Epigallocatechin-3-gallate, a green-tea polyphenol, suppresses Rho signaling in TWNT-4 human hepatic stellate cells

NOBUHIKO HIGASHI, MOTOYUKI KOHJIMA, MARIE FUKUSHIMA, SATOSHI OHTA, KAZUHIRO KOTOH, MUNECHIKA ENJOJI, NAOYA KOBAYASHI, and MAKOTO NAKAMUTA

FUKUOKA AND OKAYAMA, JAPAN

Epigallocatechin-3-gallate (EGCG), a major constituent of the polyphenoids in green tea, has been reported to possess a wide range of biologic activities, including antifibrogenesis. Activated hepatic stellate cells (HSCs) are central to hepatic fibrosis, and Rho (a small GTPase)–signaling pathways have been implicated in the activation and proliferation of HSCs. In this study, we investigated the effect of EGCG on Rho-signaling pathways in activated human HSC-derived TWNT-4 cells. EGCG inhibited stress-fiber formation, an indicator of Rho activation, and changed the distribution of α-smooth-muscle actin. These inhibitory effects of EGCG were restored by overexpression of constitutively active Rho. A pull-down assay revealed that activated Rho (GTP-bound state) was strongly inhibited by ECGC and accompanied by suppressed phosphorylation of focal adhesion kinase, which is a regulator of Rho-signaling pathways. 5-Bromo-2′-deoxy-uridine incorporation demonstrated that ECGC (100 μmol/L suppressed cell growth by 80%, and terminal deoxynucleotidyl transferase viotin-deoxyruidine triphosphate nick end-labeling revealed that EGCG (100 μmol/L) caused apoptosis in half of the total cells. EGCG also strongly inhibited lysophoaphatidic acid (an activator of Rho) and induced phosphorylation of mitogen-activated protein kinases (Erk1/2, c-jun kinase, and p38). These findings demonstrate that EGCG regulates the structure and growth of HSCs by way of Rho-signaling pathways and suggest that EGCG has therapeutic potential in the setting of liver fibrosis. (J Lab Clin Med 2005;145: 316–22)

Abbreviations: BrdU = 5-bromo-2′-deoxy-uridine; DMEM = Dulbecco's modified Eagle medium; EDTA = ethylenediaminetetraacetate; EGCG = epigallocatechin-3-gallate; FAK = focal adhesion kinase; FCS = fetal-calf serum; GDP = guanosine diphosphate; GTP = guanosine triphosphate; HSC = hepatic stellate cell; JNK = NH_2-terminal c-jun kinase; LPA = lysophoaphatidic acid; MAPK = mitogen-activated protein kinase; PAGE = polyacrylamide-gel electrophoresis; PBS = phosphate-buffered saline solution; PDGF = platelet-derived growth factor; ROCK = Rho-associated coiled-coiled forming kinase; SDS = sodium dodecyl sulfate; α-SMA = α-smooth-muscle actin; TUNEL; = terminal deoxynucleotidyl transferase viotin-deoxyruidine triphosphate nick end-labeling

Green tea contains high amounts of polyphenoids, particularly catechins,[1] which have been shown to prevent the development of proliferative diseases, including cancer and arteriosclerosis.[2,3] The major polyphenolic component of green tea is EGCG, but other, related catechins, such as epicatechin, epigallocatechin, and epicatechin-3-gallate, are present in lower levels.[1,2] EGCG has been

From the Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University; and the Department of Gastroenterological Surgery, Transplantation, and Surgical Oncology, Okayama University Graduate School of Medicine and Dentistry.

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Reprint requests: Dr Makoto Nakamuta, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-5282, Japan; e-mail: nakamuta@intmed3.med.kyushu-u.ac.jp

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reported to have several biologic effects, in particular inhibitory effects on cell proliferation.[4–6]

A small GTPase, Rho, was first identified as a regulator of actin cytoskeleton organization. It promotes the assembly of focal adhesions and actin stress fibers.[7,8] In addition to its effects on the cytoskeleton, Rho is involved in regulating the expression of c-fos, which is composed of activator protein-1 with c-jun, through the serum response element located in the promoter region.[9,10] Rho is activated by a variety of growth factors in several systems, including the G protein–coupled receptor agonists LPA, bombesin, norepinephrine, and endothelin-1,[11–14] indicating that Rho promotes cell proliferation. Recently it was shown that Rho-associated coiled-coiled forming kinase (ROCK), a direct Rho effector,[15] directly activates JNK to stimulate c-jun expression, the activity of which is independent of the ability of ROCK to promote actin polymerization.[16] Therefore the Rho-signaling pathway not only regulates the cytoskeleton but also directly regulates the expression of genes involved in cell proliferation.

