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Green Tea Polyphenols as Potent Enhancers of Glucocorticoid-Induced Mouse Mammary Tumor Virus Gene Expression

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The effect of natural and synthetic galloyl esters on glucocorticoid-induced gene expression was evaluated by using rat fibroblast 3Y1 cells stably transfected with a luciferase reporter gene under the transcriptional regulation of the mouse mammary tumor virus promoter. The glucocorticoid-induced gene transcription was strongly suppressed by synthetic alkyl esters; *n*-dodecyl gallate showed the most potent inhibition (66% inhibition at 10 μM), which was far more potent than that of crude tannic acid. *n*-Octyl and *n*-cetyl gallate also showed good inhibition, while gallic acid itself was not so active, suggesting that the presence of hydrophobic side chain is important for the suppressive effect. On the other hand, surprisingly, green tea gallocatechins, (−)-epigallocatechin-3-*O*-gallate and theasinensin A, potently enhanced the promoter activity (182 and 247% activity at 1 μM, respectively). The regulation of the level of the glucocorticoid-induced gene expression by the antioxidative gallates is of great interest from a therapeutic point of view.

Key Words: **glucocorticoid; reporter gene assay; mouse mammary tumor virus promoter; human immunodeficiency virus; green tea polyphenols; (−)-epigallocatechin-3-*O*-gallate; *n*-dodecyl gallate; gallic acid; galloylesters; regulation of gene expression.**

The mouse mammary tumor virus long terminal repeat (MMTV LTR) has been well studied and used as a model system for the regulation of steroid-induced gene transcription (1). Recently, Tanuma and coworkers have reported that the glucocorticoid-induced MMTV gene expression was suppressed by tannic acid (100 μg/ml) in mouse mammary tumor 34I cells (2). Further, promoter region analysis revealed that two elements, a 13-bp element and an ACTG motif, in a 50-bp region, contribute to the tannic acid sensitivity and the promoter repression. Moreover, the suppressive effect of tannic acid on the gene transcription was suggested to be mediated by a protein factor(s) that binds to the negative regulatory element. Very interestingly, the repressive ACTG motif is also present in the human immunodeficiency virus (HIV) promoter, the activity of which was also suppressed by tannic acid (3).

These results provided an important insight into the regulation of the level of retroviral gene expression by tannic acid, however, the tannic acid of commerce used in the studies is a crude mixture of gallotannins including galloyl (3,4,5-trihydroxybenzoyl) esters of glucose, and its chemical composition is not well defined. Therefore in order to accurately evaluate the activities of gallotannins, we now report the effect of chemically defined natural and synthetic galloyl esters on the glucocorticoid-induced gene expression, using rat fibroblast 3Y1 cells stably transfected with a luciferase reporter gene under the transcriptional regulation of the MMTV LTR (4). In agreement with the previous report, we found that the glucocorticoid-induced MMTV promoter activity was strongly suppressed by synthetic galloyl esters with hydrophobic alkyl side chain. In particular, *n*-dodecyl gallate (DG) (**2**), a widely used antioxidant food additive, showed the most potent inhibition, which was far more potent than that of the crude tannic acid. In contrast, to our surprise, green tea gallocatechins; (−)-epigallocatechin-3-*O*-gallate (EGCG) (**9**) and theasinensin A (**10**), potently enhanced the promoter activity, which was of great interest from a therapeutic point of view.

MATERIALS AND METHODS

Chemicals. Natural and synthetic esters of gallic acid were obtained as described before (5, 6). Dexathamesone and tannic acid was

