[CANCER RESEARCH 55, 5520-5523, December 1, 1995]

Advances in Brief

Human Glutathione *S*-Transferase M1 Null Genotype Is Associated with a High Inducibility of Cytochrome P450 1A1 Gene Transcription[1]

Christelle Vaury,[2] Romuald Lainé, Patricia Noguiez,[2] Pierre de Coppet,[2] Christian Jaulin,[2] Françoise Praz,[2] Denis Pompon, and Mounira Amor-Guéret[2,3]

Institut Jacques Monod, Centre National de la Recherche Scientifique, Unité mixte de Recherche 9922, 2, place Jussieu, 75251 Paris Cedex 05 [C. V., P. N., P. d. C., C. J., F. P., M. A-G.], and Centre de Génétique Moléculaire, CNRS, 91198 Gif-sur-Yvette Cedex [R. L., D. P.], France

Abstract

We investigated the transcriptional regulation of cytochrome P450 1A1 (*CYP1A1*) gene in human lymphoblastoid B cells and report that a high inducibility of *CYP1A1* gene transcription by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is associated with glutathione *S*-transferase M1 (*GSTM1*) null genotype, whereas the presence of at least one *GSTM1* allele is correlated with induction of only low levels of *CYP1A1* mRNA by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. These data underline the major importance of the *CYP1A1* inducibility phenotype associated with the homozygous *GSTM1* null genotype in chemically induced cancers.

Introduction

Interindividual variations in susceptibility to chemically induced cancers are, at least in part, associated with genetic differences in the activation and detoxification of environmental procarcinogens, which require enzymatic processing to become mutagenic. Enzymes that metabolize carcinogens are divided classically into two categories: (*a*) Phase I enzymes, which are mainly cytochromes P450 and insert one atom of oxygen into the substrate; and (*b*) Phase II enzymes, which act on oxygenated substrates, including Phase I products, and conjugate them with various endogenous moieties, such as glucuronide, glutathione, and sulfate, to produce hydrophilic products, which are excreted easily from the cells (1).

Although it is well established that TCDD[4] and PAHs such as B(*a*)P are effective in causing tumors in laboratory animals, their role in the etiology of human cancers has not been established unambiguously (2). They have been shown to induce coordinately, *via* a functional Ah receptor, at least six genes that belong to the [Ah] gene battery: two phase I genes, cytochrome P450 1A1 (*CYP1A1*) and *CYP1A2*, and four phase II genes, NADPH:quinone oxidoreductase, aldehyde dehydrogenase, UDP-glucuronosyltransferase, and *GST* (1). The [Ah] gene battery also may be involved in the cellular response to oxidative stress (3).

Our group is working on BS, a human, cancer-prone, autosomal recessive genetic disorder characterized essentially by a generalized genetic instability, including high levels of sister chromatid exchanges (4). It has been assumed that the major biochemical defect of BS cells is a chronic overproduction of oxygene radicals, which may contribute to the spontaneous chromosomal instability of BS cells (5). The genetic predisposition of BS patients to develop various types of neoplasia prompted us to investigate in BS cells the regulation of detoxicating enzymes, which respond to oxidative stress and which may influence individual cancer risk.

In this study, we have investigated the transcriptional regulation of the Phase I *CYP1A1* gene in EBV-transformed B-cell lines derived from normal individuals and from BS patients. It is well known that the *CYP1A1* gene is induced by TCDD and PAHs, and that PAHs are also substrates for the CYP1A1 enzyme (1). GSTM1, a Phase II enzyme, member of the [Ah] battery, detoxifies the mutagenic metabolites produced by CYP1A1-catalyzed oxidation of PAH (6). GSTs are a family of multifunctional enzymes (EC 2.5.1.18) that detoxify a number of mutagenic electrophiles by promoting their conjugation with reduced glutathione (6). Moreover, a homozygous null allele at the *GSTM1* locus is present in 30–60% of the general population, leading to a complete lack of the GSTM1 enzyme (7). Thus, we analyzed the *GSTM1* genotype of all the cells studied for transcriptional *CYP1A1* gene regulation.

