Rapid Communication   **Open Access**

Susceptibility of Human Lymphocytes to 2-Nitrofluorene-Induced Gene Mutation is Strongly Dependent on Metabolic Genotypes

Sai-Mei Hou[1]*, Tatiana Tvrdik[2] and Lennart Möller[1]

[1]Department of Biosciences and Nutrition, Karolinska Institutet, 14157 Huddinge, Sweden
[2]ARUP Laboratories, Salt Lake City, Utah, USA

Abstract

Genetic polymorphisms in drug metabolism contribute largely to adverse drug reactions in susceptible individuals due to excess production of reactive metabolites and active oxygen species. We conducted an *in vitro* study that clearly demonstrates that functional impact of common polymorphisms in the metabolic genes, such as N-acetyltransferase 2 (NAT2), can be easily reflected in mutant induction in the gene coding for hypoxanthine-guanine phosphoribosyl transferase (HPRT) in T-lymphocytes isolated from human peripheral blood. NAT2 is involved in the metabolic activation of 2-nitrofluorene (2-NF) to the known aromatic amide carcinogen N-acetyl-2-aminofluorene. Subsequent deactivation through glutathione conjugation involves glutathione S-transferase M1 (GSTM1). We obtained a clear dose-related increase in the HPRT mutant frequency after treating mitogen-stimulated lymphocytes isolated from a normal blood donor with 2-NF (up to 5 fold at 400 µg/mL, 24h exposure), while no HPRT mutant induction was observed using cells from another blood donor. The susceptible cells turned out to have the NAT2 rapid and GSTM1 null genotype combination (capable of activation, with insufficient deactivation), while the resistant cells had the NAT2 slow and GSTM1 positive combination. Although the contribution of GSTM1 genotype is less clear, our finding suggests that functional polymorphisms in key metabolic genes do affect induction of gene mutations at the HPRT locus and at least the NAT2 genotype plays a critical role in determining the susceptibility of human cells to genotoxicity of 2-NF. Further, human peripheral T-lymphocytes and the *in vitro* HPRT gene mutation assay can be utilized to study the functional impact of common genetic polymorphisms in drug metabolism and to identify risk genotypes susceptible for drug toxicity and somatic mutations.

Keywords: Genetic polymorphism; Adverse effects; Human lymphocytes; Gene mutation; 2-nitrofluorene; N-acetyltransferase 2; Glutathione S-transferase M1

Abbreviations: HPRT: hypoxanthine-guanine phosphoribosyl transferase; 2-NF: 2-nitrofluorene; AAF: N-acetyl-2-aminofluorene; NAT2: N-acetyltransferase 2; GSTM1: glutathione S-transferase M1

Introduction

Genetic polymorphism in drug metabolism is one of the most important reasons for inter-individual variability in responses to drug therapy [1]. Two examples of human enzyme families known to exhibit marked genetic polymorphisms are N-acetyltransferases (NATs) and glutathione S-transferases (GSTs) [2]. Acetylation is a major route of biotransformation for many aromatic amines, heterocyclic amines and hydrazine compounds. Nucleotide polymorphisms in the *NAT2* gene are highly responsible for the classical isoniazid acetylation polymorphism in humans. The frequency of the slow acetylator phenotype varies considerably among ethnic groups, ranging from 10 to 30 % in Asian populations, and from 40 to 70 % in Caucasian and African populations. GSTs are intracellular free radical scavengers conjugating reactive oxygen intermediates. At least 50 % of most Caucasian populations completely lack GST mu activity due to a homozygous deletion of the gene (*GSTM1* null genotype).

The N-acetylation polymorphism significantly affects the frequency and severity of side-effects associated with many therapeutic agents due to altered balance between metabolic activation and detoxification [3]. Sulphonamides, such as the antimicrobial sulphamethoxazole, undergo hydroxylation to reactive *N*-hydroxy metabolites that covalently bind to macromolecules and give rise to idiosyncratic adverse reactions. The formation of toxic metabolites is competitively inhibited by the alternative metabolic route, i.e. acetylation by NAT2 to non-reactive *N*-acetyl metabolites. Slow acetylators, with a higher proportion of the drug being *N*-hydroxylated, are therefore at a higher risk of sulphonamide-induced toxicity [4]. Similarly, the toxicity associated with the anti-inflammatory dapsone (diamino-diphenylsulfone) therapy occurs virtually only in slow acetylators [5].

