Original Article

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Curcumin-induced modulation of inflammatory cytokine production by circulating mononuclear cells via heme oxygenase-1

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Abstract

Curcumin (diferuloyl methane) is the active component of the spice turmeric and it exerts potent antioxidant and anti-inflammatory functions both *in vitro* and *in vivo*. The mechanism through which curcumin exhibits its biological functions is through induction of heme oxygenase-1 (HO-1). In the present study, we aimed to elucidate the direct effect of curcumin on inflammatory cytokine production by circulating monocytes. Curcumin inhibited lipopolysaccharide induced production of tumor necrosis factor-alpha (TNF-alpha) and interleukin-6 (IL-6) by peripheral blood mononuclear cells, whereas it induced significant levels of interleukin-10 (IL-10) and HO-1. The inhibitory effect of curcumin was not via cytotoxicity of the reagent because there was no significant apoptosis or cell death induced over the range of concentrations used for the assay. The inhibitory effect of curcumin was partially abrogated by adding HO inhibitor SnPP at the late phase of the culture, indicating that the curcumin induced suppression of inflammatory cytokine is partly through production of HO-1. In addition, curcumin may act on monocytes through multiple mechanisms to regulate its inflammatory cytokine production. Modulation of monocyte functions by non-cytotoxic reagent such as curcumin may offer a novel anti-inflammatory therapeutics for the treatment of various inflammatory disorders.

Key words

curcumin, heme oxygenase-1, IL-10, IL-6, TNF-alpha,

Introduction

Curcumin (diferuloyl methane) is the main component of the yellow pigment prepared from the underground stem of curcuma. Its chemical structure was determined by Roughley and Whiting in 1973, and several derivatives have been identified[1]. Its various biological activities have been reported, such as anti-inflammatory effects on allergic[2] and autoimmune[3] diseases, a preventive effect on oxidative stress-induced diabetes in rats[4], and an antitumor effect through inhibition of the oncogene MDM2[5]. It has also been suggested that curcumin exerts at least some biological activities by inducing the production of the antioxidative enzyme heme oxygenase 1 (HO-1)[6,7-11]. HO-1 is constantly produced by liver Kupffer cells[12], vascular endothelial cells[13], uriniferous tubular epithelial cells[14] and peripheral blood monocytes[15,16], and protects these cells by removing various oxidative stressors. On the other hand, details of how HO-1 production by target cells is involved in the exertion of the anti-inflammatory and antitumor effects of curcumin have not been clarified. In this study, we investigated the effect of curcumin on human peripheral blood mononuclear cells, mainly the modulation of HO-1 and inflammatory cytokine

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production. In addition, the possibility of treating inflammatory diseases by the pharmacological modulation of monocyte function is discussed based on the findings.

Materials and Methods

1. Isolation and culture of mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood collected from healthy volunteers by Ficoll-Hypaque gradient centrifugation. PBMCs were suspended in 10% FCS-supplemented RPMI 1640 medium, adjusted to 1.0×10^6 cells/ml, and cultured at 37℃ in 5% CO_2.

2. Reagents

Lipopolysaccharides (LPS) (055 : B5, Sigma-Aldrich Corp., St. Louis, MO, USA), curcumin (C7727, Sigma-Aldrich), and tin protoporphyrin IX dichloride (SnPP) (Affiniti Research Products Ltd., Mamhead Castle, UK) were added to the culture medium as needed. Curcumin was dissolved and adjusted to 300 mM with dimethyl sulfoxide (DMSO), and diluted to specific concentrations. Regarding SnPP, a 2 mM solution was prepared and diluted to the determined concentrations. Regarding LPS, a 100 ng/ml solution was prepared, and added to PBMCs at a final concentration of 10 ng/ml. Trizol Reagent (Invitrogen Corp., Carlsbad, CA, USA) was used for mRNA extraction.

3. Measurements of TNF-alpha, IL-6, IL-10, HO-1

TNF-alpha, IL-6, IL-10, and HO-1 productions by mononuclear cells were measured by ELISA using the following measurement kits: TNF-alpha and IL-6, R&D Systems Inc., Minneapolis, MN, USA ; IL-10, eBioscience Inc., San Diego, CA, USA ; HO-1, Assay Designs Inc., Michigan, USA. TNF-alpha, IL-6 and IL-10 in the culture supernatant were measured. And HO-1 in the cell lysate obtained from cultured cell pellets was measured.

