DEVELOPMENTAL EXPRESSION OF N-ACETYLTRANSFERASES IN C57BI/6 MICE

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ABSTRACT:

N-Acetyltransferases (NATs) play an important role in the biotransformation of a wide variety of arylamine drugs and carcinogens. Two genes (NAT1, NAT2) have been identified and allelic variation in NAT2 has been associated with arylamine toxicity in adults. Little information has been reported on expression of NAT genes during embryonic and fetal development although substrate specific NAT activity has been detected. The current study investigated the expression of NAT1 and NAT2 in mice pre-and postnatally. RNA was isolated from maternal liver, embryonic tissue at gestational days (GD) 10, 15, and 18, or neonates at neonatal day 3. Reverse transcription-polymerase chain reaction was performed using primers designed to amplify portions of either the NAT1 or the NAT2 gene. NAT1 and NAT2 mRNAs were detected in the embryo/placental complex at GD 10 and in GD 15 and 18 embryos. NAT2 but not NAT1 was expressed in GD 18 and neonatal day 3 hepatic tissue. These data demonstrate the differential expression of NAT genes in the mouse embryo and suggest a potential role for NAT in development.

Hepatic N-acetyltransferases (NATs) catalyze the addition of an acetyl moiety to the extracyclic amine (N-acetylation) or hydroxyamino (O-acetylation) groups of a wide variety of aromatic amines and hydrazines (see reviews Weber, 1987; Hein, 1988; Vatsis and Weber, 1997). Individuals can be classified as rapid or slow acetylators, and associations between phenotype and susceptibility to chemical toxicity have been noted (Weber, 1987; Hein, 1988; Grant, 1993). Two genes, NAT1 and NAT2, were isolated and sequenced from several species and allelic variations have been detected in both genes (Deguchi et al., 1990; Ohsako and Deguchi, 1990; Blum et al., 1990, 1991; Hickman and Sim, 1991; Vatsis et al., 1991; Martell et al., 1991; Vatsis and Weber, 1997). NAT2 has been correlated with individual susceptibility to arylamine-induced toxicity including carcinogenesis (Weber, 1987; Hein, 1988; Grant, 1993; Vatsis and Weber, 1997). In several studies, NAT2 rapid acetylators have been found to have a higher incidence of colorectal cancer when exposed to heterocyclic amines (Lang et al., 1986; Illet et al., 1987; Grant et al., 1992; Minchin et al., 1993), whereas NAT2 slow acetylators showed an increased incidence of bladder cancer, especially when exposed to aromatic amines from industrial and environmental sources (Cartwright et al., 1982; Risch et al., 1995). Individuals with the NAT1*10 allele were found to have a 2-fold higher level of 4-aminobiphenyl-DNA adducts compared with those with the NAT1*4 allele (Badawi et al., 1995).

Although there is evidence that the ability to N-acetylate or to O-acetylate aromatic amines contributes to toxicity in adults, little is known concerning acetylation and toxicity before maturity. Many biotransformation enzymes are present during gestation; however, the time of expression and specific activity vary. Prenatal activities of enzymes were frequently found to be lower than in adults (Perucca, 1987).

Early studies in rodents have suggested that NATs developed before birth and increased during the postnatal period (Pacifici et al., 1986). N-Acetylation of p-aminobenzoic acid (PABA) has been detected in fetal and neonatal guinea pigs with peak activity occurring between 3 and 8 days after birth (Sonawane, 1982). A similar postnatal pattern was seen in the kidney and liver of newborn CD1 mice (Estrada-Rogers et al., 1997). One-week-old rabbits were found to have less than 10% of the activity per gram of liver found in mature animals (Cohen et al., 1973). Adult levels were not reached until about 4 weeks of age (Cohen et al., 1973). Additionally, in the second trimester, human fetuses (Pacifici et al., 1982) and infants (Vest and Salzberg, 1965) have been shown to acetylate PABA. Determination of NAT2 phenotype with caffeine suggested that activity increased in humans from birth to 15 months (Pariente-Khayat et al., 1991). These studies demonstrated that N-acetylation occurred during development. However, because the substrates used were only selective and not specific for NAT1 or NAT2, it is unclear whether one or both enzymes were present. The current study was initiated to determine the NAT1 and NAT2 gene expression patterns during development in C57BI/6 mice.

