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Biphasic regulation of polymorphonuclear leukocyte spreading by polyphenolic compounds with pyrogallol moieties

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ABSTRACT

Green tea polyphenols have been reported to have anti-inflammatory activities, although the molecular mechanisms responsible for this effect remain unclear. In the present study, we examined the effect of green tea extract and a variety of polyphenolic compounds on spreading of peripheral blood polymorphonuclear leukocytes (PMNs) over fibrinogen-coated surfaces. Green tea extract exerted a biphasic effect on PMN spreading; it induced or suppressed spreading at low and high concentrations, respectively. We also found that pyrogallol-bearing compounds have spreading induction activity. Among the compounds tested, tannic acid (TA) had the strongest activity; the concentrations required for induction of maximal spreading were 2 μM for TA, 200 μM for (−)-epigallocatechin gallate, and 2000 μM for the other active compounds. Furthermore, TA was the only compound showing a biphasic effect similar to that of green tea extract; TA at 20 or 200 μM suppressed spreading. The spreading-stimulatory signal was still latent during PMN exposure to TA at concentrations that inhibited spreading, because the pre-exposed PMNs underwent spreading when plated after removal of free TA by centrifugation. The spreading-inhibitory effect of TA at high concentrations overcame the induction of spreading by other stimuli, including phorbol 12-myristate 13-acetate, hydrogen peroxide, denatured fibrinogen surfaces, and naked plastic surfaces. These results suggest that TA as well as green tea extract is bi-functional, having pro-inflammatory and anti-inflammatory effects at low and high concentrations, respectively. Pharmacological use of TA may thus provide new strategies aimed at regulation of PMN spreading for control of inflammation.

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1. Introduction

Green tea polyphenol catechins, which can readily be extracted by hot water, have been reported to have anti-inflammatory activities, although the molecular mechanisms responsible have been poorly investigated [1,2]. (−)-Epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) and (−)-epicatechin (EC) are the major components of green tea catechins, of which EGCG is the main one [3–5]. For example, Varilek et al. have reported that oral administration of green tea polyphenols decreases the severity of colitis in interleukin-2-deficient (IL-2(−/−)) mice [6]. Di Paola et al. have reported that green tea extract (polyphenols) attenuates the carrageenan-induced acute inflammatory response characterized by infiltration of polymorphonuclear leukocytes (PMNs) and increased production of nitrite/nitrate and tumor necrosis factor α (TNFα) [7]. They further reported that intraperitoneal administration of green tea extract attenuates the degree of zymosan-induced inflammation in mice, including peritoneal exudation and migration of PMNs, injury to the lung, liver and pancreas, and renal dysfunction [8].

PMNs, most of which are neutrophils, are the most abundant leukocytes and play a crucial role in the processes of host defense against infection. Paradoxically, however, PMNs are also implicated in tissue-damaging inflammatory reactions that are central to the pathogenesis of many inflammatory diseases [9]. Since both an excess and impairment of PMN microbicidal functions can be deleterious, PMN functions must be appropriately regulated in order to achieve host defense and avoid tissue-damaging inflammation. Development and pharmacological use of PMN-targeting agents may therefore be a good therapeutic strategy for regulation of PMN functions for control of inflammation and tissue injury.

Several recent studies have demonstrated a direct effect of tea catechins on the functions of isolated PMNs in vitro [10–13]. Hofbauer et al. reported that EGCG inhibits neutrophil migration through endothelial cell monolayers [10]. Handa et al. reported that catechins attenuate the expression of CD11b and CD18, which comprise the adhesion molecule integrin $\alpha_M\beta_2$, on PMNs [11]. Garbisa and colleagues reported that EGCG directly and potently inhibits the enzyme activity of elastase [12], and has multiple inhibitory effects on neutrophil functions including chemotaxis, generation of reactive oxygen species (ROS), and apoptosis [13].

Another aspect of the reported pharmacological action of catechins (especially EGCG) on cell cultures is inhibition of firm adhesion

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(spreading) to extracellular matrix (ECM) protein-coated surfaces [14–16]. Ogata et al. have reported that EGCG has an inhibitory effect on spreading of mouse lung carcinoma 3LL and melanoma B16F10 cells over fibronectin-coated surfaces, whereas EGC, EC, and (+)-catechin are ineffective [14]. Liu et al. have also reported that EGCG inhibits the spreading of mouse melanoma B16F3m cells over surfaces coated with fibrinectin, laminin, and collagen, associated with attenuation of the phosphorylation of focal adhesion kinase (FAK) [15]. Furthermore, it has been reported by Huang et al. that EGCG inhibits the spreading of fibroblast cells over surfaces coated with collagen, fibronectin, and fibrinogen [16]. To our knowledge, however, the effects of polyphenols on the spreading of PMNs have never been reported.

Spreading of PMNs is a pre-required step for subsequent activation of their microbicidal functions, such as hydrogen peroxide (H_2O_2) production [17,18]. At sites of inflammation and tissue injury where PMNs accumulate, deposits of fibrinogen (or fibrin), which is the major binding substrate for PMN β2-integrins ($\alpha_M\beta_2$ and $\alpha_X\beta_2$), are formed [19], enabling PMNs to spread. Studies suggesting that catechins inhibit the spreading activity of some cell lines have thus prompted us to examine whether this is indeed the case for spreading of PMNs over fibrinogen-coated surfaces.

