Expression of arylamine N-acetyltransferases in pre-term placentas and in human pre-implantation embryos

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Arylamine N-acetyltransferases (NATs) catalyse the acetylation from acetyl-CoA of arylamines and hydrazines. There are two human isoenzymes which show polymorphism, and both enzymes are involved in the activation and detoxification of environmental carcinogens and teratogens. The two human isoenzymes NAT1 and NAT2 show different tissue distribution, with human NAT2 being found in liver and intestine whilst human NAT1 is expressed in many tissues including erythrocytes, bladder, lymphocytes and neural tissue, as well as liver and intestine. It has been proposed that NAT1 has an endogenous role in the acetylation of the folate catabolite p-aminobenzoyl-L-glutamate (pABGlu) to produce the major urinary product, N-acetyl-pABGlu. The murine homologue of human NAT1 is known to be concentrated in the neural tube during development. We show here that human NAT1 but not human NAT2 is expressed in pre-implantation embryos at the blastocyst stage and show that NAT1 is also expressed in early human placenta at the earliest available stage, 5.5 weeks. We demonstrate that there is inter-individual variation in NAT1 expression. In view of the role of folate in protecting against neural tube defects, we propose that NAT1 is a candidate risk factor for susceptibility to neural tube defects.

INTRODUCTION

Throughout gestation, the fetus is exposed to all nutrients and xenobiotics in the maternal circulation via the placenta (1). Placental metabolism of these compounds can affect the abundance and nature in which they reach the fetus (2–4), and placental metabolism is important in both fetal nutrition and toxicology.

Arylamine N-acetyltransferases (NATs) are drug-metabolizing enzymes capable of the N-acetylation and detoxification of arylamine and hydrazine drugs (5–7). In addition, NATs can catalyze the O-acetylation of arylhydroxylamine substrates, increasing their toxicity (8,9). Two functional isofoms of NAT are expressed in humans, designated NAT1 and NAT2. Tissue expression of NAT2 is confined mainly to liver and intestine (10–12), while NAT1 is found in many tissues (13–16). Both NAT isofoms are encoded at polymorphic loci (17,18) and exhibit inter-individual variation in enzymic activity. The relationship between genotype and phenotype has been elucidated for NAT2 (19–21), but this relationship for human NAT1 is only established for some alleles (21–24).

The NAT1 and NAT2 proteins possess distinct but overlapping substrate specificities (25,26). NAT2 substrates include procainamide, isoniazid and some carcinogenic arylamines (6,13,27). In addition to carcinogenic arylamines (6), NAT1-specific substrates include p-aminobenzoic acid (pABA) (13,25) and the folate catabolite p-aminobenzoyl-L-glutamate (pABGlu) (28,29).

Interest in placental NAT expression was provoked initially by the potential of NATs to determine fetal exposure to certain toxic arylamines and to drugs present in the maternal circulation (30). Interest has increased following the demonstration by Minchin (28) that recombinant NAT1 and U937 cells which express NAT1 (31) mediate the N-acetylation of the folate catabolite pABGlu, indicating that NAT1 may also play a role in endogenous folate metabolism (28,29). During pregnancy, there is an increase in folate requirement in addition to the needs of the fetus (32). There is an increase also in folate catabolism (33) resulting in an increase in excretion of the folate catabolite pABGlu in its N-acetylated form, N-acetamido-p-aminobenzoylglutamate (ApABGlu) (34). The enzyme responsible for N-acetylation of the folate catabolite pABGlu has been proposed to be human NAT1 (28,29). The ability of maternal folate supplementation to protect against neural tube defects (NTDs) in the newborn is well known (35). The molecular basis for folate protection has not been resolved, although a murine model (Crooked tail) recently has been been mapped (36). Polymorphisms have also been described in the enzymes involved in the folate/cobalamin cycle, methionine synthetase (37), methionine synthetase reductase (38), methyltetrahydrofolate reductase (MTHFR) (39) and cystathionine β-synthetase (40). Polymorphism in MTHFR has been demonstrated to be associated with a small proportion of NTDs, although this is controversial (41).