4. Amplification of HO-1 and beta-actin mRNA

From isolated and cultured mononuclear cells, total RNA was extracted following the instructions supplied with Trizol Reagent (Invitrogen Corp., Carlsbad, CA, USA). Total RNA was then reverse-transcribed using Random Primer (Takara Bio Inc., Japan) and RTace (Toyobo Co., Ltd., Japan) to prepare cDNA samples. The prepared cDNA was amplified using HO-1 - and beta-actin-specific 5' - and 3' -primers (Invitrogen), followed by electrophoresis on ethidium bromide-containing 2 % agarose gel (Agarose H14 "TAKARA", Takara Bio). Photographs were taken with UV exposure. The forward and reverse primers, respectively, for these PCR reactions are the following:
CGGCTTCAAGCTGGTGATG and
GGCTGGTGTGTAGGGGATG for HO-1 and
TGGACTTCGAGCAAGAGATG and
GATCTTCATTGTGCTGGGTG for β - actin.

5. Evaluation of curcumin-induced cytotoxicity

To determine if curcumin exerts a significant level of cytotoxicity, PBMCs were cultured alone or in the presence of 20 μM of curcumin for 30 hrs. Cells were harvested at 2, 5, and 30 hrs of culture, and propidium iodide (PI) was added before analyzing the FLS and PI uptake by flow cytometry. Dead cells were determined based on the PI uptake[17,18].

6. Modulation of inflammatory cytokine production by curcumin

To investigate the concentration-dependence of curcumin stimulation, PBMCs were cultured in the concomitant presence of LPS and 0-30 μM of curcumin. To the control cells, 0.01 %v/w DMSO was added. After 18 hrs of culture, the supernatants were collected for the determination of cytokines, and stored at -80℃. TNF-alpha and IL-6 were measured by ELISA.

7. Immunohistochemical staining of HO-1

PBMCs were cultured in the presence of 0-20 μM of curcumin for 18 hrs. Cytospin preparations were dried. Immunohistochemical staining of HO-1 was performed as described by Yachie A et al[15].

8. Induction of HO-1 mRNA expression by curcumin

The concentration dependence of and time-course changes in curcumin-induced HO-1 mRNA expression were investigated. PBMCs were cultured in the presence of 0-30 μM of curcumin for 5 hrs. cDNA was prepared after culture as described above, and HO-1 and beta-actin mRNA were amplified. To investigate time-course changes in the HO-1 mRNA level, PBMCs were cultured for 0-30 hrs in the presence of 20 μM of curcumin, and

HO-1 and beta-actin mRNA expressions were similarly evaluated.

9. Induction of HO-1 and IL-10 production by curcumin

PBMCs were cultured in the presence of concomitant LPS (10 ng/ml) and 0-30 μM of curcumin or curcumin alone. For the control substance, 0.01 %v/w DMSO was used. The supernatants were collected for IL-10 measurement after culture for 18 hrs. To measure the intracellular HO-1 level, the cells were washed with PBS, and cell pellets were stored at -80℃. IL-10 and HO-1 were measured by ELISA.

10. Effect of SnPP on curcumin-induced HO-1 production

To investigate the association between the curcumin stimulation and HO-1 production, the following experiment was performed using an HO activity inhibitor, SnPP: To LPS-stimulated PBMCs, 20 μM of curcumin and 100 μM of SnPP were added, and HO-1 protein was measured in cell extracts prepared after culture for 0, 2, 4, 8, 12, and 24 hrs. Since the absolute HO-1 production level markedly varied among the donors, the HO-1 protein level was presented relative to that regarded as 1 in PBMCs cultured for 12 hrs in the presence of 20 μM of curcumin alone without LPS stimulation.

11. Influence of SnPP on the inhibitory effect of curcumin on inflammatory cytokine production

To investigate the effect of concentration-dependence of SnPP, the following experiment was performed. PBMCs were cultured without LPS for 18 hrs, and supernatant IL-6 levels were determined. Curcumin was added at 20 μM, and SnPP at 25 to 100 μM. Subsequently, LPS-stimulated PBMCs were treated with 20 μM of curcumin and 100 μM of SnPP, and IL-6 in the culture supernatant was measured at 0, 2, 4, 8, 12, and 24 hrs of culture. The IL-6 production level in each sample was presented relative to the maximum production level, regarded as 1.

12. Statistical analysis

Statistical analysis was performed by Dunnett's test or Tukey's HSD test.

