GLUCURONIDATION: AN IMPORTANT MECHANISM FOR DETOXIFICATION OF BENZO[a]PYRENE METABOLITES IN AERODIGESTIVE TRACT TISSUES

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(Received September 12, 2001; accepted December 18, 2001)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

UDP-glucuronosyltransferases (UGTs) have been implicated as important detoxifying enzymes for several major tobacco carcinogens. Because the aerodigestive tract is a primary target for exposure to tobacco smoke carcinogens, the major goal of the present study was to determine whether aerodigestive tract tissues exhibit glucuronidating activity against metabolites of benzo[a]pyrene (BaP) and to explore the pattern of expression of UGT genes in a series of aerodigestive tract tissue specimens. Glucuronidation of the phenolic BaP metabolites 3-, 7-, and 9-hydroxy-BaP was observed in all upper aerodigestive tract tissue microsome specimens tested, as determined by high-pressure liquid chromatography analysis. Glucuronidating activity toward the procarcinogenic BaP metabolite trans-BaP-7,8-dihydrodiol(±) was also detected in aerodigestive tract tissues. By semiquantitative duplex reverse transcription-polymerase chain reaction analysis, UGT1A7 and UGT1A10 were shown to be well expressed in all aerodigestive tract tissues examined, including tongue, tonsil, floor of mouth, larynx, and esophagus. UGT1A8 and UGT1A6 were expressed primarily in larynx; no expression was observed for UGTs 1A1, 1A3, 1A4, 1A5, 1A9. Of the family 2B UGTs, only UGT2B4 and UGT2B17 exhibited significant levels of expression in aerodigestive tract tissues. Of the aerodigestive tract-expressing UGTs, only UGTs 1A7, 1A8, and 1A10 exhibited glucuronidating activity against 7-hydroxy-BaP, with UGT1A10 exhibiting the highest affinity as determined by kinetic analysis (K_m = 49 μM). No UGT expression or glucuronidating activity was observed for any of the lung specimens analyzed in this study. These results suggest that several family 1 UGTs may potentially play an important role in BaP detoxification in the aerodigestive tract.

The UGT family of enzymes catalyze the glucuronidation of a variety of compounds, including endogenous compounds like bilirubin and steroid hormones, as well as xenobiotics including drugs and environmental carcinogens (Tephly and Burchell, 1990; Gueraud and Paris, 1998; Ren et al., 2000). Based upon differences in sequence homology and substrate specificity, two main families of UGTs have been identified in several species, each containing several highly homologous UGT genes. The entire UGT1 family is derived from a single locus in chromosome 2, coding for nine functional proteins that differ only in their amino terminus due to alternate splicing of the independent exon 1 regions to a shared carboxy terminus encoded by exons 2 to 5 (Owens and Ritter, 1995). In contrast to the UGT1A family, the UGT2B family is composed of several independent genes, all located on chromosome 4 (Jin et al., 1993a,b; Grove et al., 1997; Mojarrabi and Mackenzie, 1998; Strassburg et al., 1999; Guillemette et al., 2000). In previous studies, several UGTs, including UGT2B7, UGT1A9, UGT1A7, UGT1A8, and UGT1A10, were implicated in the conjugation and detoxification of metabolites of several tobacco carcinogens, including tobacco-specific nitrosamines like NNK (Ren et al., 2000), and polycyclic aromatic hydrocarbons, such as BaP (Jin et al., 1993a,b; Grove et al., 1997; Mojarrabi and Mackenzie, 1998; Strassburg et al., 1999; Guillemette et al., 2000). In addition, several studies have demonstrated that UGTs exhibit a protective effect against these carcinogens. The addition of UDPGA to the Ames test is associated with a reduction in BaP mutagenicity (Nemoto et al., 1978; Owens et al., 1979). In studies of UGT-deficient homoygous (j/j) and heterozygous (j/+ ) RHA rats versus UGT-normal (+/-) RHA controls, reduced glucuronidation of BaP metabolites in vivo was correlated with increased covalent binding to hepatic DNA and microsomal protein (Hu and Wells, 1992). In addition, a similar correlation was observed after in vitro incubations of BaP with rat liver microsomes, lymphocytes, or skin fibroblasts from UGT-deficient RHA rats (Hu and Wells, 1992; 1994; Vienneau et al., 1995). Therefore, several UGT enzymes could potentially play an important role in the detoxification of tobacco carcinogens.
Although studies examining UGT expression patterns in human tissues have been performed extensively for metabolizing organs and tissues of the digestive tract (Strassburg et al., 1998a,b, 1999, 2000), few studies have been performed for tobacco-related target tissues. For tissues of the aerodigestive tract and respiratory system, UGT1A7 was shown to be well expressed in orolaryngeal specimens (Zheng et al., 2001), and UGT1A6 was shown to be expressed in pharyngeal tissue (Ulrich et al., 1997). Family 2B UGTs were reported to be expressed in lung (Levesque et al., 1997, 1999; Hum et al., 1999), whereas several UGTs (UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B7, UGT2B10, and UGT2B15) were reported to be expressed in esophagus (Strassburg et al., 1999). No data have as yet been reported demonstrating glucuronidation activity in such target organ sites. To better assess the role of glucuronidation as a detoxification mechanism in tissues of the aerodigestive tract, the goal of the present study was to determine whether aerodigestive tract tissues exhibit glucuronidation activity to BaP metabolites and to examine the pattern of expression of UGT genes in aerodigestive tract tissues.