Our results demonstrate that a high inducibility of *CYP1A1* gene transcription by TCDD is associated with the *GSTM1* null genotype, whereas the presence of at least one *GSTM1* allele is correlated with induction of only low levels of *CYP1A1* mRNA by TCDD, with no significant differences between BS and normal cell lines. The binding of the Ah receptor to its specific DNA sequence correlates with the levels of transcription of *CYP1A1* gene induced by TCDD.

Materials and Methods

Cell Cultures and Treatment. EBV-transformed, lymphoblastoid B-cell lines Arbo, Kas, L31, Priess, Laz 388, and Lec derived from normal individuals were provided kindly by Dr. C. Raffoux (Hôpital Saint-Louis, Paris, France), Dr. F. Faure (Institut Gustave Roussy, Villejuif, France), and Dr. A. Aurias (Institut Curie, Paris, France). EBV-transformed, lymphoblastoid B-cell lines derived from BS patients were established in our laboratory (PuCh) or obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Coriell Institute for Medical Research, Camden, NJ; cell lines GM03403D and GM04408A). Cells were cultured in RPMI 1640 medium (GIBCO BRL, Cergy-Pontoise, France) supplemented with 10% heat-inactivated FCS (GIBCO BRL), penicillin (100 international units/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), vitamins, and nonessential amino acids. Cells were grown at 37°C in a 5% CO_2 humidified atmosphere.

Prior to induction with the various compounds, cells were resuspended at a density of 400,000 cells/ml. Cultures were treated for 48 h with 100 nM TCDD (ACCU Standard, Inc., New Haven, CT) dissolved in toluene, or for 24 h with 5 μM B(*a*)P (Sigma Chemical Co., Saint-Quentin-Fallavier, France) dissolved in dimethyl formamide.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated according to the method of Belyavsky *et al.* (8) after washing cells with PBS [0.01 M phosphate, 0.138 M NaCl and 0.0027 M KCl (pH 7.4)]. Denatured total RNA (20 μg/lane) was electrophoresed in a 1.2% agarose gel containing

Received 5/18/95; accepted 10/19/95.

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[1] This work was supported by grants from Ligue National contre le Cancer, Association pour la Recherche sur le Cancer No. 6642, and Centre National de la Recherche Scientifique-Actions Thématiques Incitatives sur Programmes et Equipes.

[2] Present address: Laboratoire de Génétique Moléculaire et Cancérogénèse, Institut d'Oncologie Cellulaire et Moléculaire Humaine, 129 boulevard de Stalingrad, 93000 Bobigny, France.

[3] To whom requests for reprints should be addressed.

[4] The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PAH, polycyclic aromatic hydrocarbon; GST, glutathione *S*-transferase; B(*a*)P, benzo(*a*)pyrene; 9-OH-B(*a*)P, 9-hydroxy-B(*a*)P; Ah, aryl hydrocarbon; BS, Bloom syndrome; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NRE, negative response element; XRE, xenobiotic responsive element.

formaldehyde and blotted overnight onto a nylon filter (Hybond N; Amersham France, Les Ulis, France) with 10× SSC [1× SSC, 0.15 M NaCl and 0.015 M sodium citrate (pH 7.0)] as transfer buffer. The membrane was prehybridized 2 h at 42°C, hybridized 48 h at 42°C in a buffer containing 50% formamide, 6× SSC, 1% SDS, 1× Denhardt's solution, 100 μg/ml salmon sperm DNA, and 2×10^6 cpm/ml labeled probe. Membranes were washed for 10 min, once at room temperature in 1× SSC and 0.5% SDS, once at 65°C in 1× SSC and 0.5% SDS, and once at 65°C in 1× SSC and 0.1% SDS. Membranes were exposed with intensifying screens at −80°C on Kodak XAR films for 15–48 h (Kodak, Marne-la-Vallée, France). Probes were removed from the blots by boiling in 0.1% SDS. The blots were rehybridized with either a human β-actin or rat GAPDH probe as a loading control.