Results

1. Cytotoxic effect of curcumin on PBMCs

The possibility of the direct cytotoxicity of curcumin was firstly investigated by evaluating PI uptake through flow cytometry, in which the rate of cells showing strong fluorescence due to PI uptake was calculated as the rate of dead cells. Monocytes could be confirmed as a cell population with a high FLS in the early phase (0-2 hrs), but the FLS decreased as the culture prolonged, and the population started emitting a weak PE fluorescence (30 hrs). However, this fluorescence was noted regardless of the presence or absence of curcumin treatment. PE fluorescence was generally enhanced in the presence of curcumin, which may have reflected the nonspecific fluorescence of curcumin adhering to cells. The rate of PI uptake was only 5.1% in the absence of curcumin even after culture for 30 hrs (Fig. 1A), and it was also only 4.7% in cells cultured with 20 μM of curcumin (Fig. 1B).

2. Modulation of inflammatory cytokine production by curcumin

The modulation of inflammatory cytokine production by curcumin was investigated using LPS-stimulated PBMCs. LPS induced marked inflammatory cytokine production in PBMCs. Both TNF-alpha (Fig. 2A) and IL-6 (Fig. 2B) productions were decreased by curcumin in a

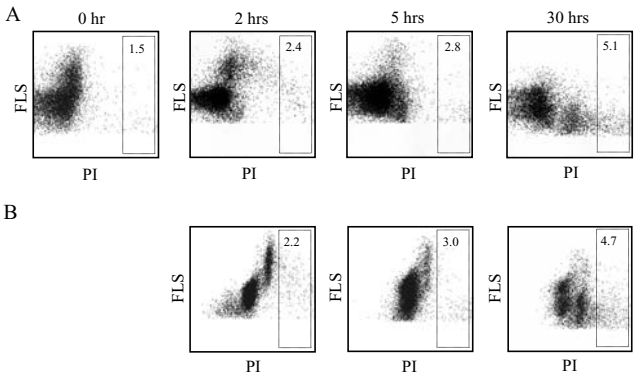

Figure 1. Cytotoxic effect of curcumin on PBMCs.
PBMCs were cultured alone (A) or in the presence of 20 μM of curcumin (B) for 30 hrs. At 2, 5, and 30 hrs of culture, cells were harvested and PI was added before analyzing FLS and PI uptake by flow cytometry. Dead cells were determined based on the levels of PI uptake. Dotted squares indicate the regions of dead cells, and the numbers denote the fractions of dead cells among each culture.

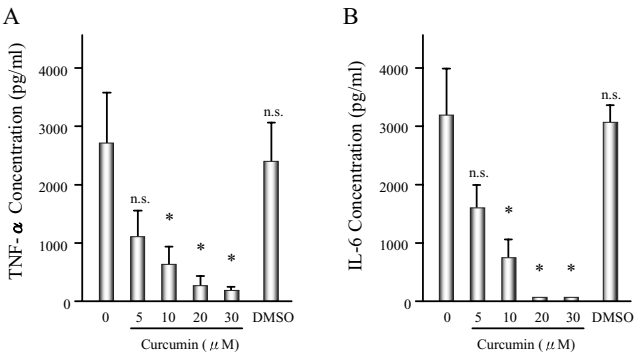

Figure 2. Effect of curcumin on LPS-induced production of inflammatory cytokines.

PBMCs were cultured in the presence of LPS (10 ng/ml) for 18 hrs. Various concentrations of curcumin were added at the start of cultures. DMSO was added at 0.01% v/w as a control. Supernatant TNF-alpha (A) and IL-6 (B) levels were measured by ELISA. The results represent the means ± SD of 3 independent experiments. Dunnett's test $^{*}P < 0.05$. n.s. indicates "not significant".

concentration-dependent manner, and the production of these cytokines was almost completely inhibited in the presence of 20 μM or higher of curcumin. In contrast, no inhibition was noted in cells treated with the control substance, DMSO.

3. Curcumin-induced HO-1 production by circulating monocytes

The possibility that the HO-1-mediated inhibition of inflammatory cytokine production by curcumin is exerted via HO-1, was investigated by employing the following experiments: Firstly, curcumin-induced HO-1 production by PBMCs was investigated by immunostaining. After washing cells treated with curcumin at various concentrations with PBS, cytospin samples were prepared and subjected to immunostaining with anti-HO-1 antibody. Cytoplasmic HO-1-positive cells appeared in the presence of curcumin, and the intensity increased in a concentration-dependent manner. HO-1 expression was limited to cells showing a monocyte morphology, but was not observed in lymphocytes (Fig. 3).