Materials and Methods

Animals. Pregnant or proven breeder C57BI/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The breeder mice were maintained at The University of Arizona Animal Care Facility under standard conditions of 12-h light/dark cycle with food and water ad libitum. Animals were mated...
and the first day after mating was considered gestational day (GD) 0. Tissue samples were collected at GD 10, 15, 18, and neonatal day (ND) 3.

**Primer Design.** NAT1 and NAT2 are genes 870 base pairs (bp) long having >80% homology. For the reverse transcription-polymerase chain reaction (RT-PCR), five primers were designed. The initial determination for NAT1 and NAT2 in GD 10, 15, and 18 used sense 5’ primer which is common (COM-S) to both NAT1 and NAT2, whereas the 3’ primer is in the untranslated region (NAT1-AS, NAT2-AS) of each gene corresponding to an area of least homology and differentiates between NAT1 and NAT2. Confirmation of NAT1 on GD 10, 15, and 18 and determination of ND 3 used the common primer in the antisense direction (COM-AS) and a small region of the NAT1 gene 5’ to the common primer (NAT1-S).

The primer sequences for NAT1 and NAT2 correspond to nucleotides 690 to 713 (COM-S), 713 to 690 (COM-AS), 155 to 180 (NAT1-S), 993 to 967 (NAT1-AS), and 975 to 952 (NAT2-AS). The sequences for the PCR primers were as follows:

COM-S: 5’-GTCTTATGGTTGGTGACACC-3’; COM-AS: 5’-GGTGGAGCCAAACAGATGAAC-3’; NAT1-S: 5’-TACAGGACATTTTTGACACATAGTA-3’; NAT1-AS: 5’-GTCGTACCAACTAATGAATCAGCTTTGGTTATGTA-3’; and NAT2-AS: 5’-ACTGTTTGCTATGTTGTACGGT-3’.

**RNA Isolation.** Tissue was obtained at GD 10, 15, 18, and ND 3. Maternal liver was isolated at each time point and used as a positive control. Littermates were pooled to obtain sufficient material. Placental and embryonic tissue were isolated together on GD 10 but separated at later time points. At GD 18 and ND 3, liver was excised from the remaining fetal/neonatal tissue. In adult and whole embryonic tissue, RNA was isolated using Trizol reagent (Gibco, Grand Island, NY) and then chloroform extraction. The aqueous phase was centrifuged at 8500 g and the RNA precipitated with 100% ethanol. The pellet was washed with 70% ethanol and resuspended in a lysing solution containing 4 M guanidium isothiocyanate, 20 mM sodium acetate, and 0.5% SDS. The RNA was purified on a 5.7 M cesium chloride gradient and centrifuged at 205,225g for 4.5 h (Futschler et al., 1992). The purity and amount of RNA was calculated from the 260/280-nm absorbance ratios. Embryonic and neonatal liver RNA was isolated in a similar fashion using only lysing solution and the same cesium chloride gradient method.

