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SHORT COMMUNICATION

In Situ Hybridization Studies of UDP-glucuronosyltransferase UGT1A6 Expression in Rat Testis and Brain

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ABSTRACT. UDP-glucuronosyltransferases (UGTs), in addition to their role in overall pharmacokinetics, play important roles in local protection of cells against toxins and in the control of endogenous receptor ligands. UGT1A6, which conjugates planar phenols, appears to be expressed in many organs, but information on cell-specific expression in these organs is controversial or absent. Therefore, a non-isotopic *in situ* hybridization method was developed and applied to localize UGT1A6 expression in rat testis and brain. It was found that UGT1A6 is expressed in Sertoli cells and spermatogonia of rat testis and in brain neurons, in particular in hippocampal pyramidal cells and Purkinje cells of the cerebellum. BIOCHEM PHARMACOL **59**;11:1441–1444, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. UDP-glucuronosyltransferase UGT1A6; *in situ* hybridization; testis; brain

UDP-glucuronosyltransferases (UGTs†) are major phase II enzymes of drug metabolism that convert a wide variety of xenobiotic and endobiotic lipid-soluble substrates into water-soluble and excretable compounds [1]. UGTs represent a supergene family of isoforms, with more than 15 isoforms expressed in rats and humans being localized to the endoplasmic reticulum and nuclear envelope of cells [2]. The isoform UGT1A6 is expressed in many rat organs, such as liver, kidney, lung, intestine, brain, and steroidogenic tissues [3–5]. It is mainly involved in the conjugation of planar phenols including endogenous compounds such as serotonin [6, 7], drugs such as paracetamol [8], and metabolites of carcinogens such as benzo(*a*)pyrene-3,6-diphenol [9]. UGT1A6 is known to be regulated in liver by arylhydrocarbon (Ah) receptor agonists [10] and antioxidant-type inducers [11], whereas in extrahepatic tissues the isoform is constitutively expressed [3]. In addition, differential induction of UGT1A6 by these two types of inducers has been found in the different zones of the liver [12].

It is known that levels of UGTs in the liver play an important role in the overall pharmacokinetics of some drugs. However, it is also increasingly recognized that these enzymes may play key roles in local protection of cells against toxins, e.g. in toxin resistance of colon carcinoma cells by UGT1A10 [13, 14]. They also appear to control endogenous ligands: treatment of prostata cancer cells LNCaP by biochanin A (a flavonoid in soybean products) was found to induce UGT2B15. The induction of this isoform decreased intracellular testosterone and androgen receptor-dependent prostata-specific antigen production [15]. UGTs are often found in metabolic barriers such as the gastrointestinal barrier [16], which protects the organism against biologically active compounds in our plant diet and decreases the bioavailability of orally administered drugs. For other barriers such as the blood–brain and blood–testis barriers, it is assumed that UGTs are expressed in cells forming these barriers. However, information on the cell types expressing UGT1A6 is absent or controversial. Therefore, a novel non-isotopic *in situ* hybridization method was developed and applied to identify the cells expressing UGT1A6 in testis and brain.

MATERIALS AND METHODS

Chemicals

Oligos for PCR were synthesized by MWG-Biotec GmbH. The AmpliTaq DNA polymerase was obtained from Perkin Elmer. The DIG RNA Labeling kit (SP6/T7), DIG RNA Labeling Mix, and DIG Nucleic Acid Detection kit were obtained from Roche.

Animal Tissues

Adult male Wistar rats (300–400 g) were anesthetized with ketamine (100 mg/kg body weight) and xylazine (15 mg/kg body weight) prior to transcardial perfusion with 4% paraformaldehyde in PBS. Testes and brain were removed immediately, snap-frozen in liquid nitrogen, cooled in isopentane, and stored at −80°.

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† *Abbreviations:* UGT, UDP-glucuronosyltransferase; PCR, polymerase chain reaction; and DIG, digoxigenin.

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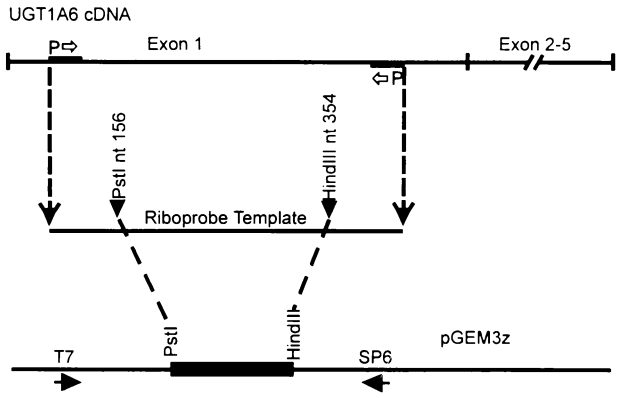

FIG. 1. Scheme used to synthesize the UGT1A6 riboprobe template. A 350-bp cDNA fragment of exon 1 of UGT1A6 was incubated with PstI and HindIII, and the resulting 197-bp fragment was cloned into the pGEM3Z vector. RNA synthesis with SP6 polymerase and T7 polymerase resulted in antisense and sense probes, respectively.