For isoniazid, acetylation precedes hydrolyzation in the liver in the formation of acetylhydrazine, but also produces the nontoxic diacetylhydrazine [6]. Without the further acetylation, acetylhydrazine is oxidized by cytochrome P450 (2E1) to produce intermediates that are hepatotoxic unless detoxified by phase II enzymes such as GSTM1. Among the metabolic genes (CYP2E1, NAT2, GSTM1, GSTT1) studied in Brazilian tuberculosis patients [7], slow acetylation status was the only independent risk factor for the occurrence of drug-induced hepatitis during treatment with isoniazid-containing schemes.

While slow acetylators are at greater risk of adverse reactions for many drugs, the opposite is true for other drugs including amonafide, an arylamine anticancer agent with DNA-intercalating and topoisomerase II-inhibiting properties. Amonafide undergoes

N-acetylation to an active metabolite that contributes to systemic toxicity including myelosuppression [8]. The clinical development of amonafide has been hampered by the highly variable toxicity that requires tailored dosing algorithms.

In vitro genotoxicity studies with human cells offer an experimental tool that can be used to study the functional impact of common polymorphisms in metabolism and repair genes, identify risk genotypes and predict individual sensitivity. One possible approach is to study the dose response relationship in the HPRT gene mutation assay utilizing human peripheral lymphocytes. The assay detects mutations in the gene for hypoxanthine-guanine phosphoribosyl transferase (HPRT), and is the best developed reporter system for studying specific locus mutation in human somatic cells in vivo and in vitro. The assay utilizes mitogen- and growth factor-dependent expansion of 6-thioguanine resistant HPRT mutant T-cell clones. Considerable variation in the in vivo background HPRT mutant frequency could be explained by age, smoking, dietary habits and common polymorphisms in metabolic genes [9-11].

To illustrate the feasibility of using human peripheral lymphocytes and the in vitro HPRT gene mutation assay to identify risk genotypes susceptible for drug toxicity and somatic mutations, we report here a study with 2-nitrofluorene (2-NF), a model compound for nitrated polycyclic aromatic hydrocarbons that requires NAT2 for metabolic activation to the known aromatic amide carcinogen N-acetyl-2-aminofluorene (AAF).

Materials and Methods

Mutant induction

The culture media and the method of studying HPRT mutant induction in human lymphocytes in vitro using the T-cell cloning assay have been described previously in detail [9,12].

Lymphocytes were purified from the buffy coats of two healthy blood donors by Ficoll-Paque density separation in UNI-SEP tubes. The two batches of cells were incubated separately at 1.5×10^6 cells/ml in nutrient medium supplemented with 0.3 % phytohemagglutinin (PHA, Difco, USA) for 23 hours.

To remove pre-existing in vivo HPRT mutants, the cells were treated with HAT (hypoxanthine, aminopterin, thymidine) supplement (Gibco BRL™) for 24 hours, washed with phosphate-buffered saline (PBS) and suspended to a cell density of 1.5×10^6/ml in a growth medium that contained 0.3 % PHA and 20 % T-cell growth factor enriched conditioned medium [9].

The cells were exposed to 2-NF (400 µg/mL, Sigma-Aldrich) for 24 hours at 37°C with 5% CO_2. After washing with PBS, the cells were suspended in growth medium to a cell density of 1.5×10^6/ml and allowed for six days mutant expression in growth medium.

Finally, the cells were subjected to mutant selection on ten 96-well plates. Each microwell received 2×10^4 target cells and 1×10^4 lethally irradiated feeder cells in growth medium supplemented with 6-thioguanine (2 µg/ml, Sigma-Aldrich). For estimation of the cloning efficiency, two microplates were made with two target cells and 2×10^4 irradiated feeder cells per well without 6-thioguanine.

All plates were scored after 14 days. Cloning efficiency was calculated from the proportion of negative wells assuming a Poisson distribution. Mutant frequency was obtained by dividing the cloning efficiency in the presence of 6-thioguanine with that in the absence of 6-thioguanine.