Materials and Methods

Tissues. For expression analysis, total RNA purified from normal human liver (19 samples), lung (32 samples), esophagus (10 samples), larynx (three samples), tongue (five samples), tonsil (three samples), and floor of mouth (three samples) specimens was obtained from the Tissue Procurement Facility at the H. Lee Moffitt Cancer Center. All RNA samples were purified from tissue specimens obtained from individual subjects undergoing cancer surgery. Accurate information on recent exposures (i.e., smoking and alcohol consumption) was not available for this study.

For glucuronidation activity assays, larynx (n = 4), floor of mouth (n = 2), tongue (n = 2), esophagus (n = 3), tonsil (two specimens from the same patient), and lung (n = 3) specimens were obtained from individual patients via the H. Lee Moffitt Cancer Center Tissue Procurement Facility. Because of the low quantities of normal orolaryngeal tissues obtained during cancer surgery, equal amounts of each specimen from each orolaryngeal site were pooled for the preparation of microsomes. Because larger specimens were obtained for lung, analysis of glucuronidation activity was performed separately in three independent lung specimens. Microsomes were prepared for all specimens by differential centrifugation, as previously described (Cougthrie et al., 1987).

All protocols involving the analysis of tissue specimens were approved by the institutional review board at the University of South Florida and were in accordance with assurances filed with and approved by the U.S. Department of Health and Human Services. Assurances were given by the Tissue Procurement Facility at the H. Lee Moffitt Cancer Center that all samples were isolated and quick-frozen at −70°C within 2 h post-surgery.

Analysis of Glucuronidating Activity in Tissue Microsomes. 3-OH-BaP, 7-OH-BaP, 9-OH-BaP, and trans-BaP-7,8-dihydrodiol(±) were obtained from the National Cancer Institute Chemical Carcinogen Repository (synthesized and characterized at the Midwest Research Institute, Kansas City, MO). BaP metabolites were dissolved in dimethyl sulfoxide and stored protected from light at −70°C. UDPGA, N-acetyl-β-D-glucosaminidase, as described above.

Orolaryngeal microsome preparations were stored in individual aliquots at −70°C, with protein concentrations determined using the bicinchoninic acid assay (Pierce Corp., Rockford, IL). Microsomes (0.1–1 mg) were incubated with 1 mM 3-, 7-, or 9-OH-BaP, or 2 mM trans-BaP-7,8-dihydrodiol(±), 4 mM UDPGA, 1 μM UDPGlcNAc (UDP [N-acetyl-β-D-glucosaminidase], as described above.

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Oral microsome preparations were stored in individual aliquots at −70°C, with protein concentrations determined using the bicinchoninic acid assay (Pierce Corp., Rockford, IL). Microsomes (0.1–1 mg) were incubated with 1 mM 3-, 7-, or 9-OH-BaP, or 2 mM trans-BaP-7,8-dihydrodiol(±), 4 mM UDPGA, 1 μM UDPGlcNAc (UDP [N-acetyl-β-D-glucosaminidase], as described above.

