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Expression of monomorphic and polymorphic *N*-acetyltransferases in human colon

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Abstract—The metabolism of sulfamethazine (SMZ) and *p*-aminobenzoic acid (PABA) by *N*-acetyltransferase (NAT) was measured in human colorectal cytosols from 12 slow and 11 rapid acetylators whose genotype was determined independently by a specific polymerase chain reaction. SMZ metabolism was significantly greater in the rapid than in the slow phenotype (192 ± 22 versus 94 ± 11 pmol *N*-acetylsulfamethazine/min/mg protein), while PABA metabolism was similar in both phenotypes (23.7 ± 4.4 versus 23.0 ± 3.9 nmol *N*-acetyl-*p*-aminobenzoic acid/min/mg protein). Both monomorphic and polymorphic NAT mRNAs were detected by the polymerase chain reaction in the colorectal mucosa of most samples. The finding that polymorphic NAT is expressed in a phenotype-dependent manner in colorectal mucosa indicates that this tissue has the capacity to participate in local bioactivation of dietary and environmental aryl- or heterocyclic amine carcinogens and may explain, in part, the phenotype-dependent occurrrence of colorectal cancer.

Key words: *N*-acetyltransferases; human colon; acetylation phenotype/genotype; sulphamethazine; *p*-aminobenzoic acid

Recent studies have elucidated the molecular basis for human arylamine NAT* polymorphism and two functional genes *NAT1* and *NAT2* have been identified [1–5]. Polymorphism of *NAT2* has been associated with a variety of disease states [6] including cancers [7, 8]. In colorectal cancer, the rapid acetylator phenotype is more prevalent [9, 10], although this association has not been found universally [11]. Epidemiological studies suggest that dietary heterocyclic amines which are subject to metabolism by NAT2 might be involved in the formation of colorectal tumours [12]. NAT2 is able to catalyse the acetylation of arylamine carcinogens, heterocyclic amine carcinogens and their respective *N*-hydroxy metabolites [13]. NAT1 also is able to acetylate many known arylamine carcinogens [13]. The proposed mechanism by which metabolic activation of such carcinogens may occur involves N-hydroxylation of the amines by cytochrome P4501A2 in the liver, followed by esterification which may be catalysed by NAT (reviewed in Ref. 14).

Several investigators have proposed a scheme to account for the formation of DNA adducts in extrahepatic tissues and in particular the colon. It has been suggested that glucuronides of the *N*-hydroxy metabolites of aromatic or heterocyclic amines are excreted in the bile and subsequently deconjugated by bacterial β-glucuronidase in the colon. This scheme requires the presence of ester-forming enzymes, such as NAT, in colorectal tissue. A number of studies have addressed this possibility. Firstly, Kirlin *et al.* [15] proposed that human colorectal NAT activity for several substrates could be used to subdivide the population sample into three phenotypes. Their probit analysis thereby suggested the presence of a polymorphism in colorectal NAT activity. Secondly, Turesky *et al.* [16] have reported that a number of polymorphic NAT2 substrates are acetylated by human colorectal cytosols. However, these data are difficult to interpret as many of the substrates used are also acetylated by NAT1 [13].

In the present study, we sought to determine clearly whether or not NAT2 is expressed in human colorectal tissue. We reasoned that polymorphic substrates should be acetylated more rapidly in rapid acetylator individuals whose genotype is determined independently, and that, if NAT2 is expressed in colorectal tissue, then NAT2 mRNA should also be detectable.

Materials and Methods

Patient and tissue samples. Patients were recruited from those attending for surgical resection of a colorectal tumour at the Sir Charles Gairdner Hospital (Perth, Western Australia) (N = 18) and the John L. McClellan Veterans Administration Hospital (Little Rock, AR, U.S.A.) (N = 5). The study protocol was approved by the Human Rights Committee of the University of Western Australia and by the Human Ethics Committee of the Veterans Administration Hospital. Blood (5 mL) anticoagulated with EDTA was obtained prior to surgery, while colorectal mucosa (dissected free of underlying muscle) was obtained at surgery. Tissue was taken from a site 2–10 cm distal to the tumour but cell morphology was not characterized histologically.

PCR of NAT1 and NAT2. DNA was isolated from blood by the method of Walsh *et al.* [17] with minor modifications. Briefly, 5 μL of blood was diluted in 1.5 mL sterile distilled water, mixed intermittently for 20 min and then microfuged for 3 min. The supernatant was discarded and the pellet resuspended in 200 μL of Chelex 100 resin (5% w/v, BIO-RAD Laboratories, Richmond, CA, U.S.A) and incubated at 56° for 20 min. After vortexing for 20 sec, the mixture was incubated at 100° for 8 min and microfuged for 8 min to sediment the resin. Supernatant (10 μL) was subjected to 30 cycles of allele-specific PCR [3] in a Perkin Elmer Cetus Thermal Cycler (final volume 25 μL) followed by electrophoresis on a 1.5% agarose gel (Nusieve 3:1; FMC BioProducts, Rockland, ME, U.S.A.) containing ethidium bromide and visualization of the PCR products with UV trans-illumination. Phenotype was assigned using the criterion that both alleles in an individual must be mutated to endow the slow phenotype [1].