4. Concentration-dependent and kinetic changes of HO-1 mRNA induction in PBMCs after curcumin stimulation

Curcumin-induced HO-1 production was investigated using mRNA expression as an index. Typical examples of HO-1 mRNA expression in PBMCs treated with 0-30 μM of curcumin are

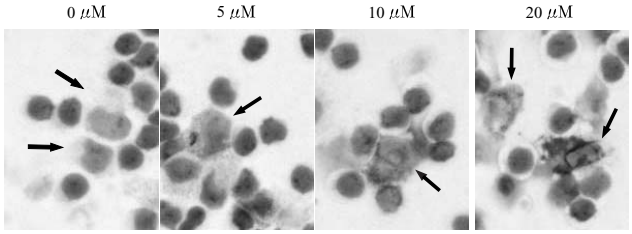

Figure 3. Curcumin-induced HO-1 production by circulating monocytes.

PBMCs were cultured in the presence of various concentrations of curcumin for 18 hrs. Cytospin preparations were dried, fixed in cold acetone, and stained with anti-HO-1 antiserum. Arrow showed the cells productive of HO-1.

shown in Fig. 4A. HO-1 mRNA expression was enhanced as the curcumin concentration increased. Typical time-course changes in HO-1 mRNA expression in cells treated with 20 μM of curcumin are shown in Fig. 4B. The HO-1 mRNA expression level increased over time, reached a maximum level at 12 hrs of culture, and decreased after 20 hrs.

5. Curcumin-induced HO-1 and IL-10 production

Curcumin-induced HO-1 and IL-10 production was investigated. Curcumin promoted HO-1 production in a concentration-dependent manner, and the production level reached a maximum at

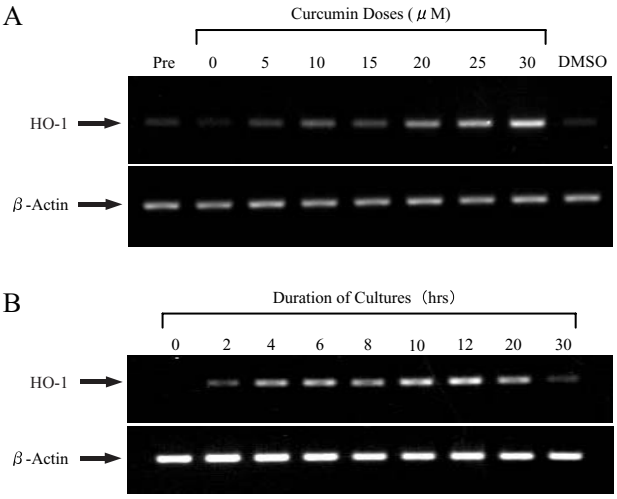

Figure 4. Concentration-dependent and kinetic changes of HO-1 mRNA induction in PBMCs after curcumin stimulation.

PBMCs were stimulated for 5 hrs at various concentrations of curcumin (A) or cultured for various periods with 20 μM of curcumin (B). After the cultures, cDNA samples were prepared and HO-1 mRNA levels were estimated based on the density of the PCR products. The levels of beta-actin were measured as a control. DMSO at 0.01 %v/w was used as a culture control.

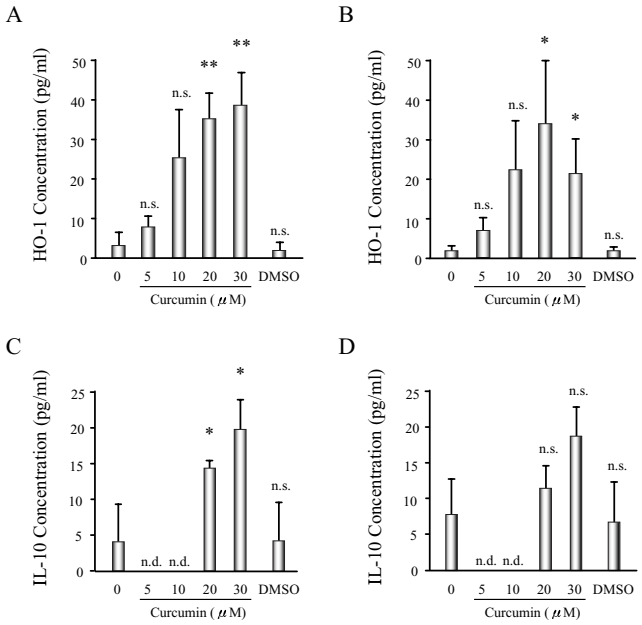

Figure 5. Curcumin induced the production of HO-1 and IL-10.