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The expression of an enzyme in early development capable of the $N$-acetylation of $pABGlu$ is an obvious candidate for folate protection, particularly when the enzyme shows inter-individual variation (15, 42–44). In order to determine whether human NAT1 is expressed in early development, we have investigated NAT1 expression in pre-term placentas from the first trimester and also in early human embryos using cDNA libraries from unfertilized human oocytes, single, 2-cell, 4-cell, 8-cell and blastocyst stage embryos and a 10-week-old fetus (45) to determine whether NAT is expressed prior to neurulation.

RESULTS

Placental NAT1 activity during gestation

All 17 pre-term placental samples (Table 1) were assayed for NAT1 activity and for $pABGlu$ $N$-acetylation activity. An aliquot of the pooled standard described in Materials and Methods was included in each assay. Total placental $N$-acetylation activities are in nmol/min/placenta as shown with $pABA$ (solid columns) and $pABGlu$ (striped columns) in relation to trimester of pregnancy. Mean placental mass is taken from (46).

The expression of an enzyme in early development capable of the $N$-acetylation of $pABGlu$ is an obvious candidate for folate protection, particularly when the enzyme shows inter-individual variation (15,42–44). In order to determine whether human NAT1 is expressed in early development, we have investigated NAT1 expression in pre-term placentas from the first trimester and also in early human embryos using cDNA libraries from unfertilized human oocytes, single, 2-cell, 4-cell, 8-cell and blastocyst stage embryos and a 10-week-old fetus (45) to determine whether NAT is expressed prior to neurulation.

pABGlu $N$-acetylation in the pre-term placenta

A sub-group of pre-term placental homogenates ($n = 12$) were investigated for their ability to $N$-acylate the folate catabolite $pABGlu$. The mean specific activity of pre-term placentas with $pABGlu$ as substrate is $72.1 \pm 38.1$ pmol $pABGlu$ $N$-acylated/min/mg protein.

Figure 1. Total placental NAT1 activity and $pABGlu$ $N$-acetylation activity according to trimester of pregnancy. Homogenates prepared from 12 pre-term placentas were assayed for NAT1 activity and for $pABGlu$ $N$-acetylation activity. An aliquot of the pooled standard described in Materials and Methods was included in each assay. Total placental $N$-acetylation activities are in nmol/min/placenta as shown with $pABA$ (solid columns) and $pABGlu$ (striped columns) in relation to trimester of pregnancy. Mean placental mass is taken from (46).

Figure 2. NAT activity in pre-term placenta. NAT1 and NAT2 activities were measured in cytosols prepared from pre-term placentas. NAT1 and NAT2 activity measurements were made using PABA and SMZ, respectively. DNA from each placenta was genotyped for NAT2* (55). Ages and genotypes of all placentas are described in Table 1. The NAT2 phenotype of each sample is indicated above the appropriate bars. The x-axis shows the type of NAT activity (NAT1 or NAT2) and the y-axis gives the specific activity in pmol of substrate $N$-acylated/min/mg protein, on a logarithmic scale.

The $NAT1^*$ and $NAT2^*$ genotypes of all 17 pre-term placentas are shown in Table 1. Three placentas of differing gestational age were investigated for their ability to $N$-acylate the NAT2-specific substrate sulfamethazine (SMZ). The NAT1- and NAT2-specific activity of the three samples is shown in Figure 2. NAT2 activity was only detectable in two of the three placentas, T12 (8.5 weeks) and T15 (32 weeks), which were genotyped as heterozygous for the fast $NAT2^*$ allele. No activity could be detected using SMZ with the other placenta, T1, which was homozygous for slow $NAT2^*$ alleles.

To investigate the relationship between NAT1 genotype and phenotype in pre-term placentas, the mean NAT1-specific activities of placental samples containing one, two or no $NAT1^*4$ alleles were calculated. There is no difference between the mean NAT1-specific activities of pre-term placental samples with one $NAT1^*4$ allele and samples with two $NAT1^*4$ alleles (both 1.7 nmol pABA $N$-acylated/min/mg protein). Only one sample contained no $NAT1^*4$ alleles ($NAT1^*10,*3$) and this sample, with a NAT1 activity of 11.4 nmol pABA $N$-acylated/min/mg protein, is in the middle of the range of pre-term placental NAT1 activities. Two samples were genotyped as heterozygous for the $NAT1^*11$ allele; both these samples have NAT1 activities (2.0 and 1.5 nmol pABA $N$-acylated/min/mg protein) which are at neither extreme of pre-term placental homogenate NAT1 activities.

pABGlu $N$-acetylation in the pre-term placenta

A sub-group of pre-term placental homogenates ($n = 12$) were investigated for their ability to $N$-acylate the folate catabolite $pABGlu$. The mean specific activity of pre-term placentas with $pABGlu$ as substrate is $72.1 \pm 38.1$ pmol $pABGlu$ $N$-acylated/min/mg protein.