Probes. The human *CYP1A1* probe used was a 1.5-kb cDNA fragment obtained by reverse transcription PCR using the following oligonucleotides: 5′-GGTGGATCCATGCTTTTCCCAATCTCCATG-3′ and 5′-CCAGGTAC-CTAGGCCTCAGGGCTCTCAAGC-3′. The human β-actin probe was a kind gift from Dr. S. Chouaib (Institut Gustave Roussy, Villejuif, France). The rat GAPDH probe, which cross-hybridizes with human GAPDH, was a kind gift from Dr. C. Nahmias (Institut Cochin de Génétique Moléculaire, Paris, France). All probes were radiolabeled with [α-^{32}P]-dCTP using a random priming technique (Amersham).

***GSTM1* Gene Deletion Analysis.** Genomic DNA was isolated from human EBV-transformed lymphoblastoid B-cell lines by standard procedures. PCR was carried on 1 μg DNA using primers hybridizing to the 5′ region of exon 4 (5′-CTGCCCTACTTGATTGATGGG-3′) and the 3′ region of exon 5 (5′-CTGGATTGTAGCAGATCATGC-3′) of GST1, as described (9).

Cytochrome P450 1A1 Enzymatic Assay. Cells treated previously for 24 h with 5 μM B(*a*)P were washed with 1× PBS and resuspended in complete RPMI 1640 medium and treated with 5 μM of B(*a*)P for 2 h. The hydroxylated metabolites of B(*a*)P were measured in the supernatants of cell cultures. Extraction of B(*a*)P metabolites was performed by adding 8 μl trifluoroacetic acid to 3 ml supernatant, followed by the addition of 1.25 μM benzo(*ghi*)-perylene used as internal standard, before the addition of 3 ml dichloromethane. Organic extracts were evaporated under a nitrogen stream, and residues were solubilized in 40 μl methanol/water (v/v). Aliquots (20 μl) were analyzed by high-performance liquid chromatography on a 10-cm RP18 column. The elution was performed at a flow rate of 1 ml/min, using a linear gradient from 30 to 35% of acetonitrile in water (containing 0.02% trifluoroacetic acid) for 6 min, followed by a 6-min gradient to 50% acetonitrile and a 9-min gradient to 75% acetonitrile. The column effluent was monitored by fluorescence, with an excitation wavelength of 375 nm and an emission wavelength of 435 nm. Identified metabolites, 9-OH-B(*a*)P and 3-OH-B(*a*)P, as well as the internal standard, benzo(*ghi*)perylene, were eluted at 14, 15, and 22 min, respectively. B(*a*)P metabolites were quantified by measuring the peak areas on chromatograms.

Gel Retardation Assay. Cells were resuspended at a density of 400,000 cells/ml and treated with 100 nM TCDD for 48 h. After washing cells in 1× PBS, nuclear extracts were prepared as described by Dignam *et al.* (10). The double-stranded Ah receptor 20-mer probe 5′-CTCTTCTCACGCAACTC-CGG-3′ was labeled at the 5′ ends using polynucleotide kinase and [γ-^{32}P]ATP (Amersham). A binding reaction was performed in 10 μl by a 15-min preincubation on ice of the mixture containing nuclear extracts (3 μg) in 10 mM HEPES (pH 7.9), 10% (v/v) glycerol, 50 mM KCl, 0.5 mM DTT, 3 mM $MgCl_2$, 4 mM spermidine, and 4 μg poly(dI·dC) (Sigma). The labeled oligonucleotide (2×10^4 cpm) was then added to the mixture in the presence or absence of competitor DNA (200-fold excess molar ratio). In some experiments, 30 or 300 ng purified human liver GST-μ antigen, obtained from Biotrin International (Dublin, Ireland), were incubated for 10 min at room temperature with the mixture, prior to the addition of labeled oligonucleotide. After a 20-min incubation at 24°C, the resulting complexes were resolved in a 5% polyacrylamide gel in Tris-borate EDTA run at 199 V. After a 2-h electrophoresis, the gel was fixed in a solution containing 10% ethanol and 10% acetic acid, dried, and autoradiographed at −80°C (Hyperfilm; Amersham) for 15 h.

