Gallotannin Inhibits the Expression of Chemokines and Inflammatory Cytokines in A549 Cells

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Received March 3, 2005; accepted June 20, 2005

ABSTRACT

Tannins are plant-derived water-soluble polyphenols with wide-ranging biological activities. The mechanisms underlying the anti-inflammatory effect of tannins are not fully understood and may be the result of inhibition of poly(ADP-ribose) (PAR) glycohydrolase (PARG), the main catabolic enzyme of PAR metabolism. Therefore, we set out to investigate the mechanism of the anti-inflammatory effect of gallotannin (GT) in A549 cells with special regard to the role of poly(ADP-ribose)ylation. Using an inflammation-focused low-density array and reverse transcription-polymerase chain reaction, we found that GT suppressed the expression of most cytokines and chemokines in cytokine-stimulated A549 cells, whereas the PARP inhibitor PJ-34 only inhibited few transcripts. Activation of the transcription factors, nuclear factor κB (NF-κB) and activator protein 1 (AP-1), was blocked by GT, whereas PJ-34 only suppressed NF-κB activation but not AP-1 activation. GT also inhibited IκB phosphorylation and nuclear translocation of NF-κB, but PJ-34 had no effect on these upstream events. In the AP-1 pathway, GT treatment, even in the absence of cytokines, caused maximal phosphorylation of c-Jun N-terminal kinase and c-Jun. GT also caused a low-level phosphorylation of p38, extracellular signal-regulated kinases 1 and 2, activating transcription factor 2, and cAMP-response element-binding protein but inhibited cytokine-induced phosphorylation of these kinases and transcription factors. GT inhibited protein phosphatases 1 and 2A, which may explain the increased phosphorylation of mitogen-activated protein kinase and their substrates. GT exerted potent antioxidant effect but failed to cause PAR accumulation. In summary, the potent inhibitory effects of GT on the transcription of cytokine and chemokine genes are probably not related to PARG inhibition. Inhibition of AP-1 activation and upstream signaling events may be responsible for the effects of GT.

Tannins are water-soluble polyphenols that are widely distributed in the plant kingdom, including food grains and fruits. So far, more than a thousand different tannins have been characterized and ordered into four major groups: 1) gallotannins (GT), 2) ellagitannins, 3) complex, and 4) condensed tannins, with gallotannins and ellagitannins considered the most widespread types. The common structural elements of all tannins include one or more polyol units (mostly α-glucose) and one or more polyphenols (gallic acid, 3,4,5-trihydroxybenzaldehyde).

The simplest hydrolyzable tannin, gallotannin, is a mixture of polygalloyl esters of glucose. Gallotannin and other tannins have been shown to exert various biological effects ranging from anti-inflammatory to anticancer and antiviral effects (Fong et al., 1972; Mota et al., 1985; Uchiumi et al., 1996; Van Mole et al., 2000; Feldman et al., 2001). The mechanisms underlying the anti-inflammatory effect of tannins include the scavenging of radicals (antioxidant effect) (Hagerman et al., 1999) and inhibition of the expression of

ABBREVIATIONS: GT, gallotannin(s); ABTS, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); PAR, poly(ADP-ribose); PARG, PAR glycohydrolase; ERK, extracellular signal-regulated kinase; PARP, PAR polymerase; NF-κB, nuclear factor κB; AP-1, activator protein-1; PP1c, catalytic subunit of protein phosphatase 1; PP2Ac, catalytic subunit of protein phosphatase 2A; IL, interleukin; RT-PCR, reverse transcription-polymerase chain reaction; CREB, cAMP-response element-binding protein; TNFα, tumor necrosis factor α; MMLV, Moloney murine leukemia virus; MAPK, mitogen-activated protein kinase(s); MEKK1, mitogen-activated protein kinase kinase 1; PBS, phosphate-buffered saline; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay; TBS, Tris-buffered saline; JNK, c-Jun N-terminal kinase; DHR123, dihydrorhodamine 123; TRE, 12-O-tetradecanoylphorbol-13-acetate-response element; ATF, activating transcription factor.
inflammatory mediators, such as some cytokines (Feldman et al., 2001), inducible nitric-oxide synthase, and cyclooxygenase-2 (Lee et al., 2003). Most of these studies focused on the effects of tannins on immune cells with special regard to mononuclear cells and macrophages, and little is known regarding the possible effects of tannins in epithelial cells.