The expression of an enzyme in early development capable of the $N$-acetylation of $pABGlu$ is an obvious candidate for folate protection, particularly when the enzyme shows inter-individual variation in NAT1 activity. The mean NAT1 activity ($\pm$ standard deviation) of the pre-term placentas was $1.7 \pm 0.6$ nmol pABA $N$-acylated/min/mg protein. Using the mean placental mass at each trimester of pregnancy (46), total placental NAT1 activity at each stage of pregnancy was calculated (Fig. 1), and shows an increase over time from the first trimester to term using both pABA and pABGlu as substrate.

**Figure 1.** Total placental NAT1 activity and pABGlu $N$-acetylation activity according to trimester of pregnancy. Homogenates prepared from 12 pre-term placentas were assayed for NAT1 activity and for pABGlu N-acetylation activity. An aliquot of the pooled standard described in Materials and Methods was included in each assay. Total placental N-acetylation activities are in nmol/min/placenta as shown with pABA (solid columns) and pABGlu (striped columns) in relation to trimester of pregnancy. Mean placental mass is taken from (46).

**Figure 2.** NAT activity in pre-term placenta. NAT1 and NAT2 activities were measured in cytosols prepared from pre-term placentas. NAT1 and NAT2 activity measurements were made using PABA and SMZ, respectively. DNA from each placenta was genotyped for NAT2* (55). Ages and genotypes of all placentas are described in Table 1. The NAT2 phenotype of each sample is indicated above the appropriate bars. The x-axis shows the type of NAT activity (NAT1 or NAT2) and the y-axis gives the specific activity in pmol of substrate N-acylated/min/mg protein, on a logarithmic scale.
N-acetylated/min/mg protein. As for NAT1 activity, the total placental pABGlu N-acetylation activity at each stage of pregnancy was calculated (Fig. 1), and shows an increase over time to term. The pattern of the increase with these two substrates differs slightly in early pregnancy. In order to explore this slight difference, the individual values of specific activity with pABA and pABGlu have been compared (Fig. 3). We demonstrate a strong correlation between the N-acetylation of pABA and pABGlu in term placenta which is shown for comparison. The two activities of the pre-term placental samples also correlate strongly, apart from two samples (Fig. 3). These two placentas, the second-trimester sample T6 and the first-trimester sample T12, show relatively greater acetylation activity with pABGlu as substrate than the other pre-term placentas. These samples are highlighted by arrows in Figure 3. Sequencing the NAT1 genes of placenta T6, which correspond to fetal DNA (44), revealed that one allele is the most common NAT1 allele referred to as NAT1*4 (21). The other is a previously unknown allele with two point mutations within the coding region compared with NAT1*4, which has now been designated NAT1*27 (21). Both mutations, T21G and T777C, are silent mutations and would not be expected to alter functional activity. NAT1*27 allele-specific PCR has shown that this allele is not present in placenta T12 (Table 1).

**NAT1 transcription in human pre-implantation embryos**

PCR screening of the cDNA libraries from pre-implantation embryos revealed that NAT1 cDNA could be detected in the cDNA libraries constructed from a 10-week-old human fetus and from the blastocyst stage, but not at earlier stages of pre-implantation (Fig. 4A–C). With primers for NAT2, a product was obtained only with cDNA from the 10-week-old fetus. This is in contrast to the cDNA for hypoxanthine–guanine phosphoribosyltransferase (HPRT) which is observed in all cDNA samples including oocytes (Fig. 4D). The HPRT primers span an intron, and serve as a control for contamination with genomic DNA. These results demonstrate that initiation of transcription of the NAT1 message occurs in the embryo at the blastocyst stage.