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UGT-specific primers (see Table 1 for primer sequences). To assure that all RT-PCR amplifications were from expressed UGT mRNA, sense and antisense primers were specific for exon 1 and exon 3, respectively, for all UGTs examined except UGT2B15 and UGT2B17, for which the antisense primer was specific for exon 2 sequences. Although the genomic sequence and gene structure of UGT2B4 is not yet known, the antisense primer sequence designed for duplex RT-PCR of UGT2B4 transcripts was homologous to sequences similar to that encoded by exon 3 for other UGT2B family enzymes. Since all family 1 UGTs share exons 2 to 5, the same exon 3-derived antisense primer was used for RT-PCR amplifications of family 1 UGTs.

The same exon 2-derived antisense primer was used for RT-PCR amplifications of UGT2B15 and UGT2B17.

The same exon 3-derived antisense primer was used for all RT-PCR amplifications of family 1 UGTs.

Primer locations are identical to those indicated in corresponding GenBank accession numbers.

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a All UGT sense primers correspond to exon 1 sequences in each of the respective UGT genes.

b Primer locations are identical to those indicated in corresponding GenBank accession numbers.

c The same exon 3-derived antisense primer was used for RT-PCR amplifications of UGT2B4 and UGT2B7.

d The same exon 2-derived antisense primer was used for RT-PCR amplifications of UGT2B15 and UGT2B17.

RT-PCR amplifications after 32, 35, 38, and 41 cycles of PCR. Appropriate sense and antisense primers (20 pmol each) were added to PCRs after the ninth sequence of RT-PCR products.

H. Lee Moffitt Cancer Center) after electrophoresis in 1.5% agarose (Wizard purified RT-PCR products were directly sequenced (Molecular Core Facility, U99508).

As shown in Fig. 1, significant levels of BaP-7-O-Gluc was detected in pooled microsomes from human esophageal microsomes. BaP-7-O-Gluc formation in esophageal microsomes was detected by both UV detection (254 nm; Fig. 1A) and UDPGA-derived [14C]glucuronic acid incorporation (Fig. 1C), and the predicted BaP-7-O-Gluc peak on HPLC was sensitive to treatment with β-glucuronidase (Fig. 1, B and D). The rate of glucuronidation of 7-OH-BaP for esophageal microsomes (16.4 nmol · mg of protein–1·120 min–1; Table 2) was similar to that observed for liver microsomes (21.2 nmol · mg of protein–1·120 min–1; results not shown). High levels of BaP-7-O-Gluc were formed in assays of pooled microsomes (0.1 mg) from all other aerodigestive tract tissues tested, including larynx, tonsil, tongue, and floor of mouth (Table 2). No difference in activities were observed for any tissue microsomes in assays with or without DL-2-lysophosphatidyl choline palmitoyl (C16:0). No activity was observed in human lung microsomes using as much as 1 mg of microsomal protein in assays with or without DL-2-lysophosphatidyl choline palmitoyl (C16:0).

Similar to that observed for 7-OH-BaP, high levels of glucuronidating activity were observed against both 3-OH-BaP and 9-OH-BaP with all aerodigestive tract tissues tested (Table 2). All aerodigestive tract tissue microsomes except larynx exhibited activity in the order of 7-OH-BaP > 3-OH-BaP > 9-OH-BaP.

Significant levels of glucuronidating activity were also detected against trans-BaP-7,8-dihydriodiol (±) for pooled microsomes (1 mg of protein) from both laryngeal and esophageal tissues (Table 2). No glucuronidating activity was detected against trans-BaP-7,8-dihydriodiol (±) in tonsil microsomes using up to 1 mg of microsomal protein. Glucuronidation of trans-BaP-7,8-dihydriodiol (±) was also not detected in pooled microsomes from both tongue and floor of mouth, but assays were performed using only 0.1 mg of microsomal protein for these tissues due to the relatively small amount of specimen obtained for each of these sites. Similar to that observed for 7-OH-BaP, no activity was detected against 3-OH-BaP, 9-OH-BaP, or trans-BaP-7,8-dihydriodiol (±) in assays of human lung microsomes using up to 1 mg of microsomal protein (Table 2).