PBMCs were cultured in the absence (A and C) or presence of 10 ng/ml LPS (B and D) for 18 hrs. Curcumin was present at different concentrations at the start of each culture. Supernatant levels of HO-1 (A and B) and IL-10 (C and D) were measured by employing ELISA. DMSO at 0.01 %v/w was used as a culture control. The results represent the means ± SD of 3 independent experiments. n.d. indicates "not detectable" or less than 3 pg/ml. Dunnett's test $^{*}P < 0.05$, $^{**}P < 0.01$. n.s. indicates "not significant"

30 μM, whereas no HO-1 production was induced in the control group treated with DMSO (Fig. 5A). HO-1 production was also increased in LPS-stimulated PBMCs with an increase in the curcumin concentration, but the level peaked at 20 μM of curcumin, and decreased at 30 μM (Fig. 5B). IL-10 production was also induced in a curcumin concentration-dependent manner, similarly to HO-1, but the profiles in the presence (Fig. 5D) and absence (Fig. 5C) of LPS were similar.

6. Effect of SnPP on curcumin-induced HO-1 production

To confirm the direct inhibition of cytokine production by curcumin-induced HO-1, an HO activity inhibition test with SnPP was performed. Considering that the effect of SnPP may be affected by time-course changes in HO-1 production after curcumin stimulation, time-course changes in curcumin-induced HO-1 production in the presence and absence of LPS were investigated. HO-1 production by PBMCs treated with 20 μM of

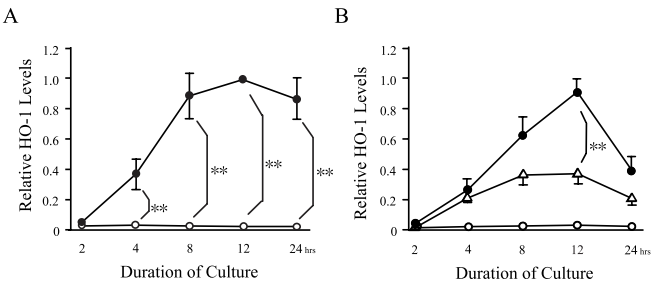

Figure 6. Effect of SnPP on curcumin-induced HO-1 production.

PBMCs were cultured with 20 μM of curcumin (A; open circles denote culture alone and closed circles are that with curcumin) or with 10 ng/ml of LPS and 20 μM of curcumin (B; closed circles) for various periods. SnPP at 100 μM was added to the cultures of PBMCs with LPS and curcumin (open triangles). LPS did not induce detectable levels of HO-1 (B; open circles). The results represent the means ± SD of 4 independent experiments. Tukey's HSD test $^{**}P < 0.01$.

curcumin reached a plateau at 12 hrs of culture (Fig. 6A, closed circles), but curcumin-untreated PBMCs produced no HO-1 protein (Fig. 6A, open circles). Curcumin also induced HO-1 production in LPS-stimulated PBMCs over time, but the production decreased after 24 hrs following reaching a peak at 12 hrs (Fig. 6B, closed circles). When 100 μM SnPP was added with curcumin, HO-1 protein production was not affected in the early phase of culture, but was slowly inhibited, and the inhibition was significant at 12 hrs of culture (Tukey's HSD test $p < 0.01$) (Fig. 6B, open triangles). In the control group treated with LPS alone, no HO-1 production was induced (Fig. 6B, open circles).

