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Specific Induction of Glutathione *S*-Transferase GSTM2 Subunit Expression by Epigallocatechin Gallate in Rat Liver

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ABSTRACT. The antitumor effect of green tea polyphenols has been well characterized in numerous papers. However, the mechanism of their action is still poorly defined. In this study, epigallocatechin gallate (EGCG), the main ingredient of green tea extract, was studied for its effect on the expression of glutathione *S*-transferases (GSTs) in rat liver to examine the mechanism of action. Liver samples were collected from Sprague–Dawley rats treated with EGCG in H_2O by portal vein perfusion and examined for total GST activity and GST expression. The results showed that the induction of GST activity by EGCG was dose- and time-dependent. GST activity was increased about 28-fold at 12 hr after treatment. Three GST subunits (GSTA1/2, GSTM1, and GSTM2) were examined by Western blot for changes in protein level affected by EGCG (1 mg/kg weight). Only GSTM2 revealed a significant time-dependent increase, with a maximal induction of ~2.0-fold. The differential effect of EGCG on GST subunit expression was also verified by immunocytochemical examination and showed strong induction of the GSTM2 (but not the GSTA1/2 and GSTM1) level in liver section. This induction occurred as early as 3 hr after treatment and extended gradually outward from the hepatic veins as treatment time increased. The change in the GSTM2 protein level was accompanied by a corresponding alteration in mRNA quantity (~2.0-fold of control). Our report is the first to demonstrate a specific induction of the GSTM2 subunit by a chemopreventor and suggests a primary influence of EGCG on *GSTM2* gene expression. BIOCHEM PHARMACOL **60**;5:643–650, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. epigallocatechin gallate; gene expression; glutathione *S*-transferase; rat liver

EGCG§ is one of the major constituents of GTPs, which have been shown to possess numerous anticarcinogenic and antimutagenic properties. Rodent studies showed that GTPS significantly inhibited tumorigenesis in the skin, lung, forestomach, esophagus, duodenum, and colon [1–5]. GTP also reduced the incidence of aflatoxin B1-induced initiation of carcinogenesis in rats [6] and inhibited the proliferation of cultured cancerous cells [7–10]. As for EGCG alone, a number of studies have demonstrated its inhibitory effects on the processes of carcinogenesis in several animal systems, such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-induced carcinogenesis in the rat glandular stomach [11] and 1,2-dimethylhydrazine-induced mice large intestinal cancers [12]. In addition, EGCG has been shown to be growth inhibitory in a number of tumor cell lines [8, 13, 14]. A recent study of Chen *et al.* further demonstrated that this effect of EGCG is cancer-specific [15].

Several mechanisms have been proposed to explain the antimutagenic and anticarcinogenic effects of EGCG and GTP, including inhibition of enzymes (e.g. cytochromes P-450) involved in the bioactivation of various carcinogens [16, 17], scavenging of reactive oxcgen species by their antioxidant properties [18, 19], and, most importantly, enhancement of the activities of phase II detoxifying enzymes such as GSTs [20, 21]. GSTs are a family of dimeric enzymes responsible for the metabolism of a broad range of xenobiotics and carcinogens [22]. Based on amino acid sequence similarities and antibody cross-reactivities, the cytosolic GSTs are grouped into six classes, termed alpha, mu, kappa, theta, pi, and sigma. The members of the *GST* supergene family have diverse structures and catalyze GSH conjugation to both identical as well as very different substrates. This conception is consistent with the requirement of their multiple functions in the metabolism of a multitude of chemical compounds and other physiologically important processes.

Although EGCG is known to increase the activity of GST overall, it is still unclear which isoform(s) is affected.

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§*Abbreviations:* CDNB, 1-chloro-2,4-dinitrobenzene; DIG, digoxigenin; ECL, enhanced chemiluminescence; EGCG, (−)-epigallocatechin gallate; GST, glutathione *S*-transferase; GTP, green tea polyphenol; IgG, immunoglobulin G; RT–PCR, reverse transcription–polymerase chain reaction; and SSC, sodium chloride and sodium citrate buffer

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A detailed understanding of the mechanism of EGCG's action would give increased confidence to its application in chemoprevention. Therefore, we investigated the influence of EGCG on the expression of GST isoforms in the rat liver.