Genotyping

The principles and details of the methods for GSTM1 and NAT2 genotyping have been described previously [13,14]. The presence or absence of the GSTM1 gene was detected by genomic PCR amplification of a short internal GSTM1 gene segment (177 bp) together with a NAT2 segment (284 bp) as an internal PCR control. Individuals with one or two copies of the GSTM1 allele were designated GSTM1 positive. Individuals with homozygous deletion of the GSTM1 allele were designated GSTM1 null.

Identification of the slow NAT2 alleles was performed by restriction analysis of a 578-bp genomic PCR product covering a large part of the intron less NAT2 coding region. The predominating NAT2*5A/B (T341C, C481T) and NAT2*6A/B (G590A) alleles were identified by loss of a restriction site for KpnI and TaqI, respectively. Individuals in whom none, or only one, slow allele could be identified by the initial restriction analysis were further analyzed by BamHI and DdeI digestions for the identification of the NAT2*7A/B (G857A) and NAT2*5C (T341C, A803G) alleles, respectively. Individuals with at least one wild-type allele were classified as rapid, and those with two slow alleles as slow.

Results and Discussion

As shown in (Table 1), 2-NF produced a clear dose-related increase in the HPRT mutant frequency in peripheral lymphocytes isolated from one of the blood donors. The increase was 5-fold at the highest evaluated concentration of 400 µg/mL 2-NF. Due to excess cytotoxicity, the top concentration 500 µg/mL was not subjected to mutant selection.

In contrast, no such mutant induction was seen when using cells from the other donor. The lowest concentration was excluded from determination of mutant frequency due to lack of cytotoxicity.

The two donors were subsequently genotyped with regard to NAT2 and GSTM1. The susceptible cells turned out to have the genotype

TREATMENT	MUTANT FREQUENCY (x 10^{-6})	
2-nitrofluorene (µg/mL)	NAT2(S)/GSTM1(+)	NAT2(R)/GSTM1(-)
0	3.48	6.39
100	n.d.	12.6
200	3.95	26.8
400	4.95	30.5
500	1.98	n.d.

NAT2(S)/GSTM1(+):N-acetyltransferase 2 slow and glutathione S-transferase M1 positive

NAT2(R)/GSTM1(-):N-acetyltransferase 2 rapid and glutathione S-transferase M1 negative

n.d. = not determined due to low or excess cytotoxicity

Table 1: HPRT mutant frequency induced by various concentrations of 2-nitrofluorene in human peripheral lymphocytes with two different genotype combinations.

combination of NAT2 rapid and GSTM1 null (capable of activation, with insufficient detoxification), while the resistant cells had the NAT2 slow and GSTM1 positive genotype combination (incapable of activation).

To our knowledge, this is the only study where gene mutation, rather than cytogenetic endpoints, is used to assess the impact of human genetic polymorphism in drug metabolism on *in vitro* genotoxicity. It's not surprising that 2-NF is found to be an efficient inducer of mutations in the HPRT gene in susceptible cells. The HPRT gene mutation assay is highly sensitive to agents interfering with DNA replication, such as acetaldehyde [12]. AAF is a potent frameshift mutagen. It reacts with guanines at the C8 position in DNA to form a structure that interferes with DNA replication. In Escherichia coli, the NarI restriction enzyme recognition sequence (G1G2CG3CC) is a very strong mutational hot spot when an AAF adduct is positioned at G3 of this sequence, causing predominantly a GC dinucleotide deletion [15]. 2-NF was also able to induce micronuclei [16] and HPRT gene mutations [17] in Chinese hamster V79 lung fibroblasts without an exogenous activating system although the responses were rather weak.

This also appears to be the only study where human primary cells are used to study the mutagenic effect of 2-NF. The clear dose-response obtained in cells with the NAT2 rapid and GSTM1 null genotypes indicates that human lymphocytes normally possess sufficient enzyme activities to convert 2-NF to AAF and to its ultimate DNA-binding metabolite. This is supported by the facts that human hepatoma cell lines can perform nitroreduction as well as ring-hydroxylation of 1-nitropyrene [18] and that human lymphocytes are able to metabolize AAF to ring- and N-hydroxy derivatives of AAF [19]. 2-NF is reduced to 2-aminofluorene by nitroreduction, followed by acetylation to AAF. The subsequent N-hydroxylation is the initial step in the metabolic activation of AAF. Further metabolism results in deactivation through glutathione conjugation [20].