Various classes of tannins have also been demonstrated to inhibit poly(ADP-ribose) (PAR) glycohydrodase (PARG), the catabolic enzyme of poly(ADP-ribose) metabolism. Poly(ADP-ribosyl)ation is a post-translational protein modification catalyzed by poly(ADP-ribose) polymerase (PARP) enzymes with PARP-1 responsible for more than 90% of the cellular poly(ADP-ribosyl)ation capacity (Burkle, 2001; Ame et al., 2001), inducible nitric-oxide synthase, and cyclooxygenases, and transcription factors. Reversible poly(ADP-ribosyl)ation regulates various cellular processes, including transcription. The inhibitory effect of PARP inhibitors on the transcription of inflammatory mediators, such as cytokines, chemokines, and inducible nitric-oxide synthase, has been made responsible for the anti-inflammatory effects of PARP inhibition (for review, see Erdélyi et al., 2005). The role of PARP in this process, however, is not fully understood. It is plausible to hypothesize that tannins may increase the amount of poly(ADP-ribosyl)ated proteins in the cell and may thus modulate transcription.

Accelerated PAR metabolism has been implicated in various oxidative stress-related lung diseases, such as asthma, reperfusion injury, acute respiratory distress syndrome, asbestosis, and shock (Virag, 2005). Many studies have demonstrated that PARP inhibitors selectively regulated the expression of cytokines and chemokines (chemotactic cytokines) in these and similar inflammatory disease models (Virag, 2005). Reduced expression of chemokines and adhesion molecules may be responsible for the reduced migration of inflammatory cells, the most common anti-inflammatory effect of PARP inhibition as observed in animal studies (Zingarelli et al., 1998; Hasko et al., 2002). However, it is not known whether macrophages or parenchymal cells are the main targets of PARP inhibitors in these diseases. Furthermore, the cellular effects of gallotannin in lung epithelial cells have not yet been characterized.

Herein, we show that immune-stimulated A549 type II lung epithelial cells express many chemokines and inflammatory cytokines. GT abolishes the expression of most chemokines/cytokines, whereas the potent PARP inhibitor PJ-34 suppressed only few transcripts. We demonstrate that, in A549 cells, 30 μM GT acts at various levels of the signal transduction cascade of the NF-κB and activated protein-1 (AP-1) pathway without causing major perturbations in poly(ADP-ribose) catabolism.

Materials and Methods

Cell Culture and Treatments. The A549 cell line was grown and maintained in a 5% CO2 incubator at 37°C using RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum and penicillin/streptomycin (Sigma-Aldrich). Cells were grown to confluence and incubated in serum-free medium for 12 h before treatment. The PARP inhibitor PJ-34 (10 μM) (Inotek Corporation, Beverly, MA.) and the PARG inhibitor gallostatin (30 μM; Fluka Chemical Corp., Ronkonkoma, NY) were added to the cultures 30 min before stimulation by recombinant human TNFα (20 ng/ml) and recombinant human IL-1β (5 ng/ml), both purchased from R&D Systems (Minneapolis, MN).

Expression Profiling with Low-Density Arrays. Profiles of TNFα/IL-1β-induced gene expression were determined by using the protocol of GEArray pathway-specific expression arrays from SuperArray (SuperArray Bioscience, Frederick, MD). After treatment with 20 ng/ml TNFα and 5 ng/ml IL-1β for 4 h, total RNA was isolated. RNA (2 μg) was reverse-transcribed into cdNA with MMLV reverse transcriptase dNTP mixture in the presence of RNasin ribonuclease inhibitor (both reagents were purchased from Promega, Madison, WI). cdNA was PCR-amplified with the Amplolabeling kit using GEArrayer mixture (supplied with the Amplolabeling kit) and dNTP mixture containing biotin-16-dUTP (Roche Hungarian Diagnostics, Budapest, Hungary). The resulting biotin-labeled cdNA probes were hybridized to gene-specific cdNA fragments on the nylon membranes according to the instructions by the manufacturer. Biotin was detected with streptavidin-alkaline phosphatase and CDP-Chemi- luminescent substrate (supplied with the SuperArray kit). The relative expression level of each gene was determined with the ImagesJ software by comparing the signal intensity of each gene in the array after normalization to the signal of a housekeeping gene. Array experiments were performed on two different experimental days, and a minimum of a 2-fold difference obtained in both experiments was considered significant.

Reverse Transcription and PCR. Total RNA was isolated using SV Total RNA Isolation System (Promega) according to the instructions by the manufacturer. Concentration and purity of the isolated RNA were measured spectrophotometrically at 260 and 280 nm. Reverse transcription was performed using MMLV reverse transcriptase (Promega). A mixture of 2 μg of total RNA and 1 μl of random primers (Promega) was incubated for 5 min in a total volume of 15 μl at 70°C and cooled on ice. After adding 5 μl of MMLV 5× Reaction Buffer (Promega), 10 mM dNTPs, 1 μl of ribonuclease inhibitor (Promega), and finally, 2 μl of MMLV reverse transcriptase in a total volume of 25 μl, the reaction mixture was incubated for an additional hour at 37°C.