MATERIALS AND METHODS

Animal Treatment and Sample Preparation

Male rats (Sprague–Dawley, 100–150 g) purchased from the National Taiwan University Hospital Animal Center were housed, three per cage, in an environmentally controlled animal room. The animals were treated with indicated dosages of EGCG (dissolved in 100 μL sterile distilled water; Sigma Chemical) by injecting the chemical through the portal vein under light ether anesthesia. Animals were allowed to recover from the anesthesia and were then killed at the indicated time points.

Liver tissue close to the portal vein area was excised from each animal. A portion of the tissue was fixed in 10% formalin and embedded in paraffin. A second portion was homogenized in a buffer containing 50 mM Tris–HCl and 0.25 M sucrose, pH 7.5. The homogenates were centrifuged at 39,000 rpm (rotor 80Ti, L-80, Beckman) for 30 min at 4°. The supernatants (cytosolic protein) were used for GST activity assay and Western blot analysis. Another portion of the liver tissue was homogenized in solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0; 0.5% sarcosyl, 0.1 M 2-mercaptoethanol; all chemicals were purchased from Sigma Chemical) and subjected to RNA isolation according to the procedures described by Chomczynski and Sacchi [23].

GST Activity Assay

Total GST activity was measured according to the method of Habig *et al.* [24] using CDNB (Sigma Chemical) as substrate. Enzyme activity was expressed as nmol of substrate–GSH conjugate produced per min per mg of cytosolic protein.

Western Blot Analysis

Cytosolic protein samples (25 μg) were subjected to SDS–PAGE separation as described by Laemmli [25] using 12% polyacrylamide gels. After proteins were electrotransferred to nitrocellulose paper (Hybond-C Extra Supported, 0.45 μm; Amersham Life Science), the blot was incubated with the rabbit anti-rat GSTM1, GSTM2, or GSTA1/2 antiserum (1:300 dilution; Biotrin International Ltd.) in PBS. The blots were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Amersham Life Science) after washing. Immunodetection was carried out using the ECL Western blotting detection kit, also from Amersham Life Science. The protein concentration was determined with the Bio-Rad Protein Assay Kit (Bio-Rad), with BSA as a standard. The detection of the α-tubulin protein level was used as an internal control. Relative protein expression levels of each subunit were quantified by densitometric measurement of ECL reactions and normalized with the values of α-tubulin.

Immunohistochemistry

Liver sections (5 μm) were dewaxed in xylene and rehydrated by passing through a series of ethanol solutions and water. The endogenous peroxidase activity was blocked by incubating with 0.6% H_2O_2 for 5 min, followed by two washes of PBS. Non-specific binding sites were blocked with 3% fetal bovine serum in PBS for 20 min at room temperature (RT). After removing excess blocking solution, the rabbit anti-rat GSTM1, GSTM2, or GSTA1/2 (1:100 dilution) antiserum was applied to tissue sections and incubated for 1 hr at RT. After rinsing off primary antibodies with two washes of PBS, the slides were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 45 min at RT. The sections were washed again, and the target proteins were visualized by incubation with freshly made diaminobenzidene (DAB; Sigma Chemical) substrate solution (0.01% H_2O_2, 0.05% DAB in 0.05 M Tris–HCl, pH 7.65) for 10 min. The substrate solution was rinsed off, and the slides were counterstained with hematoxylin, dehydrated, and then mounted. The tissue sections were examined under a microscope with a bright field condenser.

Northern Hybridization

Total RNA samples (10 μg) were separated on 1% agarose formaldehyde gels and transferred capillarily onto nitrocellulose membrane. After prehybridization, hybridization was performed at 42° overnight in a high-SDS buffer (7% SDS, 50% formamide, 2% blocking reagent from Boehringer Mannhein, 5X SSC, 50 mM sodium phosphate, pH 7.0, and 0.1% *N*-lauroylsarcosine) containing 50 ng/mL of DIG-labeled cDNA probe. After washing, the target mRNA was detected with the DIG detection system following the procedures supplied by Boehringer Mannheim. The detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. The autoradiograph results were quantitated by densitometric scanning and normalized with the values of GAPDH.