The clear difference in susceptibility to HPRT mutant induction between the blood donors used in our study confirms the crucial role of N-acetylation on the metabolic activation of 2-NF to AAF. The ability of 2-NF and N-hydroxylated AAF to revert the *lac*Z mutation in *Escherichia coli* by -2 frameshift was strongly potentiated in a strain expressing high levels of acetylation activity [21]. Likewise, Chinese hamster CHL cells stably expressing human NAT2 exhibited the highest sensitivity to the clastogenicity of 2-NF as demonstrated by micronuclei induction [22].

Although the number of donors is limited in the present study and the contribution of GSTM1 genotype is less clear, a polymorphic (bimodal) distribution of glutathione-conjugating activity corresponding to those noted for hepatic GST mu has been demonstrated in freshly isolated human lymphocytes, with 2- to 4-fold increase in activity in interleukin-2-dependent T-cell cultures [23]. Further, previous studies using cytogenetic endpoints as markers of susceptibility have shown a significant contribution of GSTM1 genotype to *in vitro* genotoxicity in human lymphocytes. The induction of sister chromatid exchanges by epoxide metabolites of 1,3-butadiene in 72-hour human whole blood lymphocyte cultures was clearly dependent on GSTM1/T1 genotypes [24]. In a study of human lymphocyte cultures with different genotypes of GSTM1, GSTT1, and GSTP1, hydroquinone was a clear inducer of both sister chromatid exchanges and micronuclei, but only the GSTM1

null genotype significantly enhanced the genotoxic response, and only in terms of micronuclei [25]. Finally, the low sensitivity of V79 cells towards 2-NF might be related to the high GST activity detected in these cells, the level being above that of freshly isolated hepatocytes [16].

Our finding suggests that functional polymorphisms in key metabolic genes do affect induction of gene mutations at the HPRT locus and at least the NAT2 genotype plays a critical role in determining the susceptibility of human cells to genotoxicity of 2-NF. It also implies that propagating human peripheral T-lymphocytes may have sufficient capability of drug metabolism if possessing adequate metabolic genotypes. The assay system may thus provide a unique tool for studying the functional impact of common genetic polymorphisms in drug metabolism, perhaps also in DNA repair, and for identifying risk genotypes/individuals susceptible for drug toxicity and somatic mutations.

References

1. Ingelman Sundberg M (2001) Pharmacogenetics: an opportunity for a safer and more efficient pharmacotherapy. J Intern Med 250: 186-200.

2. Meyer UA, Zanger UM (1997) Molecular mechanisms of genetic polymorphisms of drug metabolism. Annu Rev Pharmacol Toxicol 37: 269-296.

3. Butcher NJ, Boukouvala S, Sim E, Minchin RF (2002) Pharmacogenetics of the arylamine N-acetyltransferases. Pharmacogenomics J 2: 30-42.

4. Spielberg SP (1996) N-acetyltransferases: pharmacogenetics and clinical consequences of polymorphic drug metabolism. J Pharmacokinet Biopharm 24: 509-519.

5. Bluhm RE, Adedoyin A, McCarver DG, Branch RA (1999) Development of dapsone toxicity in patients with inflammatory dermatoses: activity of acetylation and hydroxylation of dapsone as risk factors. Clin Pharmacol Ther 65: 598-605.

6. Metushi IG, Cai P, Zhu X, Nakagawa T, Uetrecht JP (2011) A fresh look at the mechanism of isoniazid-induced hepatotoxicity. Clin Pharmacol Ther 89: 911-914.

7. Teixeira RL, Morato RG, Cabello PH, Muniz LM, Moreira Ada S, et al. (2011) Genetic polymorphisms of NAT2, CYP2E1 and GST enzymes and the occurrence of antituberculosis drug-induced hepatitis in Brazilian TB patients. Mem Inst Oswaldo Cruz 106: 716-724

8. Innocenti F, Iyer L, Ratain MJ (2001) Pharmacogenetics of anticancer agents: lessons from amonafide and irinotecan. Drug Metab Dispos 29: 596-600.