PCR reactions were performed using RedTaq polymerase (Sigma-Aldrich) in reaction mixtures containing 2.5 units of polymerase, 10 nmol of each primer, and 4 to 8 μl of cdNA and PCR buffers as supplied by the manufacturer in a total volume of 50 μl. PCR primers used for the analysis were designed based on sequences deposited in the UniGene database. Primer sequences and sizes of the PCR products are listed in Table I.

Nuclear Extract Preparation. Nuclear protein extracts were prepared from cells grown to 90% confluence in T-25 culture flasks. All of the nuclear extraction procedures were performed on ice with ice-cold reagents. Cells were washed with PBS and harvested by scraping into 1 ml of PBS and pelleted at 5000 rpm for 5 min. The pellet was resuspended in 400 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and protease inhibitors) and allowed to swell on ice for 15 min. After adding Nonidet-P-40 to a final concentration of 0.5%, the cells were vortexed for 10 s. After centrifugation at 10,000 rpm for 2 min, the supernatant was removed and the pellet was resuspended in 50 μl of buffer B (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and protease inhibitors) and incubated on ice for 20 min with occasional vortexing. Nuclear extracts were recovered after centrifugation for 10 min at 10,000 rpm. Protein concentrations were determined with Coomasie Blue Plus protein assay reagent (Pierce Chemical, Rockford, IL).

EMSA. The consensus NF-κB (5′-AGGTGAGGACTTTCCAGG-3′) and AP-1 (5′-CGCTTCGAGCTGACCGGAA-3′) probes were obtained from Sigma-Aldrich. The probes were labeled with biotin 3′-end DNA labeling kit (Pierce) as instructed by the manufacturer. Gel-shift assays were performed using LightShift Chemi-
luminescent EMSA kits (Pierce). In brief, binding reactions containing 10 μg of nuclear extracts and 1 nmol of oligonucleotide were performed for 30 min in binding buffer (2.5% glycerol, 0.05% Nonidet P-40, 50 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 10 mM Tris, pH 7.6, and 50 ng of poly(dI-dC). Protein-nucleic acid complexes were resolved using a nondenaturing polyacrylamide gel consisting of 5% acrylamide (29:1 ratio of acrylamide/bisacrylamide) and run in 0.5× TBE (45 mM Tris-HCl, pH 8.3, 45 mM boric acid, and 1 mM EDTA) for 1 h at a constant voltage of 100 V. Gels were transferred to Bio Bond-Plus nylon membrane (80 mA, 45 min; Sigma-Aldrich). DNA was cross-linked to the membrane by UV-cross-linker. DNA was incubated in blocking solution (supplied with the LightShift kit) followed by incubation of the membrane with streptavidin-peroxidase. After extensive washing, signal was detected with chemiluminescence solution (supplied with the kit).

**Western Blot Analysis.** Cells were washed once in PBS and collected by scraping into 200 μl of ice-cold lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 1 mM PMSF, 1 mM NaF, 1 mM Na₃VO₄, and protease inhibitors). The extracts were further lysed with sonication, and the supernatant was collected after centrifugation. Protein concentrations were determined with the Coomassie Blue assay. Proteins (20 μg/lane) were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 90 min. Primary antibodies against NF-κBp65 (polyclonal; Santa Cruz Biotechnology, Inc., Santa Cruz CA), phospho-c-Jun (polyclonal; Santa Cruz Biotechnology, Inc.), JNK/stress-activated protein kinase, phospho-p38 (Cell Signaling Technology Inc., Beverly, MA) and phospho-CREB (Cell Signaling Technology Inc., Beverly, MA) were applied overnight at 4°C. After three washes in TBS containing 0.05% Tween 20, secondary antibodies (peroxidase-conjugated goat anti-mouse or anti-rabbit IgG, Sigma-Aldrich) were applied for 1 h. Blots were washed in TBS containing 0.05% Tween 20 three times and once in TBS, incubated in enhanced chemiluminescence reagent (Supersignal Chemiluminescent substrate; Pierce), and exposed to photographic film. Films were evaluated by densitometry using Multi-Analysis software.