Probe Preparation

The plasmid containing the cDNA encoding for rat GSTM2 subunit [26] was constructed by inserting the cDNA into a pGEM vector in our laboratory. The cDNA insert was amplified by the RT–PCR technique using primers corresponding to the 5′ end of coding sequence 5-AGCATGGGGGATCCTCCCGACTATGACAGA and 3′ non-coding sequence 5-GTCCAGACCTAGG-GATCCTCATGAGTG. The cDNA was labeled with the DIG-labeling system following the procedures sup-

plied by Boehringer Mannheim and used for Northern hybridization.

RESULTS

Effect of EGCG on GST Activity in Rat Liver

In order to obtain enough sample for different analyses that could be correlated to each other and to observe the exact response in the liver tissue, the chemical was applied to the animals by portal vein perfusion. Although previous studies [20, 21] showed increased GST activities in the rat liver (oral feeding) or in hepatocyte cells treated with EGCG, there is no available information in the literature concerning the animal model used in this study. Therefore, the effect of EGCG on GST activity in the rat liver was investigated first. Activity measured with CDNB, a substrate for most GSTs, was increased in response to the EGCG treatment in a dose-dependent manner (Fig. 1A). The dose of 1 mg EGCG was used for a further time–course study of the cellular response. In the presence of 1 mg EGCG, GST activity in the rat liver elevated continuously with increasing treatment time (Fig. 1B).

Effect of EGCG on Protein Levels of GST Subunits

To determine the mechanism involved in the increase in GST activity by EGCG, the protein levels of several GST subunits were analyzed by Western blotting. As shown in Fig. 2, exposure of EGCG induced the cytosolic level of the GSTM2 subunit in the liver homogenates. This induction was time-dependent, reaching its maximal level (~2.0-fold of pretreatment value, Fig. 2 lower panel) at 9 hr after perfusion. On the other hand, the GSTA1/2 and GSTM1 subunits were not significantly affected by the treatment.

Immunocytochemical Examination of GST Subunits

Since EGCG was exposed to the liver by portal vein perfusion and reached cells by diffusion, there would be a time-lapse in its contact with different portions of the tissue. Because such a phenomenon was obscured in the Western blot analysis, expressions of the GST subunits were further localized by the immunocytochemical technique to observe the effect of EGCG. Before the exposure of EGCG, a low-level, evenly distributed expression of GSTM2 existed in the liver tissue (Fig. 3A). As demonstrated in the micrograph of Fig. 3B, an intense brown stain appeared in the perivascular region 3 hr after the EGCG treatment using the anti-GSTM2 antiserum for detection. This observation indicates a high-level, EGCG-induced expression of GSTM2 in the cells. The induced expression of GSTM2 extended gradually outward from the hepatic veins as the treatment time increased (Fig. 3, C–E). However, the signals in the cells localized around the vein at the 12- and 24-hr points were less profound than those observed at the 3- and 9-hr points. The examination of GSTA1/2 expression showed no significant change before