7. Influence of SnPP on inflammatory cytokine production-inhibitory effect of curcumin

To investigate the possibility that the curcumin-induced inhibition of inflammatory cytokine production is exerted via HO-1, the effect of SnPP addition was investigated. When PBMCs were cultured without LPS, and IL-6 production in the supernatant was measured after 18 hrs of culture, 20 μM of curcumin markedly inhibited IL-6 production (Dunnett's test $p < 0.01$), while no inhibition was noted in the control cells treated with DMSO. The curcumin-induced inhibition of IL-6 production was reversed by SnPP in a concentration-dependent manner, and the IL-6 production level in the presence of 100 μM of SnPP

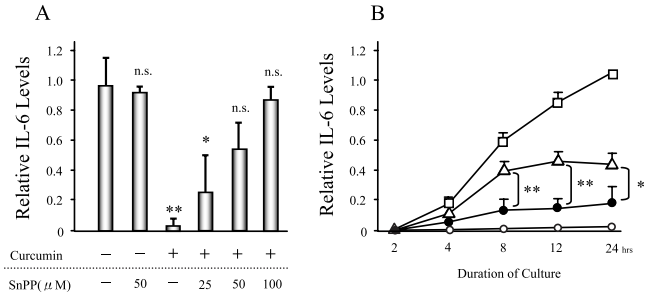

Figure 7. Reversal of curcumin's effects by SnPP.
PBMCs were cultured without LPS for 18 hrs, and supernatant IL-6 levels were determined. Curcumin was added at 20 μM and SnPP was added at 25 to 100 μM to these cultures, as shown in the figure. The results represent the means ± SD of 3 independent experiments. Dunnett's test $^{*}P<0.05$, $^{**}P<0.01$. n.s. indicates "not significant". (A) PBMCs were stimulated with 10 ng/ml of LPS for various periods either alone (open squares) or in the presence of 20 μM of curcumin (closed circles), on combinations of curcumin and SnPP (open triangles), or only 100 μM of SnPP with non-LPS stimulated PBMCs (open circles). The results represent the means ± SD of 4 independent experiments. Tukey's HSD test $^{*}P<0.05$, $^{**}P<0.01$. (B) Supernatant IL-6 levels were determined by ELISA.

was similar to that in the control cells (Fig. 7A). The inhibitory effect of curcumin on LPS-induced IL-6 production and the influence of SnPP were investigated over time. SnPP alone did not induce IL-6 production in PBMCs (Fig. 7B, open circles), and IL-6 production was induced in the presence of LPS (Fig. 7B, open squares). When 20 μM of curcumin was added to cultures with LPS, IL-6 production was markedly inhibited (Fig. 7B, closed circles). When 100 μM of SnPP was simultaneously added, the curcumin-induced inhibition of IL-6 production was apparently reversed at 8, 12 (Tukey's HSD test $p<0.01$) and 24hrs (Tukey's HSD test $p<0.05$) of culture (Fig. 7B, open triangles). However, interestingly, the reversal effect by SnPP on the curcumin-induced inhibition of IL-6 production weakened as the culture time prolonged.

Discussion

To investigate the effect of curcumin on PBMCs, the cytotoxicity of 20 μM of curcumin on PBMCs was confirmed. As described in Results, the number of dead cells after 30 hrs of culture in the presence of 20 μM of curcumin was similar to that in curcumin's absence, suggesting that curcumin exhibited no cytotoxicity at the concentration employed in this study. The level of increase in the PI fluorescence intensity in curcumin-treated cells was not high enough to suggest PI uptake, and a constant intensity was maintained even at 2 hrs of culture in a cell population with a high FLS assumed to be monocytes, and no further increase in the fluorescence intensity was noted after 30 hrs of culture, suggesting that it was a curcumin-induced nonspecific increase. These did not contradict the findings reported by Scapagnini G et al[19], whereby curcumin did not affect astrocytes at concentrations up to 30 μM. Thus, we treated LPS-stimulated PBMCs with curcumin, and investigated changes in the production of inflammatory cytokines, TNF-alpha and IL-6, and found that curcumin inhibited the production in a concentration-dependent manner. The control substance, DMSO, did not inhibit the production. As shown in Fig. 1, it was unlikely that curcumin exhibited cytotoxicity at the concentrations used. Accordingly, the inhibition of inflammatory cytokine production by curcumin was not due to its cytotoxicity, but it was assumed to be due to some pharmacological mechanism. Several reports suggested that the cell-protective and inflammation-inhibitory effects of curcumin are exerted via HO-1 production. The following experiments confirmed that the modulation of inflammatory cytokine production by PBMCs was also exerted via HO-1. We previously reported that only monocytes out of PBMCs produced HO-1 in response to stress, whereas no HO-1 production was noted in any lymphocyte subgroup[15,16], in which only monocytes produced HO-1 even when the type of PBMC stimulation was changed. Similarly, HO-1 production was detected only in monocytes among curcumin-treated PBMCs, as shown in Fig. 3. It was apparent that the curcumin-induced HO-1 production was due to the *de novo* induction of HO-1 mRNA expression based on the finding that HO-1 mRNA expression was enhanced with time in a curcumin concentration-dependent manner, as shown in Fig. 4, which did not contradict the finding reported by Rushworth SA et al[11]: curcumin was involved in antioxidant response element (ARE)-mediated