HSCs play a crucial role in liver fibrosis, which is characterized by the excess deposition of extracellular matrix components such as collagens.[17] After liver injury, HSCs undergo transdifferentiation to an activated myofibroblastic phenotype with expression of α-SMA.[18] The activated HSCs then proliferate and produce extracellular matrix proteins such as collagens. We reported previously that Rho-ROCK–signaling pathways play an important role in the activation of HSCs.[19] We also demonstrated that Y-27632, an inhibitor of ROCK, suppresses HSC proliferation and collagen production in vitro[20] and dimethylnitrosamine-induced hepatic fibrosis in vivo.[21] These reports suggested that the Rho-signaling pathway plays an important role in the activation of HSCs. In this study, we sought to investigate the effects of EGCG on the Rho-signaling pathway in HSCs in vitro.

METHODS

Cell-culture and transfection assay. TWNT-4 cells, a human cell line derived from activated HSCs, were cultured in DMEM with 10% FCS as reported previously.[22] EGCG (Kurita Industrial Co, Tokyo, Japan) was dissolved in DMEM and added to the cultures. The effects of EGCG were examined at concentrations of 50 and 100 μmol/L. We selected these concentrations on the basis of previous reports indicating that these concentrations produce biologic effects such as cell-growth inhibition on rat primary HSCs.[23,24] Expression plasmid for *Botulinum* toxin C3 transferase (pEF-myc-C3 [myc-C3]), a dominant negative mutant of RhoA (pEF-myc-RhoA-T19N [myc-Rho (T19N)], and activated mutants of RhoA (pEF-myc-Rho-Q63L [myc-Rho (Q63L)] or pEF-myc-Rho-G14V [myc- Rho (G14V)] were kindly provided by Dr Hideaki Sumimoto (Kyushu University) and Dr Yoshimi Takai (Osaka University).[25] We transfected TWNT-4 cells using FuGENE 6 (Roche, Tokyo, Japan) with the expression plasmid and cultured them for 24 hours in DMEM with 10% FCS.

Immunocytochemical studies. We maintained TWNT-4 cells in either the presence or absence of EGCG (100 μmol/L) under serum-free conditions for 24 hours. Immunocytochemical study was carried out essentially as previously reported.[19–21] After 3 washes with PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na_2HPO_4, and 1.5 mmol/L KH_2PO_4, pH 7.4), cells were fixed for 10 minutes in 3.7% formaldehyde at 37°C, permeabilized for 5 minutes in PBS containing 0.2% Triton X-100 at 37°C, washed 3 times with PBS, and blocked with PBS containing 10% FCS for 30 minutes at 37–C. The slides were then incubated with an anti–α-SMA primary antibody (Progen Biotechnik GmbH, Heidelberg, Germany) or an anti-Myc primary antibody (Roche, Tokyo, Japan) at 37°C for 60 minutes. The slides were rinsed extensively in PBS and then stained with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, Ore) mixed with Alexa Fluor 488-labeled goat anti–mouse secondary antibody (Molecular Probes). Images were visualized with an LSM 510 confocal laser scanning microscope (Zeiss, Tokyo, Japan).

Pull-down assay of GTP-bound Rho. Rho activation was analyzed with the use of a Rhotekin pulldown assay and a Rho Activation Assay Biochem Kit (Cytoskeleton Inc, Denver, Colo) in accordance with the manufacturer's instructions. TWNT-4 cells were maintained in either the presence or absence of EGCG (100 μmol/L) under serum-free conditions for 24 hours, and the cells were washed twice with and lysed in lysis buffer containing 50 mmol/L Tris, pH 7.5; 10 mmol/L $MgCl_2$; 0.5 mol/L NaCl; and 1% Triton X-100. The extracts were incubated for 1 hour at 4°C with GST-tagged Rhotekin Rho-binding domain bound to glutathione agarose beads. After washing with lysis buffer and wash buffer containing 25 mmol/L Tris, pH7.5; 30 mmol/L $MgCl_2$; 40 mmol/L NaCl; and 150 mmol/L EDTA, bound proteins were eluted in SDS sample buffer and analyzed with the use of Western blotting involving anti-RhoA monoclonal antibody.

Evaluation of phosphorylation of FAK. We performed immunoprecipitation and immunoblotting as described previously.[26] TWNT-4 cells were maintained in either the presence or absence of EGCG (100 μmol/L) under serum-free conditions for 24 hours, after which the cells were washed twice with PBS. The cells were subjected to lysis in Nonidet P-40 buffer, then centrifuged for 10 minutes at 15,000g. The supernatants were incubated with anti-FAK monoclonal antibody (Transduction Laboratories, Lexington, Ky) for 2 hours, after which the complexes were precipitated with Protein A Sepharose CL-4B (Pharmacia, Uppsala, Sweden) for 1 hour and the Sepharose beads were washed 3 times with lysis buffer and then boiled for 5 minutes in SDS sample buffer. We analyzed the eluted samples using Western blotting involving anti-FAK monoclonal antibody (Transduction Laboratories) or anti-phosphotyrosine antibody py20 (Transduction Laboratories).

Analysis of BrdU incorporation. HSC We measured the incorporation of BrdU using a cell-proliferation enzyme-linked immunosorbent assay (Roche Diagnostics GmbH,