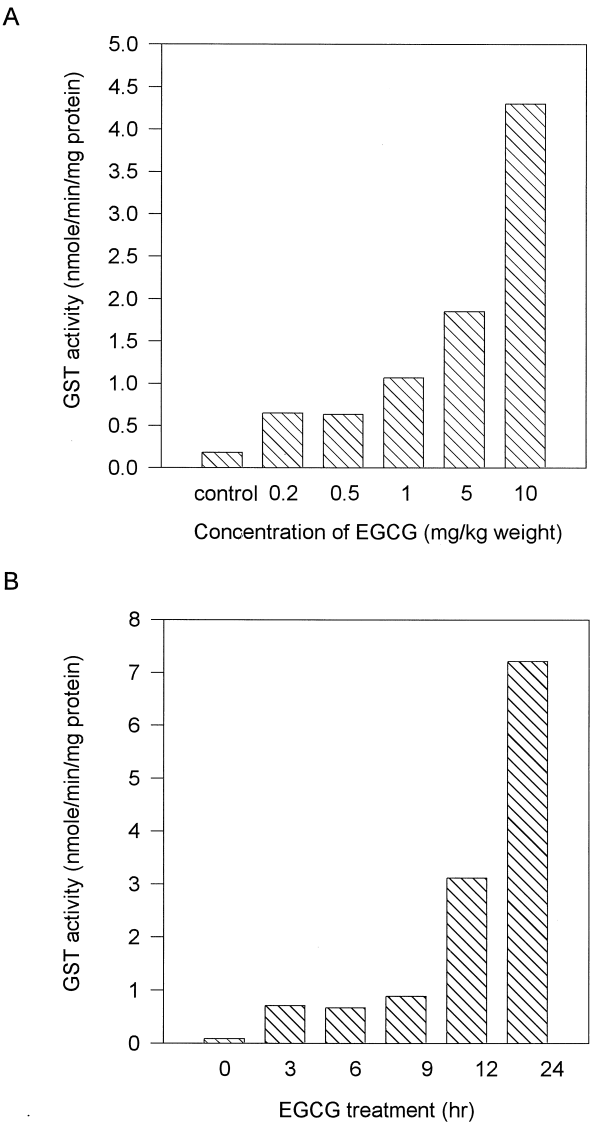

FIG. 1. Dose–response (A) and time–course (B) of the induction of GST activity by EGCG treatment in rat liver. Sprague–Dawley rats were treated with (A) the indicated dose or with (B) 1 mg/kg weight of EGCG by portal vein perfusion. The animals were killed at 12 hr (A) or at the indicated time point (B) after the application. The cytosolic protein samples were prepared from the freshly excised livers and tested for GST activity with CDNB as substrate. The figure is representative of two independent experiments with similar results.

or 24 hr after EGCG treatment (Fig. 3, F and G), although the signal also appeared to center around the hepatic veins. The result of GSTM1 detection was similar, with no evident change observed before or after EGCG treatment (data not shown).

Effect of EGCG on the mRNA Level of GSTM2

To determine whether the induction of the GSTM2 protein level by EGCG was the result of an increase in the transcript, steady-state mRNA levels were measured by Northern analysis using a specific cDNA probe for GSTM2.

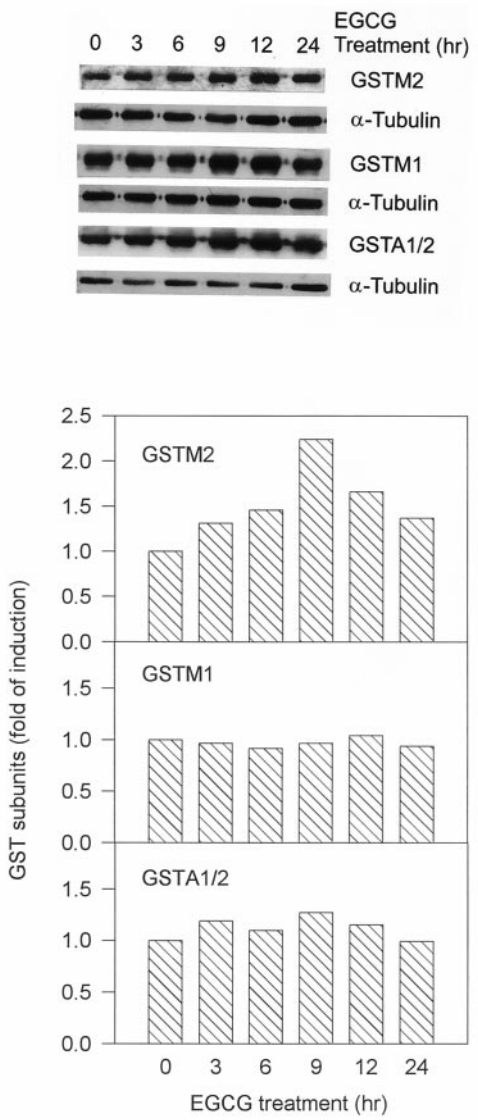

FIG. 2. Effect of EGCG on the protein levels of the GSTA1/2, GSTM1, and GSTM2 subunits. The cytosolic protein samples were prepared from the same livers as described in Fig. 1B and subjected to Western analysis. Upper panel: the Western blotting results. Lower panel: quantitative results of the autoradiograph by densitometric scanning. The data were normalized with the value of α-tubulin and presented as the induction fold of pretreatment value. This figure is representative of two independent experiments with similar results.