**TABLE 1**

<table>
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<th>Primers used in the PCR reactions</th>
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<th>Size (bp)</th>
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<td>IL-1β</td>
<td>GAA GTA CCT GAG CTC GCC AG</td>
<td>363</td>
</tr>
<tr>
<td>Forward</td>
<td>GAA GTA CCT GAG CTC GCC AG</td>
<td>363</td>
</tr>
<tr>
<td>Reverse</td>
<td>GAA GTA CCT GAG CTC GCC AG</td>
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</tr>
<tr>
<td>MIP-1α</td>
<td>GTA TAC AGC GCC GCC CCC</td>
<td>279</td>
</tr>
<tr>
<td>Forward</td>
<td>GTA TAC AGC GCC GCC CCC</td>
<td>279</td>
</tr>
<tr>
<td>Reverse</td>
<td>GTA TAC AGC GCC GCC CCC</td>
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</tr>
<tr>
<td>MCP-1</td>
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</tr>
<tr>
<td>Forward</td>
<td>GCT TCC TGG AGG TGG ATT</td>
<td>350</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCT TCC TGG AGG TGG ATT</td>
<td>350</td>
</tr>
<tr>
<td>RANTES</td>
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<td>259</td>
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<tr>
<td>Forward</td>
<td>AC ACA CCC TGC TTT GCC TAC ATT GCC</td>
<td>259</td>
</tr>
<tr>
<td>Reverse</td>
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<td>259</td>
</tr>
<tr>
<td>IL-8</td>
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<tr>
<td>Reverse</td>
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</tr>
<tr>
<td>β-actin</td>
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<tr>
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<td>CGG GAA ATC CTT GGT GAC AT</td>
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</tr>
<tr>
<td>Reverse</td>
<td>CGG GAA ATC CTT GGT GAC AT</td>
<td>712</td>
</tr>
</tbody>
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**Detection of Poly(ADP-Ribose).** Poly(ADP-ribose) was detected by immunofluorescent staining as described previously (Burk et al., 1993) with slight modifications as follows. Cells were fixed in ice-cold 10% trichloroacetic acid for 10 min and dehydrated by successive 5-min washes in 70, 90, and 100% ethanol at −20°C. Coverslips were blocked in 5% horse serum diluted in PBS-Triton X-100 for 1 h and were then incubated overnight at 4°C with 10H monoclonal anti-poly(ADP-ribose) antibody (Kawamitsu et al., 1983) diluted 1:10,000. After five 5-min washes in PBS, coverslips were incubated with biotinylated horse anti-mouse IgG diluted 1:300 for 1 h at room temperature. Excess antibody was removed by five 5-min washes in PBS. Incorporated biotin was detected by streptavidin-Allexa Fluor-488 (Molecular Probes, Eugene, OR) diluted 1:100 in PBS-Triton X-100 (30 min at room temperature). Coverslips were washed (four times for 5 min each) with PBS-Triton X-100, mounted in antifade medium, and viewed with a Zeiss Axioslab digital camera.

**ABTS Assay.** The antioxidant capacity was determined using the standard ABTS+ decolorization assay (Re et al., 1999). ABTS (Sigma-Aldrich) was used as a free radical provider and was generated by reacting this compound (7.4 mM) with potassium persulfate (2.45 mM) overnight. The solution was diluted with glycine-HCl (50 mM, pH 4.5) to obtain an absorbance of 1.5 at 414 nm. An aliquot (140 μl) of the solution was added to 10 μl of sample into a 96-well plate, and the standard curve was prepared using a similar volume of L-ascorbic acid. All of the readings were taken after 30 min of reaction time when the absorbance seemed to reach a plateau.

**DHR Assay.** Peroxynitrite scavenging effect was measured by monitoring the oxidation of dihydrorhodamine 123 (DHR123; Molecular Probes) according to the method of Koo et al. (1994). DHR123 (5 μM) was diluted in 90 mM NaCl, 50 mM Na₃PO₄, pH 7.4, and 5 mM KCl and was measured into black 96-well plates (100 μl/well). Oxidation of DHR123 by peroxynitrite (final concentration, 50 μM) was measured with a microplate fluorescence spectrophotometer with excitation and emission wavelengths of 485 and 527, respectively, at room temperature in the presence or absence of the test compounds.

**Phosphatase Activity Assay.** The catalytic subunits of protein phosphatase 1 (PP1c) and 2A (PP2Ac) were prepared from rabbit skeletal muscle and the two types of phosphatase were separated by heparin-Sepharose chromatography (Gergely et al., 1984). PP1c was purified from the heparin-Sepharose-bound fraction to homogeneity on an affinity column prepared by coupling the N-terminal PP1c-binding fragment of the myosin phosphatase target subunit to Sepharose matrix (Toth et al., 2000). PP2Ac was further purified from the heparin-Sepharose flow-through fractions on a fast protein liquid chromatography Mono Q column (Amersham Biosciences). The activity of PP1c and PP2Ac was determined with 32P-labeled 20-kDa gizzard myosin light chain substrate as described earlier (Erdodi et al., 1995).