**RT-PCR.** A 20-μl reverse transcription reaction mixture was prepared on ice to contain 1 × PCR buffer (10 mM Tris, pH 8.3; 50 mM KCl; and 1.5 mM MgCl2), 1 mM each dNTP (Boehringer Mannheim), 100 pmol of random hexamers, 20 U of RNasin (Boehringer Mannheim), 200 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim), and 2 μg of total cellular RNA. The mixture was maintained at 42°C for 3 h followed by 95°C for 10 min to denature the avian myeloblastosis virus enzyme. The newly synthesized cDNA was then amplified in a 100-μl reaction mixture containing the following concentration of 1 × PCR buffer, 200 μM of each dNTP, 1 μM of each amplification primer, and 2 U of Taq DNA polymerase. Initial denaturation at 95°C for 5 min was followed by 40 cycles of 95°C for 45 s, 58°C for 30 s, 72°C for 30 s, with a final extension at 72°C for 5 min and a quick chill at 4°C. The histone primers define a 215-bp amplicon, the NAT1 using the primer COM-S with NAT1-AS (303 bp), whereas the 3’ primer which is common (COM-S) to both NAT1 and NAT2, whereas the 3’ primer is in the untranslated region (NAT1-AS, NAT2-AS) of each gene corresponding to an area of least homology and differentiates between NAT1 and NAT2. Confirmation of NAT1 on GD 10, 15, and 18 and determination of ND 3 used the common primer in the antisense direction (COM-AS) and a small region of the NAT1 gene 5’ to the common primer (NAT1-S).

The primer sequences for NAT1 and NAT2 correspond to nucleotides 690 to 713 (COM-S), 713 to 690 (COM-AS), 155 to 180 (NAT1-S), 993 to 967 (NAT1-AS), and 975 to 952 (NAT2-AS). The sequences for the PCR primers were as follows:

COM-S: 5’-GTCTTATGGTTGGTGACACC-3’; COM-AS: 5’-GGTGGAGCCAAACAGATGAAC-3’; NAT1-S: 5’-TACAGGACATTTTTGACACATAGTA-3’; NAT1-AS: 5’-GTCGTACCAACTAATGAATCAGCTTTGGTTATGTA-3’; and NAT2-AS: 5’-ACTGTTTGCTATGTTGTACGGT-3’.

**RT-PCR products were separated by gel electrophoresis and visualized with ethidium bromide and UV light. The primers COM-S with NAT1-AS (303 bp), NAT2-AS (285 bp), and histone (H3.3) (215 bp) as a control were used. Lane 1, molecular weight markers; lane 2, minus RNA; lanes 3 to 6, maternal liver minus reverse transcriptase (3), H3.3 (4), NAT1 (5) and NAT2 (6); lanes 7 to 10, GD 10 embryonic/placental tissue minus reverse transcriptase (7), H3.3 (8), NAT1 (9), and NAT2 (10).**

**Results**

Expression of NAT1 and NAT2 during gestation was evaluated at GD 10, 15, and 18. GD 10 corresponds to the middle of the second trimester, before the formation of the liver and the tissue analyzed consisted of the embryonic/placental complex. GD 15 is the middle of the third trimester and the liver is completely formed. These tissue samples included either the placenta or the embryo. GD 18 is just before birth. At this time point and after birth at ND 3, the liver was excised from the embryo or neonate and analyzed separately. All tissue samples were obtained from pooled litters. At each time point, replicate samples from two separate litters were evaluated to ensure reproducibility of the results. There were no significant qualitative differences noted when samples of the same gestational or neonatal age were compared.

In the second trimester (GD 10), NAT1 was found to be expressed in the embryonic-placental complex (Fig. 1). By the middle of the third trimester (GD 15), NAT1 mRNA was detected in embryonic tissue as well as in the placenta (Fig. 2). At GD 18, NAT1 was found to be expressed in extrahepatic tissue, but no NAT1 mRNA was found in liver (Fig. 3) and this pattern continued through ND 3 (Fig. 4).

Expression of NAT2 was similar to that observed for NAT1 at GD 10 and GD 15 (Figs. 1 and 2). Unlike NAT1, NAT2 expression was detected in both hepatic and extrahepatic tissue on GD 18 (Fig. 3).

