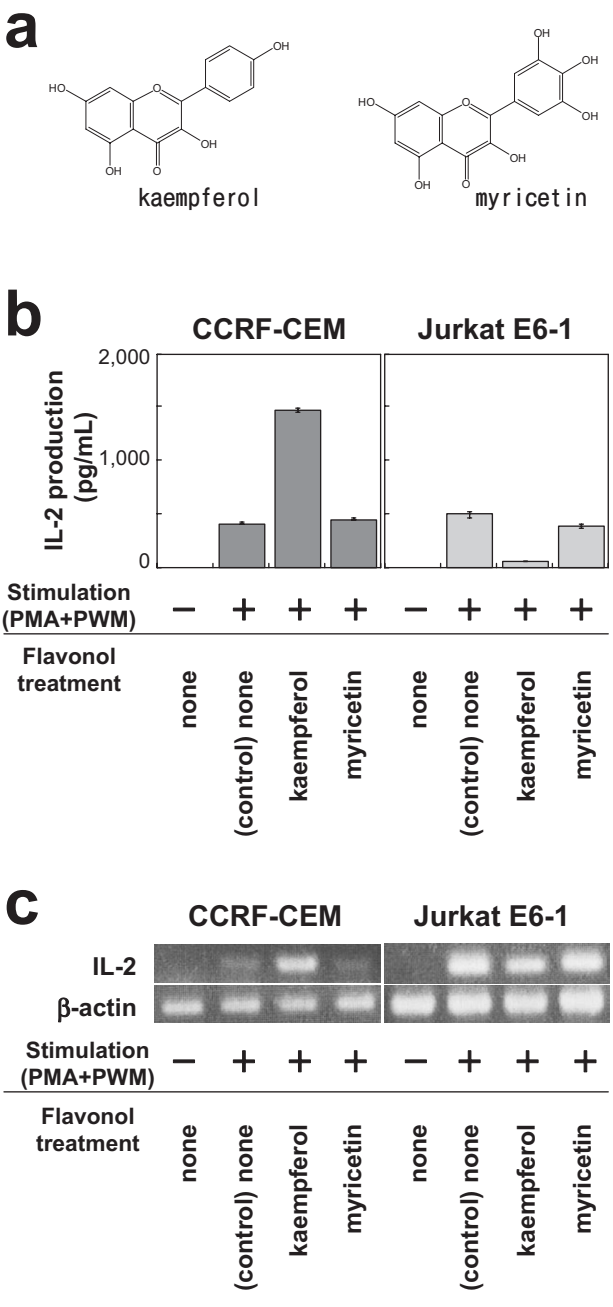

Fig. 1. Structure of flavonols and kaempferol affects IL-2 cytokine production and mRNA expression in T cell leukemia, CCRF-CEM and Jurkat E6-1

a: Structure of flavonols; kaempferol and myricetin. b: Cells (5×10^5/mL) were cultured with tea flavonols for 48 h. Cells were then washed and stimulated with PMA (10 ng/mL) and PWM (5 μg/mL) for 24 h. After harvesting the culture supernatant, the IL-2 concentration of each culture is shown with SD. Data represent the results from at least five independent experiments. c: After harvesting the cultured cells, total RNA was extracted. mRNA levels of IL-2 and β-actin were determined by RT-PCR analysis with template cDNA. Four independent experiments were performed with similar results.

myricetin were purchased from Sigma-Aldrich.

2. ELISA for measuring IL-2 cytokine production

The concentration of IL-2 in the culture supernatant was measured by ELISA using BD OptEIA™ Human IL-2 ELISA Set (BD Biosciences).

3. RNA extraction and quantitative RT-PCR analysis

Total RNA was isolated from cells using the ISOGEN reagent (Nippon Gene). Reverse transcription was carried out with Ready-To-Go™ RT-PCR Beads (Amersham Bioscience). The primers used were as follows: β-actin forward, 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3'; β-actin reverse, 5'-CTA GAA GCA TTG CGG TGG ACG ATG GAG GG-3'; IL-2 forward, 5'-ATG TAC AGG ATG CAA CTC CTG TCT T-3'; IL-2 reverse, 5'-GTC AGT GTT GAG ATG ATG CTT TGA C-3'. PCR was performed for 20 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and primer extension at 72°C for 1 min. PCR products were separated on 1.2% agarose gels and visualized by ethidium bromide staining.

4. Detection of nuclear translocation of nuclear factor expression of activated T cell (NFAT)

Nuclear extracts were prepared with a NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce) according to the manufacturer's protocol. The levels of NFATc and NFATp in the cytoplasmic and nuclear extracts were assessed by immunoblotting with anti-NFATc and anti-NFATp mAb (BD PharMingen). Band intensities were measured by NIH Image (National Institutes of Health), and arbitrary densitometric units are shown.