The amount of GSTM2 transcript in the EGCG-treated liver tissue increased during the first 6 hr, dropping to a very low level thereafter (Fig. 4). The maximal induction was about 2.0-fold of the level before treatment and was detected at the 6-hr point.

DISCUSSION

Tea is a very popular beverage worldwide. Epidemiological data have demonstrated an inverse correlation between tea consumption and the risk of several cancers [27–29], suggesting a chemopreventive effect of this beverage. Accumulating evidence obtained from both *in vitro* and *in vivo* studies also corroborates the role of green tea extract in preventing carcinogenesis and tumorigenesis. Therefore, tea is considered an excellent agent for chemoprevention and recommended for the human diet. However, it is difficult to evaluate the net effect of chemopreventors, since induction of pathways protective against one group of compound may potentiate the toxic effects of another class of toxins. For example, induction of phase II activities is thought to be beneficial in detoxification of toxins or carcinogens. Nevertheless, the up-regulation of GST pi, which is absent from normal rat and human hepatocytes, is associated with carcinogenesis [30–32] and drug resistance of tumor tissue [33–35]. The expression of GST mu, on the other hand, is related to cancer susceptibility in human studies [36, 37]. Consequently, it is essential to clarify the exact mechanism of action of chemopreventors so as to make the proper recommendation.

In this study, we used the available antibodies to specify the GST subunit that was up-regulated by EGCG in an attempt to explain its chemopreventive effect. Western data showed that EGCG treatment only induced GSTM2 subunit expression, which was the result of the increased level of mRNA, because the induction of transcript (~2.0-fold) corresponded to that of protein (~2.0-fold). The observation that the maximal level of mRNA occurred at the 6-hr point, prior to the time schedule (9 hr) of the maximal protein level, confirms this argument. The total GST activity induced by EGCG was incomparably higher than the expected value simply due to the increased expression of GSTM2. There are several possible reasons for this contradiction. First, the substrate (CDNB) used for the activity assay is a common substrate for most GST enzymes, although we only measured the protein levels of the three most abundant subunits in liver. It is possible that EGCG induces expression of other subunits in addition to GSTM2. Second, although very little is known regarding the relationship between subunit composition and the activity of GST (homo- or hetero-) dimeric isozymes, the differential induction of GSTM2 by EGCG would alter the proportion of GST isozymes, which might reflect the disproportional increase in GST activity. The third possible explanation for the disagreement between the activity and the protein level induced by EGCG is revealed by the immunocytochemical data. Apparently, the induction fold of the protein level is miscalculated from the Western result, since the amount of GSTM2 protein in cells that made contact with EGCG was much higher than the Western values. The immunocytochemical micrographs also raise the question as to the time–course of the cellular response to EGCG treatment. According to the Western and Northern data, the increase in the GSTM2 level would be a secondary response judging from the time points of the maximal levels induced by EGCG treatment. However, the immunohistochemical staining clearly showed that a strong action of EGCG on GSTM2 expression had already occurred as early as 3 hr after application. This is an important