**Discussion**

Organogenesis is commonly regarded as the stage of development most sensitive to chemically induced teratogenesis. Although susceptibility to teratogens may be affected by embryonic biotransformation, the developmental pattern of arylamine acetyltransferases has not been extensively investigated in mammals. PABA NAT activity has been found in the human mid-gestational placenta as well as fetal hepatic and extrahepatic tissue, suggesting that human NAT develops during the first trimester of pregnancy (Pacifici et al., 1986). After birth, infants were shown to acetylate PABA and caffeine (Vest and Salzberg, 1965; Pariente-Khayat et al., 1991). In neonatal mice, PABA NAT activity increased almost 4-fold from birth to day 80 in the liver and 2- to 6-fold in the kidney. Furthermore, the CD1 mouse strain was found to acetylate PABA as early as ND day 1 (Estrada-Rogers et al., 1997). The experiments presented here have shown the presence of mRNA for NAT1 and NAT2 before birth. Indeed, NAT1 and NAT2 mRNA were detected in the middle of the third trimester in embryonic tissue (GD 15) and the middle of the second trimester in the embryo-placental complex at GD 10 (Figs. 1 and 2). The presence of NATs during development may contribute to the detoxification of both endogenous and exogenous substrates. Previous
RT-PCR products were separated by gel electrophoresis and visualized with ethidium bromide and UV light. The primers COM-S with NAT1-AS (303 bp), NAT2-AS (285 bp), and H3.3 (215 bp) as a control were used. A, lane 1: molecular weight markers; lane 2: minus RNA; lanes 3 to 6: maternal liver minus reverse transcriptase (3), H3.3 (4), NAT1 (5), NAT2 (6); lanes 7 to 10: GD 15 embryonic tissue minus reverse transcriptase (7), H3.3 (8), NAT1 (9), NAT2 (10). B, lane 1: molecular weight markers; lane 2: minus RNA; lanes 3 to 6: GD 15 placental tissue minus reverse transcriptase (3), H3.3 (4), NAT1 (5) and NAT2 (6).

**Fig. 2. Analysis of NAT1 and NAT2 expression in the GD 15 embryonic tissue (A) and GD 15 placental tissue (B).**

RT-PCR products were separated by gel electrophoresis and visualized with ethidium bromide and UV light. The primers COM-S with NAT1-AS (303 bp), NAT2-AS (285 bp), and H3.3 (215 bp) as a control were used. A, lane 1: molecular weight markers; lane 2: minus RNA; lanes 3 to 6: maternal liver minus reverse transcriptase (3), H3.3 (4), NAT1 (5), NAT2 (6); lanes 7 to 10: GD 15 embryonic tissue minus reverse transcriptase (7), H3.3 (8), NAT1 (9), NAT2 (10). B, lane 1: molecular weight markers; lane 2: minus RNA; lanes 3 to 6: GD 15 placental tissue minus reverse transcriptase (3), H3.3 (4), NAT1 (5) and NAT2 (6).

**Fig. 3. Analysis of NAT1 and NAT2 expression in the GD 18 embryonic tissue without liver (A) and GD 18 embryonic liver (B).**

RT-PCR products were separated by gel electrophoresis and visualized with ethidium bromide and UV light. The primers COM-S with NAT1-AS (303 bp), NAT2-AS (285 bp), and H3.3 (215 bp) as a control were used. A, lane 1: molecular weight markers; lane 2: minus RNA; lanes 3 to 6: maternal liver minus reverse transcriptase (3), NAT1 (4), NAT2 (5), and H3.3 (6); lanes 7 to 10: GD 18 embryonic tissue without liver minus reverse transcriptase (7), NAT1 (8), NAT2 (9), and H3.3 (10). B, lane 1: molecular weight markers; lane 2: minus RNA; lanes 3 to 6: GD 18 hepatic tissue minus reverse transcriptase (3), NAT1 (4), NAT2 (5), and H3.3 (6); lanes 7 to 10: maternal liver minus reverse transcriptase (7), NAT1 (8), NAT2 (9), and H3.3 (10).