**DISCUSSION**

NAT activity in pre-term placentas has been measured to be due predominantly to NAT1 on the basis of the ability to catalyze the N-acetylation of human NAT isoenzyme-specific substrates, SMZ (NAT2) and pABA (NAT1). The NAT1 activity could be detected in the earliest placental samples available, i.e. at 5.5 weeks gestation. No NAT2 activity could be detected at 5.5 weeks although this may be due to the 5.5 week plental sample carrying a slow acetylator genotype. NAT2 activity could be detected in the first trimester in a fast acetyling 8.5 week placenta, but nevertheless the predominant NAT activity in the first trimester is NAT1. The predominance of NAT1 expression demonstrated here for pre-term placentas and in early embryos matches what has been described for term plancental samples (30,44,47) and neonates (47,48). Pre-natal NAT1 expression, as shown here, is amongst the earliest xenobiotic-metabolizing genes whose expression has been detected. Cytochrome P450 isoenzymes have been detected by RT–PCR and specific Southern blotting in 10- to 12-week-old placentas (49).

There is inter-placental variation in NAT1 activity. The inter-individual variation in NAT1 activity in pre-term placentas is comparable with that in term placentas (Fig. 3) and with previously reported variations in NAT1 activity in other adult tissues (12,15,16). We demonstrate here that apart from two samples that showed a higher than expected activity with the folate catabolite pABGlu, there is good correlation between NAT1 activity as measured with the conventional probe substrate pABA and with pABGlu as a probe substrate.

There is a growing body of evidence to support the idea that NAT1 and the murine equivalent (murine NAT2) are each responsible for the acetylation of the folate catabolite pABGlu and the generation of the major urinary catabolite ApABGlu (50). The present study (Fig. 1) illustrates that there is sufficient NAT1 activity in placenta to account for the increase in urinary excretion of ApABGlu (150 µg/day) which occurs during pregnancy (33).

NAT1 is expressed in embryos at the blastocyst stage prior to neurulation, a pre-requisite for an enzyme involved in folate protection against NTDs. Previous studies in mice also show the early expression of the murine equivalent of NAT1 at the blastocyst stage (50) and that, following neurulation, NAT expression is clearly associated with the neural tube (51) at 9.5 days and mRNA for the murine equivalent of human NAT1 is detected in 18 day fetal liver (52). Studies on congenic mice have identified co-segregation of NAT and susceptibility to cleft lip and palate (53).

We propose that human NAT1 should be considered as a candidate risk factor in NTDs.
MATERIALS AND METHODS

Collection of samples

Placentas of gestational age 5.5–34 weeks were obtained, with informed consent, from either therapeutic abortions or preterm deliveries at The Women’s Centre, John Radcliffe Hospital, Oxford (44). Term placental samples were also collected following normal vaginal deliveries (all samples were of Caucasian origin). Tissue was dissected into and rinsed with 145 mM sodium chloride, 10 mM sodium phosphate pH 7.5, then frozen and stored in liquid nitrogen. Table 1 gives the gestational age of the 17 samples included in the study.

Placental cytosols

Frozen samples were suspended in three times the mass of ice-cold potassium phosphate–EDTA [10 mM potassium phosphate pH 7.5, 1.15% (w/v) potassium chloride, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM Pefabloc (S. Black Import/Export, Hertfordshire, UK)] and homogenized using a hand-held Teflon–glass homogenizer for pre-term placental samples and with a tissue grinder for term placental samples (44). Homogenates were centrifuged at 13 500 r.p.m. at 4°C for 20 min prior to assay or storage. Pools of homogenates from six different term placental samples were made for use as standards (44). Cytosolic fractions were prepared from homogenates by centrifugation at 100 000 g at 4°C for 1 h. All samples were stored in liquid nitrogen in aliquots which were thawed only once just prior to use.

NAT activity measurements

Placental samples were assayed for their ability to N-acetylate the NAT1- and NAT2-specific substrates pABA and SMZ, respectively, according to the methods of Ward et al. (16). For assay with pABGlu as substrate, 0.22 mM pABGlu was used, and N-acetylated pABGlu was quantitated by high pressure liquid chromatography (HPLC). All reaction mixtures were loaded in a total volume of 20 µl onto a C18 HPLC column and separated using a Waters HPLC (600E solvent delivery system and a 484 absorbance detector) with a mobile phase of acetonitrile:50 mM acetic acid (10:90) and a flow rate of 2 ml/min.

