Note

Inhibition by Genistein of the Lipopolysaccharide-Induced Down-Regulation of Programmed Cell Death 4 in RAW 264.7 Mouse Macrophages

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Programmed cell death 4 (Pdcd4), a novel tumor suppressor, has recently emerged as an anti-inflammatory protein. We assessed in the present study the effects of 18 different food factors with anti-inflammatory activity on the lipopolysaccharide-induced down-regulation of pdcd4 mRNA expression in mouse RAW 264.7 macrophages. Genistein, a soy isoflavone, significantly inhibited pdcd4 down-regulation.

Key words: anti-inflammation; programmed cell death 4 (Pdcd4); macrophage; genistein; isoflavone

Although inflammation is known to be a defense mechanism against harmful substances and injury, its aberrant regulation is associated with the development of chronic diseases such as cancer.1 The application of food phytochemicals that are known to regulate inflammation, as an alternative to anti-inflammatory drugs, has therefore recently become a focus of interest, and strategies utilizing an anti-inflammatory diet to prevent chronic inflammatory diseases have recently been proposed.2

Programmed cell death 4 (Pdcd4), a novel transformation suppressor protein, is considered to be a promising target for developing anti-neoplastic therapy. Pdcd4 expression occurs ubiquitously in normal tissues, while it is down-regulated in various tumors according to the tumor grade.3,4 The protein has also been shown to inhibit tumor promoter-induced neoplastic transformation by regulating several signaling molecules, e.g., phosphoinositide 3-kinase (PI3K), c-Jun N-terminal kinase 1/2 (JNK 1/2), and activator protein-1.5 There is ample evidence that those are crucial for inflammatory responses, suggesting the possibility that Pdcd4 has anti-inflammatory effects. In fact, we recently found that Pdcd4 was capable of counteracting lipopolysaccharide (LPS)-triggered inflammatory responses, and that LPS down-regulated pdcd4 mRNA expression in macrophages via PI3K and JNK pathways for TNF-α (M. Yasuda, A. Murakami et al., manuscript in preparation).

Although numerous phytochemicals have been reported to have anti-inflammatory effects by various molecular mechanisms,2 there are no reports of those targeting Pdcd4, and there are few compounds known to be capable of inducing Pdcd4 mRNA and protein.5,6 Thus, in the present study, we investigated the effects of selected food phytochemicals on LPS-down-regulated pdcd4 mRNA expression in RAW 264.7 macrophages.

As shown in Fig. 1, treatment with LPS for 24 h dramatically suppressed pdcd4 mRNA expression in RAW 264.7 macrophages (p < 0.01). Among the 18 selected food phytochemicals reported to have anti-inflammatory activities,8–10 only genistein (GEN) inhibited pdcd4 down-regulation by 84% (p < 0.01). GEN did not affect the pdcd4 level in non-stimulated cells, whereas it suppressed LPS-induced down-regulation in a concentration-dependent manner (Fig. 2A and B).

Although we have recently demonstrated that (±)-13-hydroxy-10-oxo-trans-11-octadecenoic acid (HOA) up-regulated the pdcd4 level in mouse epidermal JB6 P+ cells,11 it showed no effects on mouse macrophages in the present study (Fig. 1). This discrepancy may have been due to differences in the cell phenotypes and stimuli used. To our knowledge, this is the first report of a food factor regulating Pdcd4 in inflammatory cells.

We subsequently examined whether GEN-related compounds could exhibit similar suppressive effects. Daidzein (DZN), the major isoflavone in soybeans, as well as GEN have shown anti-inflammatory activities in mouse macrophages.12 Since they are structurally related to 17β-estradiol (E2), both isoflavones have the potential to bind to estrogen receptors and thereby inhibit inflammatory responses in macrophages.13 The effect of herbimycin A (HEB), a protein tyrosine kinase inhibitor,13 was also examined, since GEN has been designated as a tyrosine kinase inhibitor.14 The concentrations of these agents used in the present study were determined from data presented in previous reports,15–17 and did not significantly affect the cell viability after 24 h of incubation (data not shown). As shown in Fig. 3A, neither HEB (900 nm) nor E2 (100 nm) suppressed pdcd4 down-regulation, whereas DZN showed significant suppression at a concentration of 100 μM (Fig. 3A). Our finding that the effect of DZN was weaker than that of GEN is consistent with the result of a previous report that demonstrated that DZN had a lower level of anti-inflammatory activities than GEN.11 Quercetin, which potently suppresses protein tyrosine kinase as well as GEN and HEB,15 also had no effect on the pdcd4 level (Fig. 1). Although a number of flavonoids have been reported to have anti-inflammatory

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Abbreviations: ACA, 1'-acetoxychavicol acetate; BITC, benzyl isothiocyanate; DZN, daidzein; E2, 17β-estradiol; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GEN, genistein; HEB, herbimycin A; HOA, (±)-13-hydroxy-10-oxo-trans-11-octadecenoic acid; HPRT, hypoxanthine phosphoribosyltransferase; LPS, lipopolysaccharide; JNK 1/2, c-Jun N-terminal kinase 1/2; MAPK, mitogen-activated protein kinase; Pdcd4, programmed cell death 4; PI3K, phosphatidylinositol-3-kinase; RT-PCR, reverse transcription-polymerase chain reaction
activity, 19,20) other flavonoids shown in Fig. 1 such as luteolin (flavone), quercetin and rutin (flavonol), and hesperidin (flavanone) did not affect pdc4 expression. Taken together, we conclude that GEN inhibited LPS-induced pdc4 down-regulation by a mechanism other than the inhibition of tyrosine kinase and estrogen receptor activation, and that its isoflavone structure was required for the activity. In particular, isoflavonoids with a 5-OH residue may be more effective than those without it.