**Results**

**Effects of GT and PJ-34 on the Expression Pattern of Chemokines and Cytokines in Immunostimulated A549 Cells.** We have used a nylon-based thematic low density array to investigate the effects of GT and PJ-34 on the expression pattern of chemokines and inflammatory cytokines in A549 cells. Ten positions on the arrays were occupied by positive controls (housekeeping genes), whereas six positions contained no cDNA (blank) or plasmid DNA (negative control). The remaining positions of the membrane contained 96 cDNAs of chemokines and inflammatory cytokines. Of these 96 genes, TNFα + IL-1β treatment significantly (minimum 2-fold induction) induced the expression of 13 genes and suppressed the expression of two cytokine receptor...
genes (Table 2). Pretreatment of cells with gallotannin significantly (by at least 50%) reduced these alterations with the exception of one chemokine (MIP-3α) and one chemokine receptor (CXC4). PJ-34 significantly enhanced fractalkine expression and inhibited the down-regulation of the chemokine receptors CCR4 and CCR5. To confirm our results, we also carried out RT-PCR reactions for seven genes; each reaction gave similar results (Fig. 1A). In addition, the expression of IL-8, a key neutrophil-recruiting chemokine that was not represented on the array, was also investigated with RT-PCR and found to be inhibited by both GT and PJ-34. GT (30 μM) alone did not induce any of the chemokines or cytokines tested (Fig. 1B). At a very high concentration (100 μM), GT induced IL-8 expression. Because NF-κB and AP-1 are known to regulate the expression of various inflammatory cytokines and chemokines, we have also investigated the effects of GT and PJ-34 on the activation of these transcription factors.

Effects of GT and PJ-34 on NF-κB Activation. The dimeric transcription factor NF-κB plays a central role in the transcriptional regulation of inflammatory factors and, in resting conditions, is sequestered in the cytoplasm as an inactive complex by its physical association with the inhibitor of NF-κB (IκB) (Schmitz et al., 2004). Activation of NF-κB has been shown to occur through the activation of upstream protein kinases (e.g., NF-κB-inducing kinase, MEKK1, NF-κB-activating kinase) phosphorylating the IκB kinase complex (Yamamoto and Gaynor, 2004). Activation of this complex serves to mediate phosphorylation, ubiquitination, and degradation of IκB followed by nuclear translocation of NF-κB.

Treatment of A549 cells with TNFα/IL-1β induced NF-κB activation as demonstrated by EMSA analysis (Fig. 2). Pretreatment of the cells with PJ-34 or GT markedly reduced the binding of NF-κB to its consensus oligonucleotide. TNFα/IL-1β-induced nuclear translocation of NF-κB was blocked by GT but was unaffected by PJ-34, indicating that PARP inhibition by PJ-34 may inhibit the DNA binding of the transcription factor. As for GT, we have also investigated IκB phosphorylation, an event laying upstream in the NF-κB pathway. GT abolished phosphorylation, suggesting that GT may inhibit the kinase cascade (IκB kinase or upstream kinases).

Table 2
Effect of gallotannin and PJ-34 on TNFα/IL-1β-induced chemokine and cytokine expression

<table>
<thead>
<tr>
<th>Gene Product Name</th>
<th>Common Name</th>
<th>TNFα + IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td></td>
<td>GT</td>
</tr>
<tr>
<td>MIP-1β</td>
<td></td>
<td>PJ34</td>
</tr>
<tr>
<td>RANTES</td>
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<td>ENA-78</td>
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<tr>
<td>GCP-2</td>
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<td>Fractalkine</td>
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</table>

Effects of GT and PJ-34 on AP-1 Activation. AP-1 is a collection of dimeric transcription factors that belong to the Jun, Fos, Maf, and ATF subfamilies (Kyriakis, 1999; Wisdom, 1999). Depending on the composition of the dimers, they recognize either 12-O-tetradecanoylphorbol-13-acetate-response elements (TRE, 5′-TGA(G/C)TCA-3′) or cAMP-response elements (5′-TGACGTCA-3′), which are located in the promoter regions of genes encoding cytokines, chemokines, adhesion molecules, and transcription factors. Whereas the Jun/Fos heterodimers preferentially bind to the TRE elements, dimers containing ATF2 (e.g., ATF2/ATF2 or c-Jun/ATF2 dimers) bind to cAMP-response element.

We have observed a basal AP-1 activity as demonstrated by EMSA experiments using the TRE consensus element (Fig. 3). TNFα/IL-1β treatment triggered further AP-1 activation.
GT pretreatment abolished both basal and TNFα/IL-1β-induced AP-1 activation. PJ-34 had no effect on AP-1 activation.

