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Daidzein enhances efferocytosis via transglutaminase 2 and augmentation of Rac1 activity

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ABSTRACT

Clearance of apoptotic cells, termed "*efferocytosis*", is the mechanism required to prevent secondary necrosis and release of proinflammatory cytokines. Defective efferocytosis is cumulatively regarded as one of mechanisms in the development of autoimmune and chronic inflammatory diseases. Our previous finding showed that ethanolic extract from *Glycine tomentella* Hayata (GTH) can enhance mouse macrophage RAW264.7 efferocytosis (clearance of apoptotic cells). We have demonstrated that the major components of GTH are daidzein, catechin, epicatechin and naringin. Here, we explore the potential of each component in modulating efferocytic capability. For this, RAW264.7 cells were cultured with CFDA-stained apoptotic cells and assayed by flow cytometry. We found that daidzein is the main component of GTH, and it can enhance RAW264.7 efferocytosis dose-dependently. Moreover, the enhancive effect of daidzein on macrophage efferocytic capability is accompanied by increased transglutaminase 2 (TG2) at both mRNA and protein levels. TG2 knockdown attenuated daidzein increased macrophage efferocytic capability. After treatment with daidzein, increased phosphorylation was observed in Erk, but not in p38 and JNK. Finally, we report that after daidzein treatment, Rac1 activity was markedly increased and the mitochondrial membrane potential was decreased, which may contribute to efferocytosis. Taken together, these data suggest that enhancement of macrophage efferocytic capability by daidzein treatment was mainly through up-regulation of TG2 expression and Rac1 activity. Daidzein may have the therapeutical potential in the treatment of inflammatory diseases.

1. Introduction

Apoptosis is the process of programmed cell death that plays an essential role in the development and maintenance of all mammalian tissues. The apoptotic program ensures that the damaged, aged, or excess cells are deleted in a regulated manner to maintain normal tissue homeostasis (Henson et al., 2001; Henson and Hume, 2006). The apoptotic cells are removed by phagocytosis by professional phagocytes, such as macrophages or dendritic cells; or by nonprofessional phagocytes including fibroblasts, stromal cells, and epithelial cells. The ingestion of apoptotic cells (termed "efferocytosis") by phagocytes has been shown to trigger release of molecules such as transforming growth factor β (TGF-β), interleukin-10 (IL-10), and prostaglandin E2 (PGE2), which are thought to be mediators of immunosuppression and immunological tolerance to self-antigens (Ravichandran, 2011; Savill et al., 2002; Michlewska et al., 2007). Apoptotic cells are recognized by the receptors on the phagocytes through the so-called 'eat-me' signals on the surface of the apoptotic cell (Ravichandran, 2011). The molecules downstream of these receptors are partly identified, including small GTPase Rac-1, Cdc42, and RhoG which belong

Abbreviations: TG2, transglutaminase 2; GTH, Glycine tomentella Hayata; Erk, extracellular signal-regulated kinase; TGF-β, transforming growth factor β; RT-PCR, reverse transcription-polymerase chain reaction; NFκB, nuclear factor-kappaB; siRNA, small interfering RNA; MAPK, mitogen-activated protein kinase; MDM, monocyte-derived macrophage.

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to Rho family of GTPase. Activation of these molecules results in the reorganization of the actin cytoskeleton and engulfment of apoptotic cells. In contrast, RhoA has been reported to inhibit this process (Tosello-Trampont et al., 2003). Thus, efferocytosis is tightly regulated by the small GTPase Rac-1 in the process of cytoskeletal reorganization, in which two parallel signaling pathways are involved. The first pathway is initiated by CD91 or stabilin-2 functioning as receptors, which acts in concert with the *adaptor* protein GULP, and consequently activates Rac1 (Su et al., 2002; Park et al., 2008). The second pathway involves Dock180 and ELMO functioning together as a bipartite guanine nucleotide exchange factor (GEF) for Rac1 (Brugnera et al., 2002).

A recent study has shown that tissue transglutaminase 2 (TG2) is needed for the formation of an efficient phagocyte portal during efferocytosis (Toth et al., 2009). Tissue transglutaminase belongs to the family of transglutaminases which catalyze the cross-linking between an ε-amino group of a lysine residue and a γ-carboxamide group of a glutamine residue. In addition, TG2 also has GTPase activity, suggesting that this molecule participates in G protein-mediated signaling processes in the presence of GTP (Fesus and Piacentini, 2002). Previous study reported that TG2$^{-/-}$ mice develop age-dependent autoimmune disease on account of defective efferocytosis in vivo. TG2 deficiency fundamentally impairs efferocytosis and alters release of TGF-β and other cytokines in macrophages (Szondy et al., 2003, 2011). Based on these findings, TG2 might play an important role in efferocytosis.