Results

Fig. 1 shows the hybridization of the *CYP1A1* probe on total RNA extracted from untreated and TCDD-treated human EBV-transformed lymphoblastoid cell lines derived from six different normal individuals and three unrelated BS patients. The *CYP1A1* gene is not expressed constitutively in human EBV-transformed lymphoblastoid cell lines but is highly inducible by TCDD in four of the nine cell lines tested.

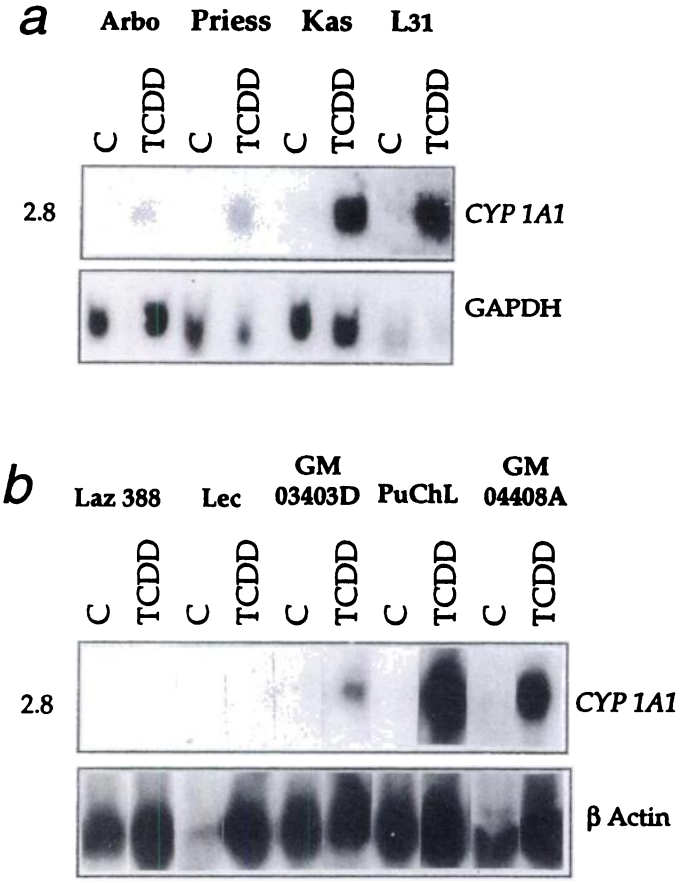

Fig. 1. *a* and *b*, Northern blot analysis of *CYP1A1* (upper panels, *a* and *b*), GAPDH (*a*, lower panel), and β-actin (*b*, lower panel) mRNA expression in EBV-transformed B-cell lines; untreated (*C*) or treated (*TCDD*), for 48 h with 100 nM TCDD.

To determine whether these differences in the inducibility of *CYP1A1* gene transcription by TCDD reflect a metabolic balance between Phase I and II enzymes, we analyzed by PCR the genotype of the cells at the *GSTM1* locus. As shown in Table 1 and Fig. 1, we found a complete correlation between the absence of the *GSTM1* gene and the induction of high levels of *CYP1A1* gene mRNA by TCDD. Furthermore, gel mobility shift assays revealed that the Ah receptor-DNA complex formed with nuclear extracts of TCDD-treated cells is strong when extracts are from cells having a *GSTM1* null genotype, whereas it is weak when extracts from cells with at least one allele of *GSTM1* are used (Fig. 2). The Ah receptor-DNA complex could not be displaced by the addition of the purified human liver GST-μ antigen (data not shown).