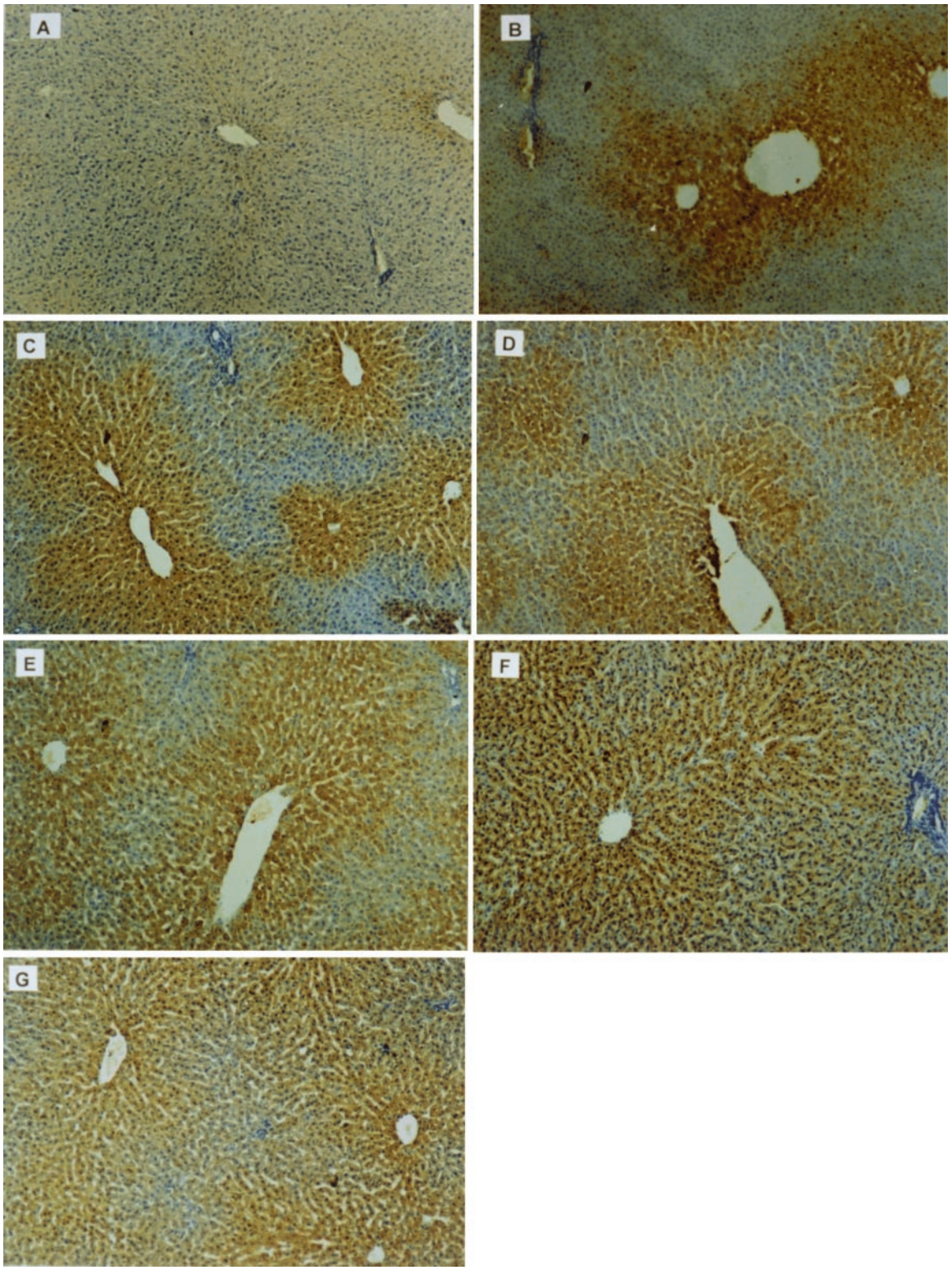

FIG. 3. Immunohistochemical examination of GST subunits in EGCG-treated liver sections. The same liver samples as described in Fig. 1B were fixed in formalin and embedded in paraffin. The GSTM2 and GSTA1/2 were localized with specific antisera. A to E: liver sections from animals treated with EGCG for 0, 3, 9, 12, and 24 hr were stained with anti-GSTM2 antibody. F and G: liver sections from animals treated with EGCG for 0 and 24 hrs were stained with anti-GSTA1/2 antibody. This figure is representative of two independent experiments with similar results.