9. Hou SM, Falt S, Steen AM (1995) Hprt mutant frequency and GSTM1 genotype in non-smoking healthy individuals. Environ Mol Mutagen 25: 97-105.

10. Hou SM, Falt S, Yang K, Nyberg F, Pershagen G, et al.(2001) Differential interactions between GSTM1 and NAT2 genotypes on aromatic DNA adduct level and HPRT mutant frequency in lung cancer patients and population controls. Cancer Epidemiol Biomarkers Prev 10: 133-140.

11. Nyberg F, Hou SM, Pershagen G, Lambert B (2003) Dietary fruit and vegetables protect against somatic mutation in vivo, but low or high intake of carotenoids does not. Carcinogenesis 24: 689-696.

12. Noori P, Hou SM (2001) Mutational spectrum induced by acetaldehyde in the HPRT gene of human T lymphocytes resembles that in the p53 gene of esophageal cancers. Carcinogenesis 22: 1825-1830.

13. Hou SM, Lambert B, Hemminki K (1995) Relationship between hprt mutant frequency, aromatic DNA adducts and genotypes for GSTM1 and NAT2 in bus maintenance workers. Carcinogenesis 16: 1913-1917.

14. Nyberg F, Hou SM, Hemminki K, Lambert B, Pershagen G (1998) Glutathione S-transferase mu1 and N-acetyltransferase 2 genetic polymorphisms and exposure to tobacco smoke in nonsmoking and smoking lung cancer patients and population controls. Cancer Epidemiol Biomarkers Prev 7: 875-883.

15. Gill JP, Romano LJ (2005) Mechanism for N-acetyl-2-aminofluorene-induced

frameshift mutagenesis by Escherichia coli DNA polymerase I (Klenow fragment). Biochemistry 44: 15387-15395.

16. Glatt H, Gemperlein I, Setiabudi F, Platt KL, Oesch F (1990) Expression of xenobiotic-metabolizing enzymes in propagatable cell cultures and induction of micronuclei by 13 compounds. Mutagenesis 5: 241-249.

17. Kappers WA, van Och FM, de Groene EM, Horbach GJ (2000) Comparison of three different in vitro mutation assays used for the investigation of cytochrome P450-mediated mutagenicity of nitro-polycyclic aromatic hydrocarbons. Mutat Res 466: 143-159.

18. Eddy EP, Howard PC, McCoy GD, Rosenkranz HS (1987) Mutagenicity, unscheduled DNA synthesis, and metabolism of 1-nitropyrene in the human hepatoma cell line HepG2. Cancer Res 47: 3163-3168.

19. McManus ME, Trainor KJ, Morley AA, Burgess W, Stupans I, et al. (1987) Metabolism of 2-acetylaminofluorene in cultured human lymphocytes. Res Commun Chem Pathol Pharmacol 55: 409-418.

20. Moller L (1994) In vivo metabolism and genotoxic effects of nitrated polycyclic aromatic hydrocarbons. Environ Health Perspect 102: 139-146.

21. Hoffmann GR, Janel Bintz R, Fuchs RP (2001) Induction of -2 frameshift mutations by 2-nitrofluorene, N-hydroxyacetylaminofluorene, and N-2-acetylaminofluorene in reversion assays in Escherichia coli strains differing in permeability and acetyltransferase activity. Mutat Res 493: 127-137.

22. Watanabe M, Matsuoka A, Yamazaki N, Hayashi M, Deguchi T, et al. (1994) New sublines of Chinese hamster CHL stably expressing human NAT1 or NAT2 N-acetyltransferases or Salmonella typhimurium O-acetyltransferase: comparison of the sensitivities to nitroarenes and aromatic amines using the in vitro micronucleus test. Cancer Res 54: 1672-1677.

23. Jones SM, Brooks BA, Langley SC, Idle JR, Hirom PC (1988) Glutathione transferase activities of cultured human lymphocytes. Carcinogenesis 9: 395-398.

24. Norppa H (2003) Genetic susceptibility, biomarker respones, and cancer. Mutat Res 544: 339-348.

25. Silva Mdo C, Gaspar J, Duarte Silva I, Faber A, Rueff J (2004) GSTM1, GSTT1, and GSTP1 genotypes and the genotoxicity of hydroquinone in human lymphocytes. Environ Mol Mutagen 43: 258-264.