Expression of UGT mRNA. To evaluate UGT gene expression in aerodigestive tract tissues, duplex RT-PCR analysis was performed for pooled total RNA samples prepared from multiple normal human tongue, tonsil, floor of mouth, larynx, and esophagus, as well as from normal human lung and liver specimens. Of the family 1 UGTs (Fig. 2A), UGT1A6, UGT1A7, and UGT1A10 were all expressed in aerodigestive tissues but not in lung. Similar to that observed by Strassburg et al. (1999), UGT1A7, UGT1A8, and UGT1A10 were not
expressed in human liver. UGT1A8 was detected specifically in larynx but not in other aerodigestive tract tissues or in lung. Although significant levels of expression was observed in liver, no expression of UGT1A1, UGT1A3, UGT1A4, or UGT1A9 was detected in lung or in any of the aerodigestive tissues examined using pooled RNA samples (Fig. 2A) or RNA from individual specimens (results not shown).
study exhibited UGT2B17 expression; one esophageal specimen exhibited UGT2B15 expression (results not shown). As shown in previous studies (Beaulieu et al., 1997; Ulrich et al., 1997; King et al., 1999; Levesque et al., 1999), family 2B UGTs were well expressed in human liver. Other than UGT2B15 expression in a single lung specimen (Beaulieu et al., 1997; Ullrich et al., 1997; King et al., 1999; Levesque et al., 1999), family 2B UGTs were well expressed in other aerodigestive tract tissues, including esophagus. Similarly, the levels of expression of both UGTs 2B4 and 2B17 were significantly higher in liver than that observed for the UGT family 2B-expressing aerodigestive tract tissues.

Activities of Aerodigestive Tract-Expressing UGTs against 7-OH-BaP. Previous studies have implicated several UGTs in the glucuronidation of BaP metabolites (Jin et al., 1993a,b; Grove et al., 1997; Strassburg et al., 1999; Guillemette et al., 2000). To better assess the relative activities of aerodigestive tract-expressing UGTs against BaP metabolites, we performed a comprehensive screening of aerodigestive tract-expressing UGT-overexpressing cell lines or baculosomes for BaP metabolite-glucuronidating activity using 7-OH-BaP as substrate. UGT1A7- and UGT1A10-overexpressing baculosomes, as well as UGT1A8-overexpressing cell homogenates, exhibited detectable levels of glucuronidation activity against 7-OH-BaP as measured by their 7-OH-BaP glucuronidation activities (Table 3). The relative affinity of each of these UGTs for 7-OH-BaP as determined by kinetic ($K_m$) analysis was 1A10 > 1A7 > 1A8. No detectable activity against 7-OH-BaP was observed for UGTs 1A6, 2B4, or 2B17 up to 5 mg of cell homogenate in glucuronidation assays; all were active against 1-naphthol (UGT1A6), clofibric acid (UGT2B4), or androsterone (UGT2B17) as test substrates (results not shown).

**Discussion.**

Glucuronidation has been implicated as a major detoxification pathway for many carcinogens, including polycyclic aromatic hydrocarbons like BaP and other tobacco carcinogens like NNK (Richie et al., 1997; Strassburg et al., 1999; Ren et al., 2000). Although recent studies have suggested that many enzymes within the human UGT superfamily are extra-hepatic, however, previous studies examining UGT enzyme expression patterns have focused primarily on sites not known as primary targets for tobacco-induced carcinogenesis, including the digestive tract, the prostate, and the brain (Cheng et al., 1999; Hum et al., 1999; King et al., 1999; Strassburg et al., 2000; Tukey and Strassburg, 2000). In the present study, all of the aerodigestive tract tissues examined exhibited glucuronidating activity against multiple BaP metabolites. This is consistent with the fact that several UGTs were expressed in all aerodigestive tract tissues examined in this study. Previous studies have shown that several UGTs, including UGT2B7, UGT1A7, UGT1A8, UGT1A9, and UGT1A10, are active against several BaP phenols (Jin et al., 1993a,b; Grove et al., 1997; Mojarrabi and Mackenzie, 1998; Strassburg et al., 1999; Guillemette et al., 2000). Of these, UGT1A7 and UGT1A10 were well expressed in all aerodigestive tract tissues examined in the present study, whereas UGT1A8 was expressed in larynx. In addition, all three of these UGTs exhibited activity against 7-OH-BaP, with UGT1A10 exhibiting the highest affinity for 7-OH-BaP as determined by kinetic analysis. None of the other aerodigestive tract-expressing UGTs
(UGT1A6, UGT2B4, and UGT2B17) exhibited activity against 7-OH-BaP. These data are consistent with results from preliminary studies in our laboratory suggesting that UGT1A7, UGT1A8, and UGT1A10 all exhibit significant activity against trans-BaP-7,8-dihydriodiol, a direct precursor of the highly carcinogenic BaP-7,8-diol-9,10-epoxide (Fang et al., 2002). Together, these data suggest that UGT1A7, UGT1A8, and particularly UGT1A10 play an important role in tobacco carcinogen detoxification in the aerodigestive tract.