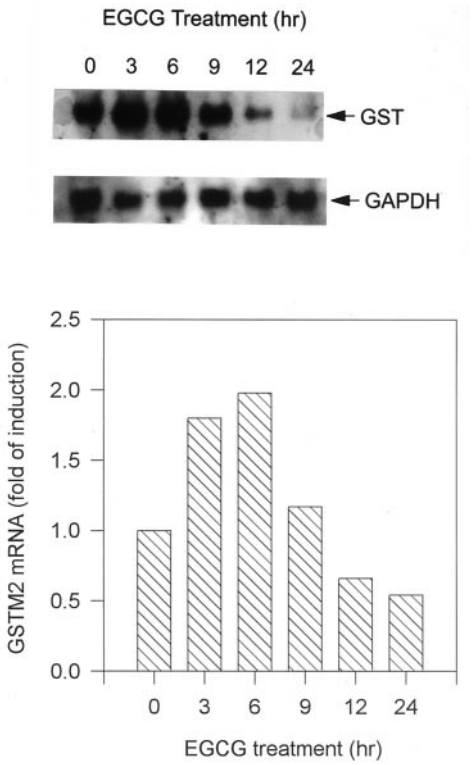

FIG. 4. Northern analysis of the induction of GSTM2 mRNA by EGCG. Total RNA samples were isolated from the same liver samples as described in Fig. 1B and subjected to Northern analysis using a cDNA probe of the GSTM2 subunit. Upper panel: the Northern hybridization results. Lower panel: quantitative results of the autoradiograph by densitometric scanning. The data were normalized to the value of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and presented as the induction fold of pretreatment value. This figure is representative of two independent experiments with similar results.

argument in favor of the idea of a direct influence of EGCG on the *GSTM2* gene.

In addition to an elevation in the GSTM2 mRNA level during the early hours of EGCG treatment, the Northern data showed a gradual decline in the transcript after the maximal induction at 6 hr (Fig. 4). This event suggests the existence of a possible down-regulation of the *GSTM2* gene by EGCG following the early up-regulation. Further studies, such as nuclear run-on experiments, are required to elucidate the mechanism responsible for the decrease in GSTM2 mRNA.

Regarding the regulation of rat GSTM2 subunit expression, very little is known about the regulatory region of the corresponding gene. To our knowledge, this report is the first to discern a specific induction of GSTM2 by a chemopreventor. Recent studies concerning the molecular mechanisms involved in the regulation of *GST* gene expression suggest that the induction of alpha and pi class *GST* genes is mediated by an antioxidant-responsive element (ARE) containing two adjacent activator protein-1 (AP-1)-like sites and activated by the Fos/Jun heterodimer [38, 39]. Using a reporter expression system containing the regulatory element of the *GSTA1/2* gene, Yu *et al.* [40] demonstrated that GTP stimulates the transcription of phase II detoxifying enzymes through the ARE. The upstream signaling pathways included mitogen-activated protein kinases, extracellular signal-regulated kinase 2, and c-Jun N-terminal kinase 1. The mRNA levels of c-*jun* and c-*fos* were also up-regulated by GTP treatment. The AP-1 dimeric complex might not be involved in the regulation of the GSTM2 subunit by EGCG, since the protein levels of c-Jun and c-Fos did not change as a consequence of the treatment (data not shown). This observation is consistent with the present Western and immunocytochemical data showing no influence of EGCG on GSTA1/2 expression. Therefore, EGCG, the most active component of green tea extract, and other components of this extract act according to different mechanisms to affect the detoxifying system of rat liver. Considering the role of human mu class GSTs in the relationship between their absence and a high susceptibility to cancer [36, 37], a specific induction of GSTM would be more beneficial in chemoprevention. Of course, many important aspects have to be taken into account when extrapolating mechanisms of chemoprotection from rodents to humans. For example, human GSTM2 is expressed in the heart and skeletal muscles but not in the liver, implying that the hepatic chemopreventive effect of EGCG may not involve this subunit. Nevertheless, EGCG can still serve to protect extrahepatic tissues through the enhancement of GST activity.

In conclusion, a clear understanding of the mechanisms of action of chemopreventors is an essential consideration before they are recommended to the public. In this study, the most active component of green tea extract, EGCG, was investigated in an attempt to determine the possible mechanism of its chemopreventive effect. As demonstrated by Western, Northern, and immunocytochemistry analyses, EGCG specifically induced the expression of the GSTM2 subunit in the rat liver. Subunits GSTA1/2 and GSTM1 were not affected by the treatment. The increase in GSTM2 protein could be observed as early as 3 hr after EGCG application, indicating a direct influence on the *GSTM2* gene by this chemical. Further study on the upstream signal transduction pathway may clarify the exact mechanism of EGCG's action.

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