In the present study, we examined the effect of green tea extract and a variety of polyphenolic compounds on the spreading of isolated porcine peripheral blood PMNs over immobilized fibrinogen surfaces. Here we report that green tea extract and polyphenolic compounds with pyrogallol moieties have spreading-inducing activity, and that among the active compounds, crude green tea extract and tannic acid (TA) have a biphasic effect, inducing PMN spreading at low concentrations, but inhibiting such spreading at higher concentrations. The molecular mechanisms of action of these polyphenolic compounds are also discussed.

2. Materials and methods

2.1. Materials

All test compounds including TA, EGCG, ECG, EGC, EC, gallic acid, pyrogallol, 3,4-dihydroxybenzoic acid (DHBA), pyrocatechol, and shikimic acid were purchased from Sigma (St. Louis, MO, USA). Porcine fibrinogen and formyl-Met-Leu-Phe (fMLP) were also from Sigma. Phorbol 12-myristate 13-acetate (PMA) and Methyl cellulose 25cP were from WAKO Pure Chemical (Tokyo, Japan). Hydrogen peroxide (H_2O_2) and dimethyl sulfoxide (DMSO) were from Kanto Chemical (Tokyo, Japan). Ficoll-Paque Plus was from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Fresh porcine peripheral blood was obtained from Tokyo Shibaura Zoki (Tokyo, Japan). Dried green tea leaves for beverage were purchased from Ocha no Maruko (Tokyo, Japan). 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl)maleimide (Gö6983) and 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazole[3,4-d]pyrimidine (PP2) were from Calbiochem (San Diego, CA, USA).

2.2. Green tea extract

Dried green tea leaves (300 mg) were mixed with 20 ml of hot distilled water and extracted by incubation for 20 min at 80 °C in a water bath, with occasional mixing. The supernatant was recovered and lyophilized after filtration. The amount of extract obtained from 300 mg of green tea leaves was termed “300 mg equivalent (mgeq)”. The lyophilized green tea aqueous extract (75 mgeq) was treated with 10 ml of methanol and re-extracted by sonication for 10 min using a water bath-type sonicator. After centrifugation for 10 min at 500 ×g, the methanol-soluble supernatant was collected, evaporated, and stored at −20 °C. The hot water-methanol extract of green tea leaves was used for experiments.

2.3. Preparation of immobilized fibrinogen surfaces

Culture microplate with immobilized fibrinogen was prepared by incubation with fibrinogen (1 mg/ml) for 2 h at room temperature (23 °C) followed by washing. In some experiments, immobilized fibrinogen was denatured by additional incubation for up to 24 h before washing [20].

2.4. PMN spreading

PMNs were isolated from porcine peripheral blood routinely, and PMN spreading over immobilized fibrinogen surfaces was assessed morphologically as described previously [21]. PMNs (1×10^6 cells/ml) suspended in HEPES-NaCl buffer (10 mM HEPES (pH 7.2), 140 mM NaCl, 5 mM glucose, 0.6 mM Mg^{2+}, and 2 mM Ca^{2+}) were plated onto immobilized fibrinogen and incubated for the indicated periods at 37 °C in the presence or absence of test compounds or other stimuli at the indicated concentrations. In some experiments, PMNs were pretreated with inhibitors at the indicated concentrations, or they were pre-exposed to test compounds followed by removal of the compounds by washing, and then they were plated onto the fibrinogen-coated surfaces. Photomicrographs were taken and cells were counted, and those that were phase dark, enlarged with irregular shapes were considered spread. Spreading data were shown as percentage of spread cells.

2.5. Chemotaxis

Chemotaxis was assessed using Chemotaxicell culture chambers (0.48 cm^2 area of membrane, 3 μm pore size; Kurabo, Osaka, Japan) as described in manufacturer's instruction. Briefly, chambers were filled with PMN suspensions (1×10^5 cells/200 μl HEPES-NaCl buffer) that had been preincubated with or without TA at the indicated concentrations. The chambers were placed into HEPES-NaCl buffer containing 50 nM fMLP in 24-well tissue culture plates. After 60 min at 37 °C, the upper surface of the chamber membranes were washed with phosphate-buffered saline (PBS), and the membranes were fixed in methanol, stained by 0.4% crystal violet to visualize the cells transmigrated to the lower surface of the membranes. Photomicrographs were taken and cell numbers on the central area of the membranes (0.14 mm^2) were counted.

3. Results

3.1. Green tea extract induces PMN spreading over immobilized fibrinogen surfaces only within a limited range of concentration

Green tea extract was prepared by extraction with hot water and methanol, as described in the Methods section. When PMNs were plated onto plastic microplate wells coated with fibrinogen and incubated in the presence of green tea extract at various concentrations, they were induced to spread markedly by green tea extract at a concentration of 0.1 mgeq/ml (Fig. 1A). At 0.01 mgeq/ml, only faint induction of spreading activity was observed. Notably, the effect of green tea extract was biphasic; PMNs did not spread when the concentration of the extract was increased to 1 mgeq/ml or further (Fig. 1A). The morphology of PMNs exposed to green tea extract at 0, 0.1, and 1 mgeq/ml, respectively, is shown in Fig. 1B.

3.2. Polyphenolic compounds with a galloyl and/or pyrogallic group induce spreading of PMNs

We next examined whether catechins, the main constituents of green tea extract, and related compounds have PMN-spreading induction activity. The chemical structures of the compounds we tested here, and the results of the PMN spreading assay, are shown in Figs. 2 and 3, respectively.

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