Placental samples were assayed on two to four separate occasions with each substrate, and the specific activity is the mean of the separate activity measurements. An aliquot of the pooled placental samples described above was used as a standard in each assay performed. For calculation of specific activity, the protein concentration of each sample was determined using a dye-binding procedure with γ-globulin (Bio-Rad Laboratories, Hertfordshire, UK) as standard.

Cloning and sequencing of NAT1

Genomic DNA (gDNA) was extracted from a 50 µl sample of placental homogenates by proteinase K digestion and phenol/chloroform extraction (54). The NAT1 genes of sample T6

Table 1. Age and genotypes of pre-term placentas

<table>
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<th>Sample*</th>
<th>Gestational age (weeks)b</th>
<th>Trimester</th>
<th>Specific pABA N-acetylation activityc</th>
<th>Specific pABGlu N-acetylation activityd</th>
<th>NAT1* genotype</th>
<th>NAT2* genotype</th>
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<td>Mean 1.7 ± 0.6</td>
<td>Mean 72.1 ± 38.1</td>
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*Samples highlighted by an asterisk were not assayed with pABGlu as substrate.

bGestational age is given according to the date of the last menstrual period.

cnmol of pABA N-acetylated/min/mg protein determined using homogenates. Mean is shown ± standard deviation.

dpmol of pABGlu N-acetylated/min/mg protein determined using homogenates. Mean is shown ± standard deviation.
were PCR amplified using primers N-376 and N1177 (23), and cloned for sequencing across the coding region of this intronless gene using automated primer walking using primer N-376, N1177, N714, N769 (23), NatHu31 (5'-GATGTGTTGGAGG-GGTATG-3') and NatHu37 (5'-CATGTTGTTGGCACAAGC-3'). In addition, the region between nucleotides 398 and 203 was sequenced by automated primer walking using primer N-376 and N1177 (23).

**NAT1 genotyping**

Samples were genotyped for the presence of the NAT1* alleles NAT1*4 (most common allele), NAT1*3, NAT1*10 and NAT1*11 (12,23) and for NAT2* alleles as previously described (55). In addition, allele-specific PCR was used to investigate the presence of the NAT1*27 allele in placenta T12. Sense primers were designed to anneal specifically to the region of NAT1 next to the nucleotide substitutions present in the NAT1*27 allele. These primers N21-Mutant (5'-TGGAC-CATTGAAGGACTTCTGAGT-3') and N777-Mutant (5'-AGAG-CTCGAACACAGCAGTC-3') were used in conjunction with primers NatHu37 (described above) and N1113 (23), respectively.

**Screening of cDNA libraries for NAT**

NAT1 transcription in early human embryos was investigated by screening cDNA libraries representative of pre-implantation human embryos by PCR. The libraries were constructed using mRNA extracted from four unfertilized human oocytes, 2-cell, 4-cell, 8-cell and blastocyst stage embryos and a 10-week-old whole fetus. These and similar cDNA libraries have been shown to be representative of human mRNA expression at this stage of development (45).

Initial PCR amplification used primers NatHu31 and NatHu37 as described above. Amplification (with 1.5 mM MgCl2) was for 35 cycles with denaturation at 95°C (0.5 min), annealing at 56°C (1 min) and extension at 72°C (1 min), with a final extension time of 5 min. Amplification products were separated by agarose gel electrophoresis and visualized by UV transillumination, or were transferred to a Hybond N+ membrane (Amersham), hybridized to a NAT1-specific probe (56) and visualized by autoradiography. Nested PCR was carried out on the products of the initial amplification, using primers N539 and N714 as described (12). Amplification of NAT2 was carried out with NatHu7 and NatHu8 (20), except that two rounds of amplification were carried out.

Semi-nested amplification was also performed with HPRT primers, with the initial amplification using sense primer 5'-AATTATGGACAGGACTGAC-3' and antisense primer 5'-GGCGATGTCAATAGGACTCCAGATG-3' and alleles as previously described (55). In addition, allele-specific PCR was used to transfer to a Hybond N+ membrane (Amersham), hybridized to a NAT1-specific probe (56) and visualized by autoradiography. Nested PCR was carried out on the products of the initial amplification, using primers N539 and N714 as described (12). Amplification of NAT2 was carried out with NatHu7 and NatHu8 (20), except that two rounds of amplification were carried out.

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