Mitogen-activated protein kinases JNK, p38, and ERK1/2 play key roles in cytokine-induced signaling (Johnson and Lapadat, 2002). Because formation of the c-Jun/c-Fos heterodimer of AP-1 is induced by JNK-mediated phosphorylation of c-Jun (Kyriakis and Avruch, 2001), we have also investigated the effects of GT and PJ-34 on these upstream events of the AP-1 pathway. TNFα/IL-1β induced a rapid phosphorylation of JNK detectable as early as 2.5 min, peaking between 5 and 10 min and fading 40 min after the cytokine treatment (Fig. 3). It is surprising that GT stimulated basal JNK phosphorylation that was not further increased by the cytokines. PJ-34 had no effect on JNK phosphorylation. Phosphorylation of c-Jun has shown a prolonged...
Pattern with signals detectable even in unstimulated cells. Whereas PJ-34 had no effect on c-Jun phosphorylation, GT treatment, even in the absence of cytokines, induced maximal c-Jun phosphorylation that was not further enhanced by TNFα/IL-1β. c-Jun can heterodimerize with ATF2 that is regulated mainly by p38 MAPK. Therefore, we also sought to determine whether GT and PJ-34 affect the p38-ATF2 pathway. TNFα/IL-1β induced a rapid phosphorylation of p38, which did not fade during the 40-min period tested (data not shown). PJ-34 had no effect on p38 phosphorylation (Fig. 4A). Although GT alone caused a low-level phosphorylation of p38, the TNFα/IL-1β-induced signal was reduced by GT. Phosphorylation of ATF2 was similarly affected by the two drugs with no effect of PJ-34 and inhibition of cytokine-induced ATF2 phosphorylation by GT (Fig. 4A).

Although MAPK ERK1/2 is mainly involved in the regulation of cell proliferation (Johnson and Lapadat, 2002), it has also been implicated in transcriptional regulation of inflammatory mediators (Neff et al., 2003; Lecureur et al., 2005). One of the downstream events in the activation of the ERK1/2 (and p38) pathway is the phosphorylation of the transcription factor CREB (Yang et al., 2003). Phosphorylated CREB has been shown to be involved in the transcriptional regulation of inflammatory mediators. We found that ERK1/2 and CREB are regulated by GT the same way as seen with p38 and ATF2 (Fig. 4B). Whereas PJ-34 had no effect on the basal and cytokine-induced phosphorylation of ERK1/2 and CREB, GT induced the phosphorylation of these proteins but inhibited their further activation by cytokines (Fig. 4B).

Considering that GT increased the phosphorylation state of many proteins (JNK, c-Jun, p38, ATF2, ERK1/2, and CREB), we hypothesized that GT may interfere with protein phosphatase activity. We have determined the effect of GT on the activities of protein phosphatases 1 and 2A and found that GT inhibited both phosphatases in a concentration-dependent manner (Fig. 5). GT also inhibited the PP1 catalytic subunit associated with a regulatory subunit as assayed with myosin phosphatase holoenzyme (data not shown), indicating that regulatory subunits do not mask the gallotannin-binding site on the catalytic subunits.

**Effect of GT on Poly(ADP-Ribosylation).** Based on the known PARG inhibitory effect of gallotannin and the anti-inflammatory effect of a recently developed nontannin PARG inhibitor (Genovese et al., 2004), we have also investigated the effect of GT on poly(ADP-ribose) metabolism as a possible mechanism underlying the anti-inflammatory effect of GT. Treatment of the cells with the cytokines for various time intervals results in a rapid increase in the poly(ADP-ribosylation) of several proteins, including the transcription factor CREB. We found that GT inhibited the poly(ADP-ribosylation) of CREB and other proteins in a concentration-dependent manner (Fig. 5). GT also inhibited the PP1 catalytic subunit associated with a regulatory subunit as assayed with myosin phosphatase holoenzyme (data not shown), indicating that regulatory subunits do not mask the gallotannin-binding site on the catalytic subunits.

**Fig. 4.** Phosphorylation of p38, ATF2, ERK, and CREB in A549 cells. A549 cells were pretreated for 30 min with 30 μM GT or 10 μM PJ-34 and were then stimulated with the cytokines TNFα and IL-1β (CM, cytokine mixture). After 2.5 to 5 min, lysates were prepared and phosphorylation of the proteins was detected in Western blots. Bands were evaluated by densitometry and density values of three independent experiments are presented as the mean ± S.E.M.