gene activation including HO-1 expression in human peripheral blood monocytes. The induction of HO-1 production is known to induce the production of an anti-inflammatory cytokine, IL-10[20]. These findings suggest that the curcumin-induced inhibition of cytokine production is limited to inflammatory cytokines, such as IL-6 and TNF-alpha, and IL-10 production also accompanies the induction of HO-1 production. As expected, curcumin induced the production of not only HO-1 but also IL-10. However, in LPS-stimulated PBMCs, curcumin-induced HO-1 production peaked at 20 μM, and then decreased at 30 μM, but the cause was unclear. Similarly, curcumin-induced HO-1 production decreased after a certain period in the presence of LPS (Fig. 6B), suggesting that monocytes were already strongly activated in the presence of LPS-induced marked inflammatory cytokine production, and their HO-1 production ability was limited to some extent, but details of the mechanism remain to be elucidated. In any case, these findings clearly showed that curcumin induces antioxidative responses and anti-inflammatory reactions of PBMCs, not contradicting the findings reported by Jagetia GC et al[21]. It was clarified that curcumin inhibited inflammatory cytokine production, and, at the same time, induced HO-1 and IL-10 production. We then attempted to clarify whether the cytokine production was inhibited by an HO-1-mediated mechanism. In the presence of LPS, SnPP directly inhibited curcumin-induced HO-1 production. Although the mechanism is unclear, one of the explanations is that LPS enhanced the direct cytotoxic effect of SnPP. In any case, it should be paid attention to on evaluating findings on culture with SnPP. Thus, in the subsequent experiment, we evaluated its influence on IL-6 production in the absence of LPS, and found that the curcumin-induced IL-6 production was completely reversed, showing that HO-1 activity was directly involved in the inhibition of IL-6 production. Hsu HY et al[8] reported the HO-1-mediated biological activity of curcumin in human peripheral blood monocytes, which did not contradict our findings regarding the biological activity of curcumin on human PBMCs. We also investigated the time-course rate of the SnPP-induced reversal of the curcumin-induced inhibition of IL-6 production. The degree of the reversal decreased after 12 hrs of culture, suggesting that curcumin-induced HO-1 is more closely involved in the inhibition of IL-6 production in the early phase of culture, while other factors, such as NFκB, become more closely involved, in addition to HO-1, in the late phase[22,23]. It was clarified that curcumin strongly modulated the inflammation-related cytokine-producing ability of human peripheral mononuclear cells, and the inhibition was exerted via HO-1 mRNA and protein production. The elucidation of these pharmacological effects of curcumin may lead to the development of new methods to treat inflammatory diseases.

Acknowledgments

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ヘムオキシゲナーゼ１による クルクミン誘導末梢血単球炎症性サイトカイン産生の制御

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要　　旨

クルクミン(diferuloyl methane)は香辛料ターメリックの活性成分であり、試験管内あるいは生体内において強い抗酸化あるいは抗炎症作用などを示すとされているが、このような生物活性の機序としてheme oxygenase-1 (HO-1)の誘導を介することが知られている。本研究では、末梢血単球の炎症性サイトカイン産生におけるクルクミンの関与を明らかにすることを目的として行った。その結果、クルクミンは lipopolysaccharide により末梢血単核球に誘導されたTNF-alpha や IL-6の産生を抑制した一方で、IL-10やHO-1活性を有意に増強した。また、本研究で用いたクルクミンの濃度範囲において、アポトーシスや細胞死はほとんど認めなかったことから、クルクミンによる炎症性サイトカインの抑制効果はクルクミンの細胞毒性に起因したものではないといえた。さらに、HO活性抑制物質SnPP添加により単核球の培養後期においてクルクミンの抑制効果は部分的に解除された、このことから、クルクミンはHO-1産生を介した炎症性サイトカインの抑制を部分的に誘導することが示唆された一方で、他機序を介した単球への関与についても示唆された。クルクミンのような非細胞毒性を示す薬剤を利用することによる単球機能の調節は、さまざまな炎症性疾患の新しい抗炎症治療への可能性が期待される。