A crude extract from immature green tea (*Camellia sinensis*) leaves promotes Toll-like receptor 7-mediated interferon- α production in human macrophage-like cells

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Received: 11 September 2011 / Accepted: 12 November 2011 / Published online: 29 November 2011
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Abstract Toll-like receptor 7 (TLR7) senses viral single-stranded RNA (ssRNA), induces the production of type I interferons (IFNs), IFN- α and - β , in macrophages such as dendritic cells (DCs), and its immune system protects the host from virus infection. Here, we found that a crude extract from immature green tea leaves (iTPS) containing a macromolecule with ssRNA fragments, induces IFN- α production in human macrophage-like cells. In addition IFN- α production was inhibited by treatment with TLR7 inhibitors or a phagocytosis inhibitor.

Keywords Green tea · Crude extract · Interferon- α · Toll-like receptor 7

Introduction

LPS and β -glucan are well-known polysaccharides having immunomodulating activities, and induce innate immunity via activation of CD14/TLR4 and

Dectin-1/TLR2, respectively, on antigen-presenting cells such as dendritic cells (DCs) and macrophages (Miyake 2003; Gantner et al. 2003). Recently, we found that iTPS containing a macromolecule (above 10^6 M.W.) with ssRNA fragments, activated the phagocytosis of human macrophage-like cells, but its activity was inhibited by TLR7 inhibitors (Monobe et al. 2010); however, the mechanism by which iTPS activated TLR7, an intracellular receptor residing in lysosomes, is not clear. TLR7 is a receptor for virus ssRNA and plays important roles in antiviral immunity (Akira and Takeda 2004; Diebold et al. 2004), and its activation induces a phagocytic gene program (Doyle et al. 2004) and type I IFN production in DCs and so on via an MyD88-dependent signaling pathway (Diebold et al. 2004). Therefore, TLR7 agonists such as imidazoquinolines, including imiquimod and resiquimod, are used as antiviral drugs. Imidazoquinolines show antiviral activity through the induction of IFN- α in immunocompetent cells such as DCs and natural killer (NK) cells, unlike traditional antiviral drugs that act by directly interfering with virus replication. IFN- α also has antitumor activity (Smyth et al. 2004); therefore, imidazoquinolines not only have antiviral activity but also antitumour activity (Stanley 2002). Imidazoquinolines are recognized as substances having an antiviral function via the regulation of TLR7; however, very few drugs have the same function. Here we investigated the possibility of inducing IFN- α in macrophage cells by iTPS.

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Materials and methods

Preparation of iTPS

iTPS was prepared as previously described (Monobe et al. 2008). Briefly, dried tea leaves were boiled in distilled water (DW). The extract was precipitated using ethanol (final concentration 70%). The sediment was washed with ethanol, and dried. Buds, first and second, and third leaves were used as immature tea leaves.

Agarose gel electrophoresis

The gel was prepared by melting 2% agarose S (Wako Pure Chemicals, Osaka, Japan) in MOPS buffer (20 mM MOPS, 8 mM sodium acetate, and 1 mM EDTA) containing 0.5 µg/mL of ethidium bromide in a microwave oven, pouring the solution into the mould of a Mupid-21 Mini-Gel Electrophoresis System (Advance, Tokyo), and allowing it to solidify at room temperature with an inserted comb. One µL of 10 mg/mL TPS solution or size marker (RNA Size Standard Marker IV; Nippongene co. Ltd., Toyama, Japan) were loaded into the wells. Electrophoresis was carried out in MOPS buffer using a Mupid-21 electrophoresis system (Advance). The gel was visualized under UV light.

Cytokine analysis

To differentiate human monocytic leukemia cells (THP-1) along the monocytic pathway, the cells were seeded at densities of 5×10^5 cells/mL in growth medium supplemented with 120 nM 1,25-dihydroxyvitamin D3 (VD3; Wako Pure Chemical Industries, Ltd., Osaka, Japan), and differentiation was allowed to proceed for 2 days. Differentiated THP-1 cells (10^6 cells/250 µL/well) were seeded into 48-well plates (Falcon 351178; BD Biosciences, Franklin Lakes, NJ). The cells were treated with 25 µL iTPS for 8 h, and brefeldin A (Wako) was added 2 h before harvest. The cells were fixed in paraformaldehyde, permeabilized and labeled with anti-IFN- α antibody (PBL Interferon Source, Piscataway, NJ). The expression of intracellular IFN- α was detected by flow cytometry (Cell Lab Quanta; BeckmanCoulter,

Brea, CA), and analyzed by EPICSXL software (BeckmanCoulter).