We have previously reported that a total extract of *Glycine tomentella* Hayata (GTH) could enhance efferocytosis and TG2 mRNA up-regulation during this process (Yen et al., 2010). Daidzein is a plant-derived diphenolic isoflavone found in a number of plants and herbs like *Trifolium pratense* (red clover) or *Pueraria mirifica*, as well as in food sources such as soybeans and soy products like tofu (Wang et al., 2008; Hamalainen et al., 2007). It has been reported that in murine macrophage J774 and RAW 264.7 cells daidzein could inhibit expression of inducible nitric oxide synthase (iNOS) and nitric oxide production which may account for the its anti-inflammatory effects (Franke et al., 1998). Daidzein has also been shown to inhibit lipopolysaccharide (LPS)-induced NF-κB transcriptional activity in mouse macrophage cells and fibroblasts (Vanden Berghe et al., 2006). In another study, daidzein reduced osteoclastic differentiation in the murine RAW264.7 cell model (Garcia Palacios et al., 2005). Moreover, daidzein reduced myocardial injury in a rat ischemia/reperfusion model by inhibiting NF-κB activation (Kim et al., 2009). These studies suggest that daidzein modulates cell signaling pathways and subsequently production of inflammatory cytokines.

In this study, we demonstrate that daidzein enhances efferocytic capability in a dose-dependent manner by up-regulation of TG2 and Rac1. Daidzein may be a potent plant-derived substance for enhancing efferocytosis and may have therapeutic potentials for autoimmune and inflammatory diseases.

2. Materials and methods

2.1. Cell culture and isolation

Normal human PBMCs were isolated from five healthy donors by density Histopaque®-1077 sterile-filtered (density: 1.077 g/ml) gradient centrifugation. The cells were then washed and resuspended in Dulbeco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), 2 mM glutamine, 1 mM pyruvate, 1% non-essential amino acid, 100 U/ml penicillin, 0.0025 mg/ml amphotericin, and 1 mg/ml streptomycin (Biological Industries). Monocyte-derived macrophages (MDM) were isolated from PBMCs by plastic adherence. Phenotypical and functional characterizations of MDM was performed after 6–7 days. RAW264.7 cells were purchased from Bioresource Collection and Research Center (HsinChu, Taiwan) and cultured in DMEM.

2.2. Cell viability

Cell viability was assessed by using annexin V and propidium iodide stain. Measurement of early and late apoptosis was performed by flow cytometry using annexin V: FITC assay kit (AbD Serotec, Oxford, UK) according to the manufacture's instructions. The stained cells were analyzed by FACSCAN laser flow cytometry (Becton Dickenson, San Jose, CA, USA).

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from RAW264.7 cells by using the Trizol reagent protocol (Sigma, St. Louis, MO, USA). Two micrograms of total RNA were denatured at 65 °C with 1 μl oligo-dT (Promega, Madison, WI, USA), and 4 μl dNTPs (10 mM) for 5 min in 12 μl final volume. The primer-RNA mixture was cooled on ice, and 1 μl Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA, USA), 1 μl RNase inhibitor (Promega) and 4 μ l 5× RT-buffer were added for a total volume of 20 μl. PCRs were performed under the following conditions: 94 °C for 5 min, annealing at 54 °C for 1 min, and DNA synthesis at 72 °C for 2 min, followed by 28 cycles. To assess the TG2 mRNA expression of RAW264.7 cells, RAW264.7 cells were incubated with indicated component for 24 h and the mRNA was analyzed by RT-PCR. The amplified PCR products were subjected to electrophoresis in a 2% agarose gel. Sequences for the PCR primers were: 5′-TGATGACCGGGAGGACATCA-3′, 5′-GATTC TCC AGGTA G AGATCTC-3′ (forward and reverse mouse TG2 primers), 5′-TCACTCAA GATT GT CAGCAA-3′, 5′-AGATCCACGACGGACACATT-3′ (forward and reverse mouse GADPH primers).

2.4. TG2 expression constructs and stable transfection

Full length human transglutaminase 2 was cloned into the pGene/V5-HisB vectors (Invitrogen). RAW264.7 cells were transfected with the pSwitch vectors containing the mifepristone-inducible system, and then co-transfected with pGene/V5-HisB TG2 or pGene/V5-HisB vector alone using TurboFect (Fermentas) with several weeks of dual-select with zeocin and hygromycin B.

2.5. siRNA treatment

TG2 siRNA was purchased from Santa Cruz Biotechnology. Transfection was performed with TurboFect (Fermentas) according to the manufacturer's instructions. Cells were then treated with daidzein for an additional 24 h.

2.6. Phagocytosis assay

The human keratinocyte cell line (HaCat) was offered by Professor Jen-Hung Yang of the Department of Dermatology at Chung Shan Medical University in Taichung, Taiwan. For induction of UV-induced apoptosis, HaCat cells were exposed to UV radiation at 400 J/m^2 using the Spectroline UV Crosslinker (Spectroline, New York, NY, USA). For phagocytosis of apoptotic cells, the UV-irradiated HaCat cells were labeled with carboxy-fluorescein diacetate (CFDA) followed by incubation with RAW264.7 cells at 10:1 target/macrophage ratio at 37 °C for 4 h. After washing, cells on the dish were resuspended in phosphate buffered saline (PBS) solution and 10,000–20,000 cells were analyzed for fluorescence intensity by flow cytometry (Becton Dickenson, San Jose, CA, USA).