These results led us to investigate whether the rate of transcription of the *CYP1A1* gene induced by TCDD is correlated with CYP1A1 enzyme activity. Thus, the amount of hydroxylated metabolites of B(*a*)P, 9-OH-B(*a*)P, and 3-OH-B(*a*)P, were measured by high-performance liquid chromatographic analysis in the supernatants of cell cultures after a 48-h treatment with TCDD, an unmetabolizable inducer of the CYP1A1 enzyme, followed by a 2-h treatment with B(*a*)P as a substrate. In these experimental conditions, very low levels of B(*a*)P metabolites were detected, probably because TCDD binds to the active site of the CYP1A1 enzyme, resulting in B(*a*)P metabolism

Table 1 *Hydroxylated metabolites of benzo(a)pyrene [addition of 9-OH-B(a)P and 3-OH-B(a)P products] accumulated in the supernatant of cells treated for 24 h with 5 μM B(a)P measured in two independent experiments*

Cell lines	*GSTM1* genotype[a]	B(*a*)P metabolites (pmol/10^6 cells)	
		Experiment 1	Experiment 2
KAS	–	19.7	27.2
L31	–	7.2	7.2
PuChL	–	20.4	15.6
GM04408A	–	9.5	nd[b]
Arbo	+	16.1	4.3
Priess	+	0.9	0.9
Laz 388	+	1.5	nd[b]
GM03403D	+	1.5	1.5

[a] Cells were genotyped at the *GSTM1* locus by PCR and scored as: –, homozygous deletion; and +, presence of at least one allele.
[b] nd, not determined.

inhibition. For this reason, we investigated the effects of B(*a*)P on the induction of *CYP1A1* gene transcription and showed that it behaves similarly to TCDD (data not shown). The amounts of 9-OH-B(*a*)P and 3-OH-B(*a*)P metabolites were determined in the supernatants of cells that had been pretreated for 24 h with B(*a*)P and washed extensively before a subsequent 2-h treatment with B(*a*)P as a substrate. As shown in Table 1, with the exception of the Arbo cell line, the production of B(*a*)P metabolites by cells with very low transcriptional inducibility of the *CYP1A1* gene by TCDD was hardly detectable. On the other hand, cells with high transcriptional inducibility of the *CYP1A1* gene by TCDD produce variable amounts of B(*a*)P metabolites.

Discussion

Our results demonstrate that a high *CYP1A1* mRNA inducibility in response to TCDD is associated with a strong binding of the Ah receptor to its specific DNA sequence and *GSTM1* null genotype. Conversely, a relationship between low *CYP1A1* mRNA inducibility, formation of less Ah receptor-DNA complexes, and the presence of at least one allele of *GSTM1* was observed. The levels of 9-OH-B(*a*)P and 3-OH-B(*a*)P metabolites accumulated in the supernatants of cells treated with B(*a*)P do not correlate strictly with the amount of *CYP1A1* mRNA. Nevertheless, with the exception of the Arbo cell line, amounts of accumulated B(*a*)P metabolites are higher in *GSTM1* null genotype cell lines than in cell lines with at least one *GSTM1* allele. Amounts of 9-OH-B(*a*)P and 3-OH-B(*a*)P metabolites in the supernatants of B(*a*)P-treated cells result from a metabolic balance between Phase I and Phase II enzymes, including *CYP1A1* and *GSTM1*, as well as other P450 enzymes (11). Thus, accumulated B(*a*)P metabolites do not reflect CYP1A1 enzyme activity *per se*, which is illustrated by the results obtained with the Arbo cell line.