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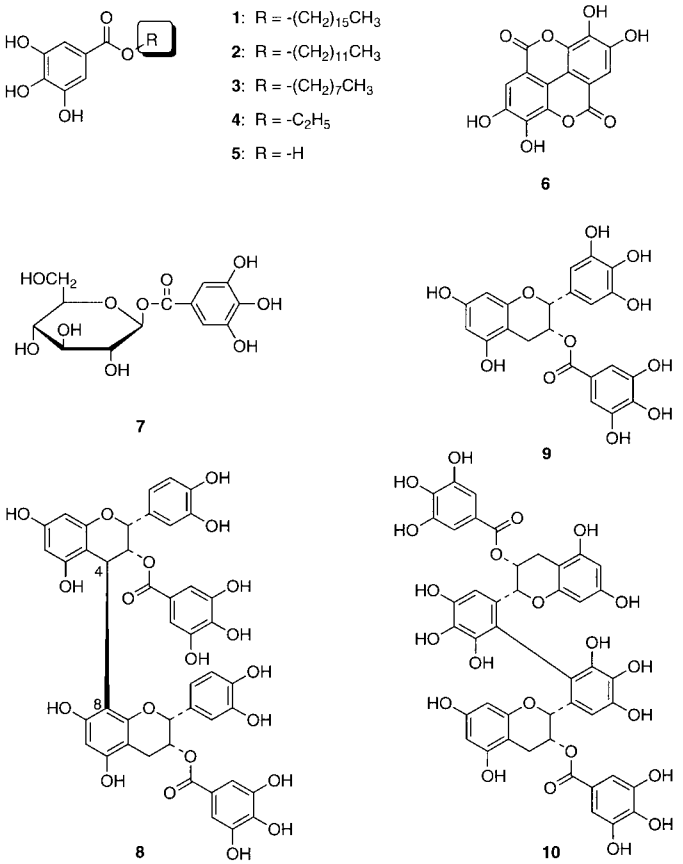

SCHEME

purchased from Nakarai Chemicals (Kyoto, Japan) and Wako (Tokyo, Japan), respectively.

Cell culture. Rat fiblobrast 3Y1 cells (American Type Culture Collection, Rockville, USA) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml kanamycin). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Plasmid construct. Luciferase reporter gene pBV2-MMTV-LUC was constructed by inserting the 1.5-kbp *Hind* III-*Sma* I fragment of the LTR region of the MMTV gene (Pharmacia Biotech, USA), into the *Sma* I site of pBV2 basic vector plasmid (Toyo Ink, Tokyo).

Stable transfection and assay. The pBV2-MMTV-LUC plasmid and pCAGGS-bsr plasmid with blasiticidin S resistant marker (7) were cotransfected into 3Y1 cells by using the OptiMEM/lipofectamin (Gibco, USA) according to manufacturer's instruction. The blasiticidin S resistant, stably transfected 3Y1 cells were selected and cultured routinely at 37°C in humidified chambers at 5% CO_2 in DMEM supplemented with 10% FBS and 2 mM L-glutamine. For the luciferase assay, cells were first serum starved for 24 h on DMEM supplemented with 0.5% FBS. Cells were plated at a density of 20,000 cells per well in 96 well plates (Costar, USA), totally opaque in 100 μl of DMEM supplemented with 0.5% of FBS. Then, dexamethasone (200 nM) and test compound were added to the culture medium. After 12 h of incubation, the cells were washed twice with phosphate-buffered saline and incubated with 20 μl of lysis buffer for 10 min at room temperature. Luciferase activity was measured in a luminometer (Labsystems Luminoskan RS), with automatic injection of 100 μl luciferin per well, and luminescence generated from each well was integrated over a 10 second interval. All data points represent the mean of quadruplicate experiments against luciferase activity. Error bars represent the standard deviation of the mean.

RESULTS AND DISCUSSION

The effect of natural and synthetic galloyl esters on glucocorticoid-induced gene expression was evaluated by using rat fibroblast 3Y1 cells stably transfected with a luciferase reporter gene under the transcriptional regulation of MMTV LTR (4). In the 3Y1 cells, dexamethasone, a synthetic glucocorticoid, induced luciferase activity in dose dependent manner with a detection limit of 0.1 nM, an EC_{50} of 5 nM, and maximum induction of 30-fold relative to solvent controls. Natural and synthetic steroid hormones such as 17β-estradiol, tamoxifen, testosterone propionate and flutamide did not induce the gene transcription, whereas hydrocortisone and dexamethasone acetate induced luciferase activity to a maximal equal to dexamethasone, indicating that the gene transcription is primarily regulated through the endogenous glucocorticoid receptors (4).