Treatment of inhibitors

VD3-differentiated THP-1 cells were incubated in the presence of both iTPS and inhibitors at 37 °C in 5% CO₂ for 8 h.

Results and discussion

Crude tea polysaccharide (TPS) has been found to be mainly composed of uronic acids, especially galacturonic acid (Wang et al. 2001; Chen et al. 2004), and iTPS includes many ssRNAs as compared with TPS from mature tea leaves (Monobe et al. 2010). The size of ssRNAs in iTPS is around 120 bp (Fig. 1). In a previous study, we found that iTPS activated phagocytosis through TLR7 in human macrophage-like cells. TLR7 is a receptor for ssRNA (Akira and Takeda 2004; Diebold et al. 2004), and its activation induces a phagocytic gene program (Doyle et al. 2004) and type I IFN production in DCs and so on (Diebold et al. 2004). It has been previously suggested that IFN- α induction may be more specific to TLR7 (Gorden et al. 2005); therefore, first we investigated the possibility of inducing IFN- α by iTPS. As shown in Fig. 2a, iTPS induced IFN- α in VD3-differentiated THP-1 cells. Next, we investigated the involvement of TLR7 in the induction of IFN- α by iTPS using TLR7 inhibitors. As shown in Fig. 2b and c, the induction of IFN- α by iTPS was inhibited by both TLR7 inhibitors of chloroquine phosphate (an inhibitor of intracellular TLR signaling; LKT Laboratories, Inc., Minnesota), and immunoregulatory DNA sequences (IRS) 661 (Barrat et al. 2005) (a specific inhibitor of human TLR7; 5'-TGCTTGCAAGCTTGCAAGCA-3'; Nippon EGT, Toyama, Japan). These results show that iTPS promotes TLR7-mediated interferon- α production in human macrophage-like cells.

A macromolecule with ssRNA in iTPS is cell-impermeable; therefore, we inferred that the macromolecule with ssRNA uptake by phagocytosis recognizing a macromolecule might stimulate intracellular TLR7. Then, we investigated the relationship of phagocytosis on IFN- α induction by iTPS. As shown in Fig. 3, the induction of IFN- α by iTPS was

Fig. 1 Agarose gel electrophoresis of TPS. *Lane 1*, RNA molecular marker; *lane 2*, immature leaves TPS. One μL of 10 mg/mL TPS solution or size marker were loaded into the wells

inhibited by cytochalasin B, a well-characterized inhibitor of phagocytosis. This result shows that a macromolecule in iTPS is taken up by phagocytosis, ssRNA in the macromolecule stimulates TLR7 residing in lysosomes, and induces IFN- α in macrophage-

Fig. 2 IFN- α production induced by iTPS and its inhibition by TLR7 inhibitors. VD3-differentiated THP-1 cells were incubated in the absence (*open histogram*; unstimulated control) or presence (*grey histogram*) of iTPS (100 $\mu\text{g/mL}$); **a** Absence of inhibitor; **b** Chloroquine phosphate (5 $\mu\text{g/mL}$); inhibitor of intracellular TLR signaling; **c** IRS661 (5 μM ; specific inhibitor of human TLR7). Representative histograms from four independent experiments are shown. Inhibitors-induced cell reduction or expansion was not observed by Flow cytometer

like cells. We infer that a macromolecule in TPS is a carrier of ssRNA.

TLR7 agonists other than imidazoquinolines and viperin (Saitoh et al. 2011) have not been discovered. TLR7 is involved in sequence-specific sensing of ssRNAs in human macrophages (Gantier et al. 2008). Although it has been known that immature green tea extract contains micro RNAs (Prabu and Mandal 2010), its details are unknown; however, at least the ssRNA is likely to have a specific sequence for human TLR7. Although further studies are needed to clarify the molecular mechanisms concerning the activation of the immune system by iTPS, iTPS might be a potential TLR7 activator from natural products.

Fig. 3 Inhibition of IFN- α production by cytochalasin B. VD3-differentiated THP-1 cells were incubated in the absence (*open histogram*; unstimulated control) or presence (*grey histogram*) of iTPS (100 μ g/mL); **a** Absence of inhibitor; **b** Cytochalasin B (5 μ M). Representative histograms from four independent experiments are shown. Inhibitor-induced cell reduction or expansion was not observed by Flow cytometer

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