These data are of major importance, because accumulated evidence suggests that the increased risk of smoking-induced bronchogenic carcinoma is associated with the high *CYP1A1* inducibility phenotype and with the homozygous *GSTM1* null genotype (12, 13). Furthermore, moderate to high constitutive *CYP1A1* expression also has been shown to correlate with a poor prognosis in human breast cancer independent of smoking (14). Such data underline the importance of *CYP1A1* expression in the etiology and prognosis of some human cancers. The magnitude of *CYP1A1* expression depends on the interplay between positive regulatory factors such as the Ah receptor and negative regulatory factors, which would act via NREs (1, 15). Several models, which involve either the NRE or the Ah receptor, have been proposed for negative regulation of the *CYP1A1* gene in mammalian cells. First, Puga *et al.* (16) and Raychaudhuri *et al.* (17) reported constitutive high levels of murine cytochrome P450 1A1 (*Cyp1a-1*) mRNA expression in a B(*a*)P-resistant mouse Hepa-1 cell line, *c37*, a mutant deficient in CYP1A1 activity. Transfection of *c37* mutant cells with a wild-type *Cyp1a-1* or *CYP1A2* expression vector suppressed endogenous *Cyp1a-1* mRNA expression (16, 17), suggesting the existence of CYP1A1/CYP1A2-dependent repression acting via the NRE (18). Another mechanism involving the NRE is proposed by Boucher *et al.* (15), who identified a *cis*-element (NRE_{275}), composed of two subfragments (NRE_{105} and NRE_{170}) that down-regulate the *CYP1A1* promoter. This down-regulation is observed in the absence of either the XRE or activated Ah receptor and does not depend on CYP1A1 enzyme activity (15). Last, another mechanism involving the Ah receptor was proposed by Watson *et al.* (19), who reported that *c31*, a dominant mutant of Hepa-1 cells, expresses a repressor that prevents TCDD-dependent stimulation of *Cyp1a-1* transcription. These authors suggested that the repressor associates with the Ah receptor to prevent its binding to the XRE. Our data showing that the

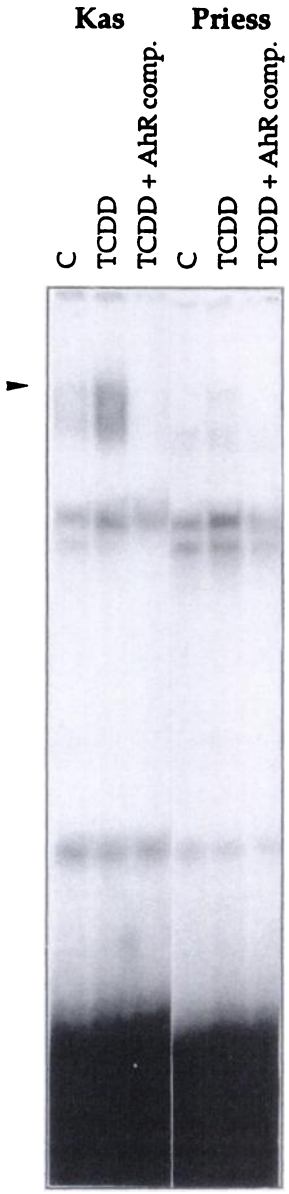

Fig. 2. Induction of the Ah receptor by TCDD. Gel mobility shift assays with nuclear extracts from GST^+ (*Priess*) and GST^- (*Kas*) cells untreated (*C*) or treated (*TCDD*) for 48 h with 100 nM TCDD. Each binding reaction contained 3 μg nuclear proteins. To confirm the identity of the band containing the Ah receptor (*AhR comp.*), we carried out binding reactions in the presence of a 200-fold excess of the competing 20-mer 5′-CTCTTCTCACGCAACTCCGG-3′.

presence of at least one allele of the *GSTM1* gene correlates with a decrease in TCDD-dependent induction of *CYP1A1* transcription supports the model proposing the existence of a repressor that associates with the Ah receptor (19). Although this repressor has not been characterized yet, our results suggest that it is not the gene product of *GSTM1* itself, which, however, may be involved in its activation.

Our study demonstrates that the absence of the *GSTM1* gene correlates with the induction of high levels of *CYP1A1* mRNA by TCDD and with high to moderate accumulation of B(*a*)P derivatives, which is in agreement with the data suggesting an increased risk for lung carcinoma among smokers without the GSTM1 enzyme. Furthermore, we found a correlation between the presence of at least one *GSTM1* allele and the induction of low levels of *CYP1A1* mRNA by TCDD, which parallels a very low accumulation of B(*a*)P metabolites in three cell lines. Our data suggest that, in some metabolic environments, the presence of the GSTM1 enzyme may participate in the inhibition of TCDD-dependent stimulation of *CYP1A1* transcription. Because less Ah receptor-DNA complex formation was observed in cells with at least one allele of the *GSTM1* gene, we suggest that such inhibition results probably from impairment of Ah receptor binding to the XRE. In this case, the GSTM1 enzyme would play a protective role against lung carcinoma among smokers.

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