In consistent with the earlier report (2), the dexamethasone-induced MMTV gene transcription was suppressed by crude tannic acid (25% inhibition at 17 μg/ml) in our assay system (Fig. 1). More potent inhibition was attained by synthetic alkyl esters of gallic acid, the basic structural unit of gallotannins. Among them, *n*-dodecyl (C_{12}) gallate (DG) (**2**) showed the most potent inhibition (66% inhibition at 10 μM, 3.4 μg/ml). *n*-Octyl (C_8) gallate (**3**) and *n*-cetyl (C_{16}) gallate (**4**) also showed good inhibition, while ethyl gallate (**4**) and gallic acid (**5**) were not so active, suggesting that the presence of hydrophobic alkyl side chain is important for the potent suppression of the MMTV gene transcription. Here it should be noted that within the concentration range used for the assay, there was essentially no effect on proliferation of the cells, which was confirmed by counting the number of cells by the standard alamarBlue assay. On the other hand, addition of more than 20 μg/ml of alkyl gallates strongly retarded the growth of the rat fiblobrast 3Y1 cells. As described below, the lipid derivatives of gallic acid have been reported to inhibit proliferation of lymphocyte cells and induce apoptosis in tumoral cell lines (8, 9).

In addition, ellagic acid (**6**), the basic unit of ellagitannins, also showed good suppression of the glucocorticoid-induced luciferase activity (30% inhibition at 10 μM). It has been reported that oligomeric ellagitannins including nobotanin B (30 μM) potently suppressed the glucocorticoid-induced MMTV gene expression in mouse mammary tumor 34I cells (10).

In contrast, 1-*O*-galloyl-β-D-glucose (**7**) did not suppress the MMTV gene transcription, instead significantly enhanced the promoter activity (141% activity

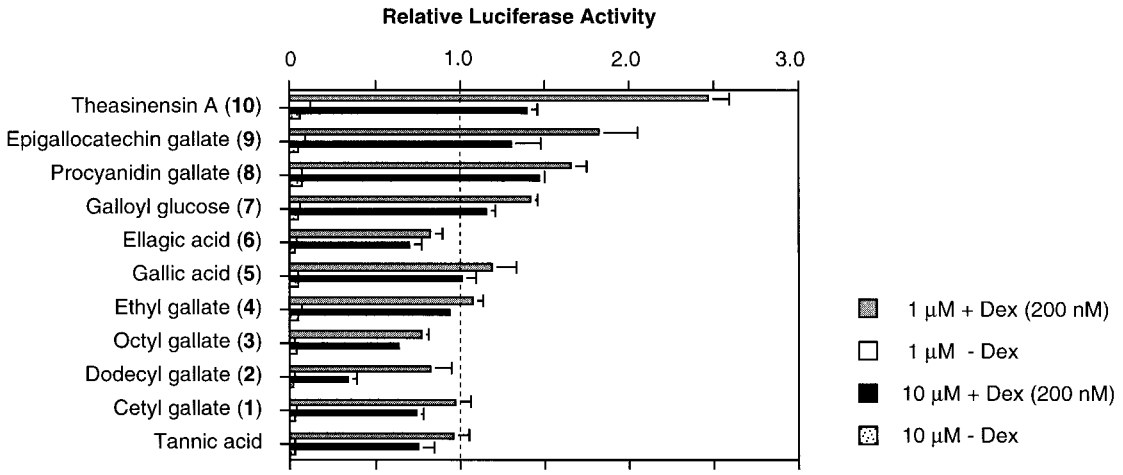

FIG. 1. The effect of natural and synthetic galloyl esters on the dexamethasone-induced luciferase activity in the stably transfected rat fibroblast 3Y1 cells. The cells were treated with 1 μM or 10 μM of test compound for 12 h in the presence or absence of 200 nM of dexamethasone. The relative luciferase activities to that of a control experiment (the cells were treated with 200 nM of dexamethsone only) are illustrated. Results are shown as the average of quadruplicate experiments. Error bars represent the standard deviation.