Results

1. Production of IL-2 cytokine and expression of IL-2 mRNA

Two forms of human T cell leukemia, CCRF-CEM and Jurkat E6-1 were used. IL-2 production was detected in cells stimulated with PMA and PWM. We investigated the effects of kaempferol on IL-2 cytokine production in these cells. CCRF-CEM IL-2 production was enhanced by kaempferol treatment. However, Jurkat E6-1 IL-2 production was significantly inhibited by kaempferol treatment. IL-2 production in CCRF-CEM and Jurkat E6-1 cells remained almost unchanged with myricetin treatment against non-flavonol treatment and PMA and PWM stimulation groups (control) (Fig. 1b).

We next examined the capacity of flavonols to change IL-2 mRNA expression levels. To determine the

level of IL-2 mRNA in these cells, we performed quantitative competitive RT-PCR. In CCRF-CEM, the IL-2 mRNA level was up regulated by kaempferol treatment, however, in Jurkat E6-1, its expression was inhibited by kaempferol treatment (Fig. 1c). The potency of flavonols in the changing steady-state IL-2 mRNA level was similar to that observed in the change of IL-2 protein synthesis.

2. NFAT nuclear translocation

To investigate the point at which different signaling cascades of kaempferol change in IL-2 cytokine production, we examined the NFAT. The transcription factor, NFAT, is a critical regulator of IL-2 gene transcription during T cell activation. The nuclear translocation of NFATc and NFATp, which is thought to be a result of T cell activation, was assessed in flavonol treatment of CCRF-CEM and Jurkat E6-1 cells.

As can be seen in Fig. 2a, NFATc was not detected in either nuclear or cytoplasmic extracts before stimulation, and after stimulation for 24 h, a substantial amount