Data have suggested that human NAT1 catalyzes the acetylation of the folate breakdown product p-aminobenzoyl-L-glutamate (Minchin, 1995; Ward et al., 1995) and that the amount of p-aminobenzoyl-L-glutamate is a balance between NAT1 activity and the intracellular concentration of folate. It has been proposed that folate deficiency syndromes seen during pregnancy may be related to NAT1 activity (Ward et al., 1995). Comparison of the substrate specificities and deduced amino acid sequences of mouse NAT2 and human NAT1 indicated that mouse NAT2 may be homologous to NAT1 (Martell et al., 1992). Mouse NAT2 had higher deduced amino acid identity with human NAT1 (80%) than with human NAT2 (74%). Furthermore, mouse NAT2 acetylated p-aminobenzoyl-L-glutamate, whereas NAT1 did not (Estrada-Rogers et al., 1998). Thus, the role of the mouse NAT2 and human NAT1 enzyme may be to ensure the turnover of folate in embryonic cells. This is supported by the detection of NAT2 mRNA but not NAT1 in liver pre- and postnatally (Figs. 3 and 4).

The presence of NAT1 and NAT2 mRNA in embryonic tissues also suggests the possibility of biotransformation by the embryo to activate or detoxify aromatic amines. Other classes of chemicals have been shown to undergo embryonic biotransformation. For example, the teratogenesis of phenytoin has been linked to the formation of a reactive intermediate via embryonic prostaglandin H synthase (Wells and Winn, 1996). Embryonic biotransformation of benzo(a)pyrene, attributed to CYP1A1, has been detected as early as the preimplantation stage in mice (GD 4) (Filler and Lew, 1981). The hydroxylated metabolites formed by CYP1A1 can be eliminated through glucuronidation in adults, which avoids alternative bioactivation to electrophilic and free radical intermediates. However, UDP-glucuronosyltransferases were low or absent in fetal tissues (Dutton, 1971; Burchell, 1973) resulting in the inability of the embryo to detoxify the products through glucuronidation. For aromatic amines, the initial step in bioactivation is N-hydroxylation, followed by acetylation, leading to the formation of reactive products (Hein, 1988). Thus, the expression of NATs during development may increase the potential fetal toxicity of aromatic amines.

Detoxification and bioactivation may also occur in the placenta, which is derived from embryonic and maternal tissue. The placenta contains both phase I and phase II enzymes. The ability to perform conjugation reactions may serve to protect the embryo by capturing reactive intermediate crossing the placenta. PABA NAT activity has been detected in the human mid-gestational placenta (Pacifici et al., 1986) and both PABA and sulfamethazine activities were present in term placental tissue (Smelt et al., 1998). In guinea pigs, placental acetylation of PABA has a discontinuous developmental pattern, rising rapidly to a maximum value by four-fifths of the way through the gestational period then decreasing to a minimum at birth (Sonawane, 1982). The current study shows mouse NAT1 and NAT2 mRNAs in the embryo-placental complex at mid-gestation (GD 10) as well as in the placental tissue on GD 15 (Figs. 1 and 2).

Although, in general, xenobiotic biotransformation is low in the neonatal relative to the adult, this capacity quickly increases postnatally. Kidney and liver PABA NAT2 activities in CD1 mice were present at birth, increasing 3.7-fold over 80 days, although hepatic NAT protein remained constant from day 1 to 80 postnatally (Estrada-Rogers et al., 1997). The data presented here extend these findings, showing embryonic NAT1 and NAT2 mRNA expression before birth. Since NAT1 was not expressed in liver isolated at GD 18 or ND 3, activity in that tissue is probably due to NAT2.

The expression of NAT1 and NAT2 during development may allow...
the embryo/fetus to activate or detoxify both endogenous and exogenous aromatic amines. Further studies to quantify NAT1 and NAT2 mRNAs and to demonstrate catalytic activity will be necessary to determine the association between NAT and the developmental toxicity of aromatic amines.

References


Minchin RF, Kadlubar FF and Ilett KF (1993) Role of acetylation in colorectal cancer. Mutat Res 290:35–42.