Differences were observed for aerodigestive tract tissues in their glucuronidating activity against different BaP metabolites in the present study. This may be due in part to differences in levels of expression of BaP metabolite-glucuronidating UGTs. For example, unlike that observed for microsomes from other aerodigestive tract tissues, laryngeal microsomes exhibited higher activity against 9-OH-BaP than 3-OH-BaP. This may be a result of the fact that in addition to UGT1A7 and UGT1A10, which were expressed in all aerodigestive tract tissues examined, UGT1A8 was also expressed in larynx.

The detection of glucuronidating activity in aerodigestive tract tissues and that specific UGTs are expressed in these tissues are consistent with recent data demonstrating that such UGTs could play an important role in tobacco-related cancer risk. Zheng et al. (2001) have demonstrated that UGT1A7 allelic variants coding for variant UGT1A7 isoforms with decreased activity against BaP phenols significantly contribute to increased risk for orolaryngeal cancer, an association that was linked to smoking. Studies are currently underway examining whether such associations may also be present for other aerodigestive tract tissue-expressing, BaP phenol-metabolizing UGTs (i.e., UGT1A10 and UGT1A8). Preliminary studies have shown that there exist at least three independent amino acid-altering polymorphisms present in the coding region of the UGT1A10 gene (Z. Zheng and P. Lazarus, unpublished results). The presence of these polymorphisms may be particularly important in risk assessment studies of aerodigestive tract cancer risk given the activity of UGT1A10 toward BaP metabolites. The functional significance of these polymorphisms and their potential role in aerodigestive tract cancer risk is currently being assessed.

The data presented in this study strongly suggest that glucuronidation is not a major metabolic pathway/detoxification mechanism in human lung. Similar to that observed in other studies of other substrates, including the NNK metabolite 4-((methyl nitrosamino)-1-(3-pyridyl)-1-butanol and 4-nitrophenol (Ren et al., 2000), no glucuronidating activity was observed in microsomes from human lung specimens against any BaP metabolite tested. Analysis was performed using up to 1 mg of lung microsomal protein and was performed for three normal lung specimens from three individual subjects. These data are consistent with the fact that none of the UGTs screened in this study were expressed in lung tissue, as determined by duplex RT-PCR of pooled RNA samples. These data are consistent with previous studies demonstrating that UGT2B15 and UGT2B17 expression was not detected by analysis of pooled samples in the present study. This may be due in part to differences in levels of expression of certain UGTs screened in this study were expressed in lung tissue, as determined by duplex RT-PCR analysis, certain UGTs exhibit polymorphic expression (Strassburg et al., 1998a, 2000). Multiple UGTs were shown to exhibit differential expression in normal gastric (Strassburg et al., 1998a), duodenum, jejunum, ileum (Strassburg et al., 2000), and esophagus (Strassburg et al., 1999). Although UGT2B15 and UGT2B17 expression was not detected by analysis of pooled samples in the present study, expression of UGT2B17 was detected in esophageal tissue for 2 of 10 subjects when RNA samples were analyzed individually, whereas UGT2B15 was detected in one esophageal and one lung specimen. These data support results from previous studies suggesting that certain UGTs are either inducible or may exhibit polymorphic expression.

In summary, the data presented in this study demonstrate that aerodigestive tract tissues exhibit significant glucuronidating activity against BaP metabolites and that UGT enzymes with activity against these metabolites are expressed in aerodigestive tract tissues. These results are consistent with recent studies suggesting that specific UGTs play an important role in the detoxification of tobacco carcinogens and in risk for aerodigestive tract cancer (Zheng et al., 2001). Further studies are currently being performed examining whether BaP metabolite-glucuronidating UGTs may play a similar role for other cancers of the digestive tract (i.e., colon) in which BaP exposure is also etiologically important.

Acknowledgments. We are grateful of the Tissue Procurement Facility of the H. Lee Moffitt Cancer Center for access to tissue specimens and patient chart data.

References


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