at 1 μM). To our surprise, the augmentation of the luciferase activity was further eminent with (−)-epigallocatechin-3-*O*-gallate (EGCG) (**9**) (182% activity at 1 μM), the major component of the green tea polyphenols, and gallocatechin dimers, procyanidine B-2 3,3′-di-*O*-gallate (**8**) (165% activity at 1 μM) and theasinensin A (**10**) (247% activity at 1 μM). Among them, theasinensin A, a very minor component of green tea, showed particularly strong enhancement (247% activity at 1 μM, 0.9 μg/ml). Interestingly, in these cases, more potent activities were obtained with 1 μM than with 10 μM concentration.

Alkyl gallates including *n*-octyl gallate and DG are widely used as antioxidant food additives due to their scavenging activity against reactive oxygen species. Further, the lipid derivatives of gallic acid are known to be potent inducers of apoptosis in tumoral cell lines and inhibitor of lymphocyte proliferation (the IC_{50} value for DG is dependent on the cell type varying from 0.6 to 19. 5 μM) (8, 9). In addition, DG showed good inhibition toward human spleen protein tyrosine kinase (IC_{50} = 5 μM) (11). We have also reported that DG (IC_{50} = 0.061 μM) along with other galloyl esters are potent enzyme inhibitors of recombinant rat squalene epoxidase, a flavin monooxygenase and a rate-limiting enzyme of cholesterol biogenesis (6).

Presumably, the highly antioxidative, hydrophobic DG would have better permeability of the cell membrane and better interaction with the nuclear proteins and/or enzymes involved in the glucocorticoid-induced gene transcription. As described, previous studies have demonstrated that two elements, a 13-bp element and an ACTG motif, in a 50-bp region of the MMTV LTR, contribute to the tannic acid sensitivity and the promoter repression (2). Moreover, the gene suppression has been suggested to be mediated by a specific protein factor(s) that binds to the negative regulatory element (2). Although more detail of the suppression of the MMTV gene transcription is not well understood at present, one of the possible mechanisms may involve inhibition of protein tyrosine phosphorylation of the transcriptional coactivator proteins by the antioxidative galloyl esters. On the other hand, it has been also suggested that the gene suppression may be result from inhibition of poly(ADP-ribose) glycohydrolase, for which ellagitannins showed more potent inhibition activity than gallotannins (10). Very interestingly, the tannic acid-responsive ACTG motif of the MMTV promoter is also present in a 30-bp element of the HIV LTR, the activity of which was also suppressed by tannic acid (3). Therefore, the gene suppression by DG and its analog compounds would possibly contribute to the development of therapeutic agents for the retrovirus-associated diseases.

For green tea polyphenols (GTPs), a variety of biological activities including cancer prevention (12), suppression of angiogenesis (13), cholesterol lowering effect (5), and induction of apoptosis (14), have been reported. The above described potent enhancement of the glucocorticoid-induced gene expression by GTPs adds another beneficial feature of green tea. In particular, this is interesting from a therapeutic point of view, since glucocorticoids are involved in a variety of physiological processes that range from the regulation of the stress response and the control of the immune system to modulation of behavior. In addition, glucocoticoids are the most widely used anti-inflammatory and immunomodulatory agents, whose mechanism of action is based mainly on interference with the activity of transcription factors (15). Although the mechanism for the enhancement of the gene expression is not clear at present, GTPs would possibly accelerate these phys-

iological processes in our body. As previously reported, green tea can be consumed in much higher doses without any toxicological effects, and orally administered EGCG was shown to be absorbed from intestinal tracts into the circulation system; 1 h after single oral administration, the EGCG concentrations of plasma and liver tissue could reach up to 12.3 μM and 48.4 nmol/g, respectively (16, 17). Further analysis of the regulation of the level of the glucocorticoid-induced gene expression is now in progress in our laboratories.

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