Riboprobe Preparation

Total RNA from livers of adult male rats was isolated according to the method of Chomczynski and Sacchi [17] and reverse-transcribed into cDNA [18]. The probe template was generated by cloning a specific 350-bp cDNA fragment of UGT1A6 (spanning nucleotide [nt] 125–474; Genbank accession numbers J02612 or D83796) into the polylinker site of the pGEM3Z vector (Promega). pGEM3Z and the UGT1A6 fragment were incubated with PstI and HindIII and the vector was ligated to pGEM1A6, containing a 197-bp fragment, specific for UGT1A6 (Fig. 1). Antisense or sense templates were generated by PstI or HindIII linearization of pGEM1A6, respectively. The following primers for PCR of UGT1A6 were used: forward primer spanning nt 125–148, 5′-ATGGCTTGCCTTCT-TCCTGCTGCT-3′ and reverse primer spanning nt 451–474, 5′-AGAGTACAGGAACAACATGATTGT-3′. DIG-labeled riboprobes were synthesized using the DIG RNA Labeling kit and DIG RNA Labeling Mix (10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-11-UDP), according to the instructions of the manufacturer (Roche). No significant homology with other UGTs was found when the oligos were checked against Genbank using the BLASTN software. Sequencing of the PCR fragments confirmed the published sequence of rat UGT1A6.

Non-Isotopic In situ *Hybridization*

Cryostat (5-μm thick) sections of rat testes and paraffin sections (2-μm thick) of rat brain were placed on Superfrost Plus slides (Menzel Gläser, Roth). Cryostat sections were postfixed for 1 hr in 4% paraformaldehyde–phosphate-buffered saline (PFA–PBS) and washed in PBS. Paraffin sections were dewaxed for 5 min and postfixed in 4% PFA–PBS for 30 min at 37°. They were then treated with pepsin (10 μg/mL), fixed for 20 min in 4% PFA–PBS, and washed in PBS. Both cryostat and paraffin sections were acetylated with 0.25% acetic anhydride for 15 min in 0.1 M triethanolamine buffer, pH 8.0. Thereafter, they were washed in PBS, dehydrated in ethanol, and air-dried for 1 hr. The sections were marked with a silicon pen (DAKO Diagnostika). Thereafter, hybridization was carried out with hybridization mix (30 μL/slide) that contained 100 ng riboprobe, 3 mM NaCl, 10% dextrane sulfate, and 1× basic mix (10× basic mix: 0.2 M Tris–HCl, pH 7.5, 10 mM EDTA, 20% 50× Denhardt's solution, tRNA from baker's yeast (5 mg/mL), polyA-RNA (1 mg/mL), and DNA from fish sperm (0.1 mg/mL)). The hybridization mixture was heat-denatured at 80° for 10 min in formamide and shock-frosted on ice. Finally, the slides were sealed with plastic coverslips (Appligene-Oncor). The slides were placed in a humid chamber and hybridized at 56° for 16–20 hr. Posthybridization washes of unbound cRNA consisted of the following: 1× standard sodium citrate (SSC) at room temperature for 30 min, 1× SSC/50% formamide at 56° for 15 min, 1× SSC at room temperature for 15 min. Thereafter, the slides were treated with RNase A (40 μg/mL) in 500 mM NaCl, 20 mM Tris–HCl, pH 7.4, 1 mM EDTA (=STE) at 37° for 30 min followed by 3 washes in STE at room temperature. For immunological detection with the DIG Nucleic Acid Detection kit, the slides were washed for 1 min with buffer 1 (150 mM NaCl, 100 mM Tris–HCl, pH 7.4), blocked for 30 min in 1% blocking reagent (Roche), and then incubated for 60 min with alkaline phosphatase-labeled anti-DIG antibody (1:400). Afterwards, the slides were washed in buffer for 1 to 5 min and then for 10 min in 100 mM NaCl, 100 mM Tris–HCl, pH 7.4, 50 mM EDTA on a rocker. Thereafter, the staining solutions NBT (nitroblue tetrazolium chloride; 0.34 mg/mL) and BCIP (5-bromo-4-chloro-3-indolyl phosphate-*p*-toluidine; 0.18 mg/mL) were added and the slides were incubated at 4° overnight (16–21 hr). The color reaction was stopped by washing the slides in 10 mM Tris–HCl, pH 7.4, 1 mM EDTA. Counterstaining was carried out with methylene green for 10 min at 60°. Negative controls were performed in parallel using sense riboprobes. The slides were stored in the dark.