As already mentioned, we have recently found that the PI3K and JNK 1/2 pathways and TNF-α were involved in LPS-induced pdc4 down-regulation in macrophages. As shown in Fig. 3B and C, GEN attenuated LPS-induced P3K, JNK 1, and c-Jun activation at the time point of 6 h (p < 0.01). The molecular target(s) of GEN for regulating pdc4 may therefore be located upstream of PI3K and JNK 1. GEN has been reported to suppress the production of pro-inflammatory cytokines, including IL-2 and TNF-α, in immune cells. 21,22) Although the involvement of Pdc4 in these mechanisms has yet to be demonstrated, it is notable that PI3K played a key role in pdc4 regulation by IL-2, 23) and that GEN may target PI3K (Fig. 3B and C). Those findings together with the present data raise the possibility that the anti-inflammatory mechanisms for GEN are associated, at least in part, with pdc4 regulation.

In conclusion, our results show GEN to be a distinct phytochemical capable of inhibiting the down-regulation of pdc4 induced by an inflammatory agent in macrophages, and that the isoflavone structure was important for this activity. The molecular mechanism by which it suppresses PI3K/JNK activation is now being investigated in our laboratory.

Experimental

DMEM and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). LPS from Escherichia coli 0127:B8 was obtained from Sigma-Aldrich (St. Louis, MO, USA). RAW 264.7 mouse macrophages were purchased from American Type Culture Collection (Manassas, VA, USA) and grown in DMEM supplemented with 10% heat-inactivated FBS, as well as penicillin (100 U/ml) and streptomycin (100 μg/ml). LPS from Escherichia coli (Osaka, Japan), unless specified otherwise. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) assays and western blot analyses were performed as previously described. 9,24) The primers used and PCR conditions were as follows: mouse anti-HPRT (5'-CAATGAAGCTTCTTCTCC-3') and 5'-TTGAGGCTTGCTGTTTCTG-3'), 29 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 45 s; and hypoxanthine phosphoribosyltransferase (HPRT) (5'-gTAATgATCATTCACgAeggggAC-3') and 5'-CCAgCAgCATTgCgAA-3'), 29 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 45 s; and hypoxanthine phosphoribosyltransferase (HPRT) (5'-gTAATgATCATTCACgAeggggAC-3') and 5'-CCAgCAgCATTgCgAA-3'), 29 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 45 s; and hypoxanthine phosphoribosyltransferase (HPRT) (5'-gTAATgATCATTCACgAeggggAC-3') and 5'-CCAgCAgCATTgCgAA-3'), 29 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 45 s; and hypoxanthine phosphoribosyltransferase (HPRT) (5'-gTAATgATCATTCACgAeggggAC-3') and 5'-CCAgCAgCATTgCgAA-3'), 29 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 45 s; and hypoxanthine phosphoribosyltransferase (HPRT) (5'-gTAATgATCATTCACgAeggggAC-3') and 5'-CCAgCAgCATTgCgAA-3'), 29 cycles at 95°C.
Fig. 3. Effects of GEN-Related Compounds on Pdcd4 Expression (A) and Suppression by GEN of PI3K and JNK/c-Jun Phosphorylation (B and C).

A. RAW 264.7 macrophages (7.5 × 10^5 cells/well) were incubated with the vehicle (0.5% DMSO) alone, HEB (900 nM), E2 (100 nM), DZN (10, 25 or 100 μM), or GEN (25 μM) for 30 min, then stimulated with LPS (100 ng/ml) for 24 h, after which total RNA was isolated for the RT-PCR assays. None of the phytochemicals showed significant cytotoxicity during incubation (data not shown). B. RAW 264.7 macrophages and bars not sharing a letter differ, separate experiments for the data shown in Fig. 3B. A two-way analysis of variance was performed, followed by Fisher’s protected least-square mean t-test. C. Histograms show the densitometric analysis of phosphorylated protein expression normalized to α-tubulin, and the data are presented as the mean ± SD of three separate experiments for the data shown in Fig. 3B. A two-way analysis of variance was performed, followed by Fisher’s protected least-square difference test to determine whether the differences among the groups were significant. The figure is representative of three separate experiments with each similar results. C, Histograms show the densitometric analysis of phosphorylated protein expression normalized to α-tubulin, and the data are presented as the mean ± SD of three separate experiments for the data shown in Fig. 3B. A two-way analysis of variance was performed, followed by Fisher’s protected least-square difference test to determine whether the differences among the groups were significant. The figure is representative of three separate experiments with each similar results.

72 °C for 45 s. The Pdcd4 mRNA expression levels were normalized by those of HPRT mRNA, and the pdc4/HPRT ratios were calculated by a densitometric analysis with Scion Image 0.4.0.3. (Scion Corporation, Frederick, MD, USA).

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References