**Fig. 5.** Effect of gallotannin on the activity of PP1c and PP2Ac. Gallotannin was assayed on the phosphatase activity at concentrations of 0.5, 2, 10, and 50 μM. Gallotannin was preincubated with PP1c or PP2Ac for 5 min, and the reaction was initiated by the addition of 32P-labeled 20-kDa gizzard myosin light chain. Assays were performed at 30°C, and the 32P released from the substrate was determined. Phosphatase activity of PP1c (○) or PP2Ac (●) in the absence of gallotannin was taken as 100%. Values represent the means ± S.E.M. (n = 7–9).
periods (5 min to 4 h) caused no elevation in cellular PAR content as determined by immunofluorescence (Fig. 6) or Western blotting (Fig. 7) using the anti-PAR monoclonal antibody. Hydrogen peroxide used as a positive control triggered PAR elevation in the nucleus as demonstrated by immunocytochemistry (Fig. 6). On Western blot, the lysates of hydrogen peroxide-treated cells contained many positive bands with most immunopositivity found in the region above 116 kDa (the molecular mass of PARP-1) corresponding to automodified PARP-1 (Fig. 7). Treatment of cells with GT in the absence or presence of the cytokines caused no elevation in the cellular PAR content.

Antioxidant Effects of GT. Another feature that could, at least in part, explain the effect of gallotannin on cytokine/chemokine expression is the well known antioxidant effect of tannins (Ho et al., 1999; Riedl and Hagerman, 2001). Considering that both NF-κB and AP-1 are redox-sensitive transcription factors (Schulze-Osthoff et al., 1995), modification of the cellular redox state by GT could be responsible for the described effect of GT. We have studied the radical-scavenging effect of GT and PJ-34 in the ABTS decolorization assay. Using ascorbic acid as positive control, we have determined the ABTS-scavenging effect of GT and PJ-34 (Fig. 8A). In this assay, GT displayed an even more potent radical-scavenging effect compared with ascorbic acid. However, PJ-34 did not scavenge the radical (Fig. 8A). We have also used a pathophysiologically relevant oxidant called peroxynitrite. Peroxynitrite oxidizes DHR123 into fluorescent rhodamine. The addition of GT and ascorbic acid inhibited peroxynitrite-induced DHR oxidation, with GT being the more potent antioxidant. PJ-34 had no effect (Fig. 8B).

Discussion

Several laboratories have demonstrated that tannins exert potent anti-inflammatory effects. Most cellular studies aiming at revealing the mechanism of these anti-inflammatory effects used macrophages. However, in epithelial cells, the effect of tannins is not well characterized. We hypothesized that PARG inhibition by gallotannin may contribute to this anti-inflammatory effect because poly(ADP-ribose) polymerase-1 has been shown to regulate the expression of inflammatory mediators.

In A549 lung epithelial cells, our current study revealed no major role of poly(ADP-ribosyl)ation as indicated by the lack of effect of PJ-34 on the expression of most chemokines, with the exception of IL-8, CCR4, CCR5, and fractalkine. However, this finding does not exclude the possibility that PARP-1 regulates inflammatory gene expression via protein-protein interaction as previously demonstrated in experi-
ments using PARP-1 knock-out cells (Ha et al., 2002, 2004; Carrillo et al., 2004). To investigate this possibility in epithelial cells, studies using PARP-1 antisense or small interfering RNA will be needed. PJ-34 has previously been shown to inhibit chemokine expression in macrophages (Hasko et al., 2002), a finding also confirmed by us (data not shown). This finding emphasizes the importance of cell type- and stimulus-dependent differences in the requirement of PARP activity for transcriptional regulation.

In contrast to PJ-34, GT exerted a robust suppression of inflammatory gene expression. This effect is not caused by a general suppression of gene expression, because GT also prevented the cytokine-induced down-regulation of three chemokine receptors. In theory, the effects of GT could be attributed to hyperpoly(ADP-ribosylation) of PARP-1 or other poly(ADP-ribose) acceptors, including the transcription factors NF-κB and AP-1. Oliver et al. (1999) identified deficient NF-κB activation in PARP-1−/− mice, and it was later proposed that PARP-1 physically interacts with the NF-κB p50; however, the DNA-binding and catalytic activity of PARP-1 was found not to be required for the NF-κB coactivator function (Hassa et al., 2001). In certain cellular systems, however, PARP inhibitors did inhibit NF-κB activation (Ha et al., 2002; Hasko et al., 2002). Our data showing normal nuclear translocation but decreased DNA binding of NF-κB in PJ-34-treated cells indicate that, in cytokine-stimulated A549 cells, DNA binding of NF-κB requires poly(ADP-ribosylation). GT also blocked the NF-κB pathway. However, GT targeted an event upstream of IκB phosphorylation. Nonetheless, the inhibition of NF-κB activation by GT does not fully explain the marked effects of GT on cytokine/chemokine expression because PJ-34, which has also inhibited NF-κB, failed to affect cytokine expression. Therefore, we have considered the possibility that GT also interferes with the activation of AP-1, the other key transcription factor-regulating inflammatory gene expression.