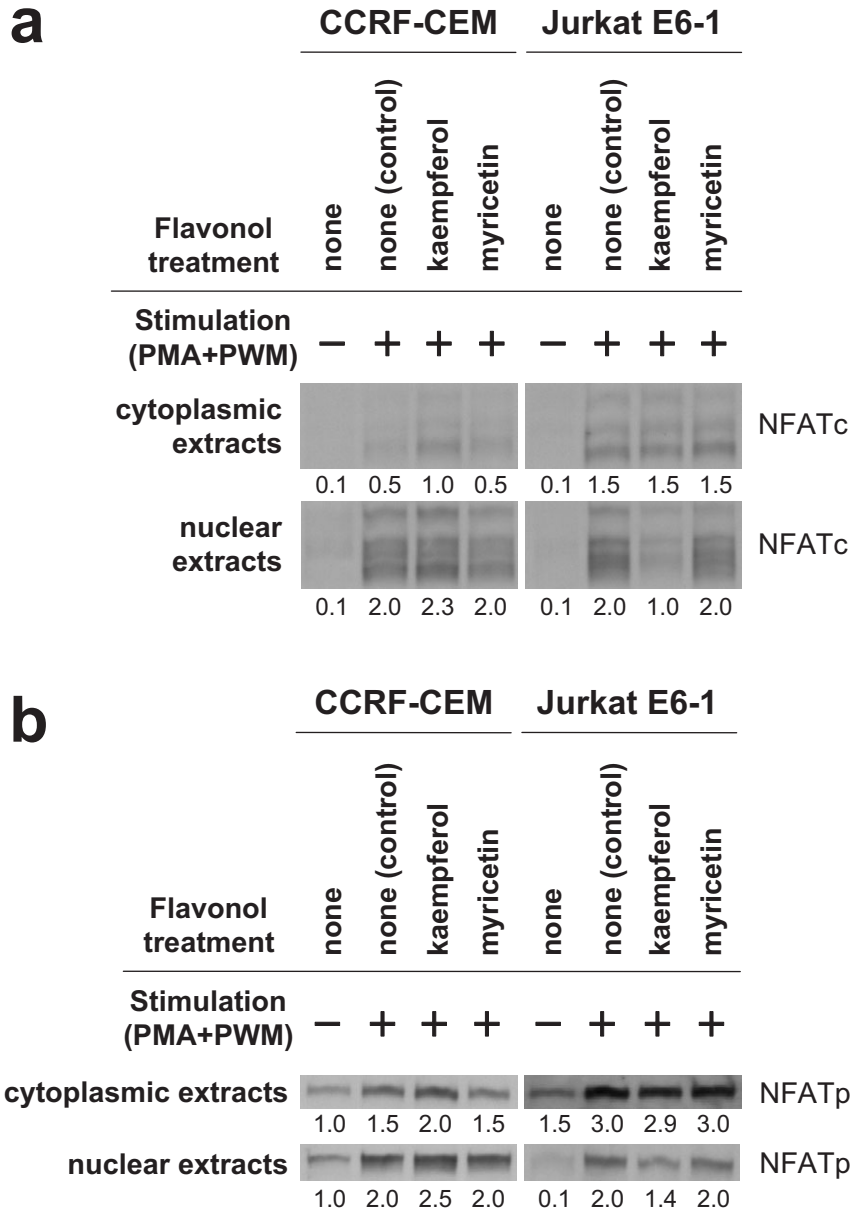

Fig. 2. NFAT nuclear translocation in T cell leukemia, CCRF-CEM and Jurkat E6-1

Cells (5×10^5/mL) were cultured with flavonols for 48 h. Cells were then washed and stimulated with PMA (10 ng/mL) and PWM (5 μg/mL) for 24 h. After cultured cells (2×10^6/lane) were harvested, the extracts were subjected to immunoblotting with anti-NFATc (a) and NFATp (b) mAbs. Arbitrary densitometric units of NFAT bands are indicated. Representative results of four independent experiments are shown.

was found in the nuclear extract of CCRF-CEM and Jurkat E6-1. As expected, in kaempferol-treated CCRF-CEM cells, NFATc was intensely detected in nuclear and cytoplasmic extracts. On the other hand, a significant decrease in NFATc in nuclear extracts was detected in kaempferol-treated Jurkat E6-1 cells, however, the amount of NFATc in cytoplasmic extracts was not significantly decreased. In myricetin-treated cells, the amount of NFAT in nuclear and cytoplasmic extracts did not change against the non-flavonol treatment group (control). This shows that, in kaempferol-treated Jurkat E6-1 cells, NFATc could be induced in the cytoplasm but did not translocate to the nucleus. Furthermore, the same results were obtained with NFATp (Fig. 2b). NFATc and NFATp were the main NFAT family in T cells. It was subsequently determined that there is a family of four related proteins that have similar properties and functions[10].

Discussion

To clarify the immunoregulatory effects of tea flavonols, particularly kaempferol, on T cells, we analyzed IL-2 cytokine production, the IL-2 mRNA level and NFAT nuclear translocation using human T cell leukemia, CCRF-CEM and Jurkat E6-1. When these cells were stimulated with PMA and PWM, they produced IL-2. It is essential that IL-2 regulates T cell proliferation and survival for correct homeostasis in the immune system[14].

We previously reported that kaempferol increased IL-2 production and mRNA levels in CCRF-CEM cells. We therefore estimated that kaempferol might increase IL-2 production in T cell leukemia, and we performed the same experiments using Jurkat E6-1 cells, which is the same source of CCRF-CEM cells. We initially expected that identical results would be derived from these cells, however, when these cells were treated with kaempferol, the opposite results were revealed. IL-2 production and mRNA levels were increased in CCRF-CEM, but in Jurkat E6-1, they decreased by kaempferol treatment, whereas with myricetin treatment, IL-2 production and mRNA levels were not changed in these cells.

To clarify the different influences observed between CCRF-CEM and Jurkat E6-1, we analyzed T cell signaling, especially the transcription factor, NFAT. T cell activation and cytokine gene induction are largely attributed to a family of transcriptional regulators referred to as NFAT[13]. Activated NFAT transcription factors were located in the cytoplasm, resulting in their dephosphorylation and subsequent translocation into the nucleus[5,6]. Once in the nucleus, NFAT functions as an important co-inducer of cytokine gene expression. As shown in Fig. 2, in kaempferol-treated CCRF-CEM, NFATc and NFATp levels in cytoplasmic and nuclear extracts worked together and translocated to the normal nucleus. It was therefore considered that, in CCRF-CEM, kaempferol affects the upstream T cell signaling cascade of NFAT. We examined the upstream protein phosphorylation of the T cell signaling cascade, but it was not detected in this system using PMA and PWM as stimulation reagents, and we therefore researched the detection system using antibodies as stimulation reagents. However, in kaempferol-treated Jurkat E6-1, NFATc and NFATp levels in the cytoplasmic extract were normal or not decreased, and the amount of nuclear extract was significantly decreased. This suggested that kaempferol inhibited the nuclear translocation of NFAT in Jurkat E6-1. We did not clarify why different influences of kaempferol were observed between CCRF-CEM and Jurkat E6-1, although it was clear that this was not the reason for differences between *in vivo* and *in vitro*. Although CCRF-CEM and Jurkat E6-1 had the same T cell leukemia origin, their cell-surface molecule types or levels were different[7,18], so we suggest that different influences were detected. We are currently attempting to clarify the mechanisms using cell-surface molecules.

Tea contains the main three flavonols, kaempferol, myricetin and quercetin. We analyzed the effect of quercetin on T cell leukemia, CCRF-CEM and Jurkat E6-1. However, we could not detect the signal transduction in quercetin treatment of these cells and we didn't know why we could not detect it, so in this paper we left out data of quercetin.

In conclusion, kaempferol affected the T cell signaling cascade of IL-2 production. This is the first report to show the interaction between T cell signal transduction and a flavonol, kaempferol. We are currently extending our physiological studies into tea in a further effort to clarify the mechanisms of flavonol influence on cells.

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