Total RNA was extracted from approximately 1 g of colorectal mucosa (powdered under liquid nitrogen) by the guanidinium thiocyanate method [18] and contaminating DNA was removed essentially as described previously [19]. The ethanol-washed pellet was dried under vacuum,

* Abbreviations: NAT, *N*-acetyltransferase; NAT1, *N*-acetyltransferase 1; NAT2, *N*-acetyltransferase 2; PABA, *p*-aminobenzoic acid; PCR, polymerase chain reaction; SMZ, sulfamethazine.

Table 1. Acetylation of SMZ and PABA by colorectal cytosols from slow and rapid acetylators

Patient identification	Sex/age	Genotype	Substrate metabolism SMZ*	PABA†
Slow acetylators				
WH	M,81	M1/M1	42	22.2
AR	F,91	M1/M1	75	28.9
SA	M,85	M1/M1	174	17.0
NG	F,61	M1/M2	82	48.5
JF	M,54	M1/M2	102	11.3
JP	M,72	M1/M2	130	13.4
SP	M,51	M1/M2	44	12.0
LM	M,77	M1/M2	74	13.3
WA	M,48	M1/M2	114	33.3
RO	M,48	M1/M3	79	21.5
AG	M,63	M2/M2	128	45.5
RR	M,62	M2/M2	85	9.1
Mean ± SEM			94 ± 11	23.0 ± 3.9
Rapid acetylators				
JC	M,63	WT/WT	140	0.9
TH	F,64	WT/M1	324	39.1
JO	F,60	WT/M1	264	17.3
HK	M,71	WT/M1	107	19.7
CB	M,86	WT/M2	186	27.5
CR	F,73	WT/M2	177	53.2
BC	F,51	WT/M2	188	19.1
VM	F,77	WT/M2	109	25.4
MS	M,71	WT/M2	294	9.0
BF	F,91	WT/M2	127	34.3
KG	F,79	WT/M2	201	14.7
Mean ± SEM			192 ± 22‡	23.7 ± 4.4

* pmol *N*-acetyl SMZ/min/mg protein.
† nmol *N*-acetyl PABA/min/mg protein.
‡ $t = 4.1$, $P < 0.01$ for comparison of slow versus rapid.

resuspended in 0.1 mL 0.05% diethylpyrocarbonate and treated with DNase. The remaining RNA was re-extracted with phenol:$CHCl_3$:isoamyl alcohol, precipitated with ethanol, dried under vacuum and resuspended in 0.05% diethylpyrocarbonate solution. Purity of the RNA was assessed by the A_{260}/A_{280} ratio. A 100 ng aliquot of each RNA sample was reverse transcribed using a poly$(T)_{16}$ primer and subjected to PCR using a Gene Amp RNA PCR kit (Perkin Elmer-Cetus). NAT2 cDNA was amplified using the specific primers described above, while NAT1 cDNA was amplified using the following primers: forward primer; 5′-GGGAGGGTATGTTTACAGCA-3′, reverse primer; 5′-AAATCTATCACCATGTTTGGG-3′.

Preparation of cytosols and metabolism of SMZ and PABA. Homogenates of colon mucosa (approximately 30%) were prepared in a buffer (pH 8) containing Tris–HCl (10 mM), sucrose (0.25 mM), EDTA (0.1 mM), dithiothreitol (0.1 mM) and butylated hydroxytoluene (20 μM) using a Polytron homogenizer (2 × 25 sec) followed by a Potter–Elvehejm homogenizer (2 passes). Homogenates were centrifuged at 10,000 *g* for 15 min and the resulting supernatants were then centrifuged at 100,000 *g* for 60 min to yield a cytosol for *in vitro* assays. Protein was measured by the Biuret method and adjusted to 5–10 mg/mL. *In vitro* metabolism of SMZ (0.5 mM) and PABA (0.1 mM) was measured under linear conditions (acetylcoenzyme A, 0.5 mM) as described previously [20, 21]. *N*-Acetyl metabolites of SMZ and PABA were quantified by HPLC [20, 21].

Statistical evaluation of data. Differences between mean *in vitro* substrate metabolism values for slow and rapid acetylators were examined by use of Student's *t*-test, while values for the metabolism of SMZ and PABA in individual patients were subjected to correlation analysis.

Results and Discussion

The patients entered into the study comprised nine females and 14 males with an age range of 48–91 years. Twenty-two of the subjects were of Caucasian and one of African American (LM, Table 1) ethnic origin. As assessed from their genotype, 12 patients were classified as having the slow acetylator phenotype (3 M1/M1, 6 M1/M2, 1 M1/M3, 2 M2/M2) and 11 as having the rapid acetylator phenotype (1 WT/WT, 3 WT/M1, 7 WT/M2). In the present study, we did not test for the M1 variant allele with an A → G transition at position 803 [4]. Nevertheless, this does not impair our ability to identify the sequence which results in the slow phenotype for M1 and its variants as there is a 98% association of the M1 mutation at position 481 with the mutation at position 803 [22]. One patient had a diagnosis of severe polyposis while the remaining 22 had a diagnosis of colorectal cancer.