The redox-sensitive transcription factor AP-1 is composed of a mixture of heterodimeric protein complexes derived from the Fos and Jun families. AP-1 heterodimers bind to DNA on a serum-response element with the 5′-TGA(C/G)TCA-3′ sequence. AP-1 is regulated at the level of both jun and fos gene transcription and by post-translational modifications of their gene products. MAPK with special regard to JNK play a key role in AP-1 activation by phosphorylating c-Jun (Kyriakis and Avruch, 2001; Johnson and Lapadat, 2002). Zingarelli et al. (2004) reported increased basal JNK activity and c-Jun phosphorylation but decreased AP-1 DNA binding in PARP-1 knock-out cells. It is noteworthy that, in A549 cells, we found similar effects with GT but PJ-34 had no major effect on the AP-1 pathway.

Our current data suggest that AP-1, rather than NF-κB, plays a key role in the regulation of cytokine/chemokine gene expression in A549 cells. It is noteworthy that suppression of AP-1 DNA binding by GT was paralleled by maximal activation (phosphorylation) of JNK and c-Jun, even in the absence of cytokines. To elucidate the mechanism by which GT “uncouples” phosphorylation of JNK and c-Jun from DNA binding of AP-1 requires further investigation. It is possible that GT triggers the JNK-c-Jun pathway by an unknown mechanism (e.g., by inhibiting protein phosphatases) and, independent of this, it also interferes with the DNA binding of AP-1. Decreased AP-1 DNA binding in GT-treated cells may result from the inhibition of the p38-ATF2 pathway that is also important in the TNFα/IL-1β-induced inflammatory gene expression. The MAPK ERK can also regulate inflammatory gene transcription by indirectly activating CREB. The ERK-CREB pathway and the p38 pathway seem to be similarly affected by GT. GT inhibited cytokine-induced activation of both ERK and CREB, but GT alone caused a moderate phosphorylation of these proteins.

Gallotannin-induced phosphorylation of MAPK and MAPK targets may be due to interference of GT with protein phosphatases. Our data indicate that GT inhibits the catalytic subunits of protein phosphatases 1 and 2A. This inhibitory activity could also be observed on the phosphatase holoenzyme. PP1 and PP2A have been proposed to regulate the MAPK pathways in various systems (Garcia et al., 2002; Kim et al., 2003). Therefore, inhibition of PP1 and PP2A by GT may contribute to the increased phosphorylation level of MAPK in GT-treated cells. MAPK phosphatases also play a key role in dephosphorylation of MAPK. Whether MAPK phosphatases are also inhibited by GT remains to be seen.

We also sought to determine whether the transcriptional regulatory effect of GT is related to PARG inhibition. Considering that a basal PARP activity is usually present in cultured cells (Bakondi et al., 2002), we expected GT to cause PAR accumulation. Our data showing the lack of PAR accumulation in GT-treated cells suggest that no major alterations of PAR metabolism occur in response to GT treatment.
This is in line with previous reports from Falsig et al. (2004), demonstrating that GT inhibits PARG in a cell-free assay but has no effect on PARG activity in intact cells. Moreover, the cytokine exposure stimulated no PAR synthesis in either the absence or presence of GT. In light of these data, it seems unlikely that PARG is the major target of GT in our system. Furthermore, a GT concentration of 50 \( \mu M \) or higher was previously shown to be required for PAR accumulation in cell lysates (Keil et al., 2004), whereas the marked transcriptional inhibitory effects in our current study required lower concentrations. Nonetheless, PAR accumulation on certain low abundance proteins may remain undetected in Western blots or immunocytofluorescent stainings and may be important for the regulation of transcription. Recent generation of PARG-deficient mice (Cortes et al., 2004; Koh et al., 2004) will certainly accelerate research on the role of PARG in transcriptional regulation.

Considering that both NF-\( \kappa \)B and AP-1 are regarded as redox-sensitive transcription factors, the antioxidant effect of GT may explain its effect on inflammatory gene expression. Our data showing potent antioxidant effects of GT at relatively low concentrations (30 \( \mu M \)) support this hypothesis.

### Conclusion

As opposed to macrophages, where GT seems to act as a proinflammatory stimulus (Rohrbach et al., 1989; Rapizzi et al., 2004), in epithelial cells, it acts as an anti-inflammatory agent. This effect results from the inhibition of the AP-1 pathway and, to a lesser extent, the NF-\( \kappa \)B pathway. Unlike in macrophages, in A549 epithelial cells, poly(ADP-ribose)ylation is not a crucial mechanism in the regulation of inflammatory gene expression and PARG is probably not the target of GT in this system.

### Acknowledgments

We thank Dr. Shiao-Li Oei (Free University Berlin) for providing the 10H hybridoma.

### References


Uchiimi F, Maruta H, Inoue J, Yamamoto T, and Tanuma S (1996) Inhibitory effect of tannic acid on human immunodeficiency virus promoter activity induced by...


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