RESULTS AND DISCUSSION

Cells expressing UGT1A6 in rat testes could be visualized (Fig. 2A). All cell populations of the basal seminiferous epithelium (Sertoli cells, spermatogonia, primary spermatocytes) showed strong signals for UGT1A6. There were no signals detectable in cells present in the lumen such as spermatids. Moreover, cells in the interstitial space were negative. UGT1A6 expression in rat brain is shown in Fig. 2B. Dense staining was found in the cerebellum, in particular in the cytoplasm of Purkinje cells (Fig. 2B, a–c). Strong signals were also obtained in pyramidal cells of the hippocampus (Fig. 2B, e–g). Reaction of the control sections with the sense riboprobe in testis (Fig. 2A, d) and brain (Fig. 2B, d and h) were negative. The present results

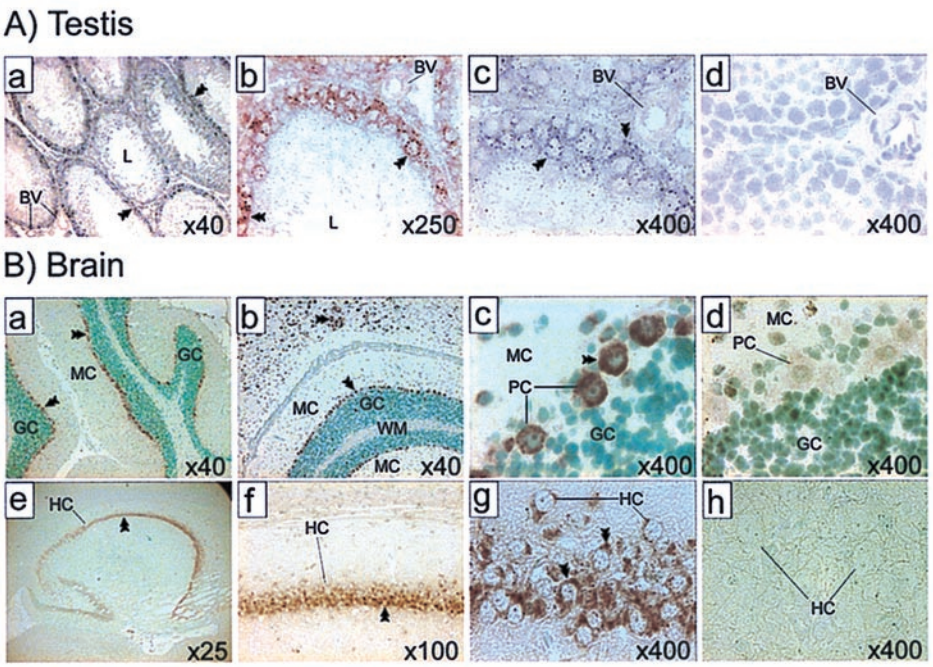

FIG. 2. UGT1A6 expression in rat testis (A) and brain (B) detected by non-radioactive *in situ* hybridization. Cryostat (A) and paraffin sections (B) were hybridized with UGT1A6 antisense and sense riboprobes and stained with alkaline phosphatase-labeled anti-DIG antibodies as described in Materials and Methods. (A) a–c, UGT1A6 detection in seminiferous tubules; d, sense riboprobe control. Arrows indicate cytoplasmic staining of cells of the basal seminiferous epithelium such as spermatogonia. (B) a–c, paraffin sections of the cerebellum; e–g, sections of the hippocampus; d and h, sense riboprobe controls. Arrows indicate Purkinje cells (PC) and hippocampal pyramidal cells (HC). L, lumen of seminiferous tubules. BV, blood vessel. GC, granular cell layer; MC, molecular cell layer; WM, white matter.

demonstrate that UGT1A6 expression in rat testes is not restricted to Sertoli cells which form the blood–testis barrier; UGT1A6 is also present in spermatogonia and in primary spermatocytes. Expression of UGT1A6 in spermatogonia may protect these important cells against lipid-soluble phenolic toxins which are likely to penetrate the Sertoli cell layer by passive diffusion.

Detection of UGT1A6 expression in rat brain is particularly interesting, since it confirms and extends earlier immunohistochemical findings [5] by a novel and selective *in situ* hybridization method. Glucuronide conjugation of 7-hydroxycoumarin was detected in the effluent of isolated perfused rat brain in studies on the metabolism of 7-ethoxycoumarin [19], suggesting glucuronidation of phenolic compounds in the brain *in vivo*. Again, the present results demonstrate (in contrast to the situation in the gastrointestinal barrier in which enterocytes express UGT1A6) that—inside the tissue protected by the blood–brain barrier—important neuronal cells such as Purkinje cells of the cerebellum and hippocampal pyramidal cells are expressing UGT1A6. Since the limit of detection of our method is unknown, we cannot exclude the presence of UGT1A6 in the blood–brain barrier and in astrocytes [20, 21]. Rat UGT1A6 has been shown to be involved in the glucuronidation of serotonin [6]. Hence, UGT1A6 may, apart from protecting against toxins, be involved in the control of important neurotransmitters. However, sulfation may also regulate serotonin levels. Further studies are required to determine the role of glucuronidation in the control of cellular serotonin. Interestingly, human UGT1A6 has also been shown to glucuronidate serotonin and is expressed in brain [7]. Identification of UGT1A6 in cells of rat testis and brain by *in situ* hybridization should encourage further studies to localize UGT1A6 and other UGT isoforms in various tissues and to identify the cells expressing UGTs.

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