The metabolism of SMZ and PABA by cytosols from the rapid and slow acetylators is summarized in Table 1. The metabolism of SMZ was significantly greater in the rapid than in the slow phenotype ($t = 4.1$, $P < 0.01$), while metabolism of PABA was independent of phenotype. There was no significant correlation between the metabolism of SMZ and PABA ($r = 0.05$, $P > 0.05$). The presence of mRNAs for both NAT1 and NAT2 in the colorectal mucosa

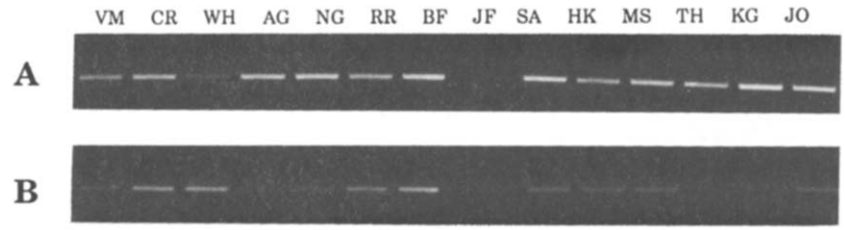

Fig. 1. PCR products for NAT1 mRNA (A) and NAT2 mRNA (B). Patient identification is the same as that in Table 1.

was examined in 14 patients, where sufficient tissue was available (Fig. 1A and B). There were five slow (three males and two females) and nine rapid (three males and six females) acetylators. NAT1 mRNA was detected in all samples, although only faintly in WH and JF. This may be due to tissue handling. For NAT2, mRNA was present in all samples except that from AG and TH. Only a very faint band was evident with patients KG and JF. From the present study, it was not possible to determine to what extent this apparent variation in NAT2 mRNA was influenced by the sample preparation. A comparison of data from Table 1 and Fig. 1 suggests little relationship between NAT activity and the ability of the NAT mRNAs to be amplified by PCR.

Previous studies have investigated the acetylation of various substrates in human colorectal cytosols. Kirlin *et al.* [15] investigated the metabolism of the NAT substrates PABA, 4-aminobiphenyl, 2-aminofluorene and β-naphthylamine using surgical samples from 25 cancer patients and 12 controls. Patients or controls were phenotyped as slow, intermediate or rapid acetylators on the basis of the *in vitro* metabolic rates for the various substrates. While the distribution of phenotypes was similar between patients and controls, no attempt was made to phenotype the subjects by traditional *in vivo* methods, or to correlate phenotype classification with the four different substrates. In addition, phenotype assignment in this study was made on the basis of probit analyses assuming a normal distribution of metabolic activities. Close examination suggests that the data are log-normally distributed and this would result in curvi-linear probit plots. Turesky *et al.* [16] measured acetyltransferase activity for *N*-hydroxy-4-aminobiphenyl, *N*-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, *N*-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, and *N*-hydroxy-2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole in colorectal cytosols from eight patients with colorectal cancer. However, unlike that seen with liver cytosols, no clear polymorphism was demonstrated with any of these substrates and *in vivo* phenotype data were not available. Due to the overlapping substrate specificities for the two NAT isozymes, assignment of phenotype on the basis of *in vitro* substrate metabolism by cytosols may be fraught with difficulties [1, 2, 13]. In the present study we assigned phenotype from a knowledge of the patient's individual genotype and correlated this description with colorectal cytosol metabolism of SMZ and PABA. For SMZ, which is metabolized primarily by NAT2 in humans [1, 13], rates of metabolism were significantly higher in rapid than in slow acetylators. On the other hand, when PABA, a highly selective substrate for NAT1 in humans [1] was used, there was no significant difference in metabolism between slow and rapid phenotypes. The lack of a correlation between the metabolism of SMZ and PABA is predictable on the basis of their isozyme preference.

NAT1 and NAT2 mRNAs were expressed in the colorectal mucosa of both slow and rapid phenotypes in humans. There was no apparent correlation between the presence of the respective mRNAs and enzyme activity. However, this is not surprising considering the qualitative nature of PCR and its variation with tissue handling. The finding that *NAT2* message is present in the colorectal mucosa and that NAT2 activity is phenotype-dependent supports our overall hypothesis on the mechanisms by which rapid acetylators are over-represented in colorectal cancer. Thus phenotype-dependent local activation of *N*-OH-arylamines or *N*-OH-heterocyclic amines delivered to the colon via biliary excretion is a viable option. However, further studies are required to demonstrate that local NAT activity is involved in the etiology of colorectal cancer in humans.

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