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A comparison of the genotoxicity of benzo[*a*]pyrene in four cell lines with differing metabolic capacity

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ABSTRACT

Benzo[*a*]pyrene (B[*a*]P) is a known genotoxin and carcinogen, yet its genotoxic response at low level exposure has not been determined. This study was conducted to examine the interplay of dose and metabolic capacity on genotoxicity of B[*a*]P. Investigating and better understanding the biological significance of low level chemical exposures will help improve human health risk assessments. The genotoxic and mutagenic effects of B[*a*]P were investigated using human cell lines (AHH-1, MCL-5, TK6 and HepG2) with differential expression of the CYP450 enzymes CYP1A1, 1B1 and1A2 involved in B[*a*]P metabolism. MCL-5 and HepG2 cells showed detectable basal expression and activity of CYP1A1, 1B1 and 1A2 than AHH-1 which only show CYP1A1 basal expression and activity. TK6 cells showed negligible expression levels of all three CYP450 enzymes. *In vitro* micronucleus and HPRT assays were conducted to determine the effect of B[*a*]P on chromosome damage and point mutation induction. After 24 h exposure, linear increases in micronucleus (MN) frequency were observed in all cell lines except TK6. After 4 h exposure, only the metabolically competent cell lines MCL-5 and HepG2 showed MN induction (with a threshold concentration at 25.5 μM from MCL-5 cells) indicating the importance of exposure time for genotoxicity. The HPRT assay also displayed linear increases in mutant frequency in MCL-5 cells, after 4 h and 24 h treatments. Mutation spectra analysis of MCL-5 and AHH-1 HPRT mutants revealed frequent B[*a*]P induced G to T transversion mutations (72% and 44% of induced mutations in MCL-5 and AHH-1 respectively). This study therefore demonstrates a key link between metabolic capability, B[*a*]P exposure time and genotoxicity.

1. Introduction

Genotoxins are chemicals that induce DNA mutations that can lead to cancer. Mutation in genes essential to perform normal regulatory processes like controlled cell signalling, cell cycle checkpoints and apoptosis, can lead to cancer development [1]. Some genotoxic chemicals, when released into the environment, can remain intact for long periods of time and cause adverse effects. It is important to assess the genotoxic potential of such chemicals for a better understanding of their carcinogenicity in order to reduce the potential risk of human cancer and other diseases involving acquired mutations in the somatic cells.

The link between environmental chemical exposure and human disease was first recorded in 1775 with the discovery of chimney sweeper's cancer (soot wart), a squamous cell carcinoma of the skin of the scrotum, which originated from the components of soot [2]. The first report of the induction of squamous cell papilloma on rabbit ears after repeated exposure of coal tar, was published in 1918 [3]. Kennaway, in 1930, found that tumours in mouse skin could be produced by pure 1, 2, 5, 6 dibenzanthracene [4]. Later on in 1932, Cook and colleagues successfully isolated polycyclic aromatic hydrocarbon, benzo[*a*]pyrene (PAH, B[*a*]P) from coal tar, a highly carcinogenic crystalline compound [5].

B[*a*]P belongs to a family of chemicals that can act like dioxins, based on a common mechanism of toxic action. Like dioxin compounds, PAHs have biologic responses mediated via binding to the aryl hydrocarbon receptor (AhR) which is a specific high-affinity cellular reporter protein [6] despite PAHs being quite different structurally from dioxins. Due to their potential risk to humans and animals, the United States Environmental Protection Agency (US-EPA) has designated 126 PAHs as priorities for environmental concern and B[*a*]P is one which is frequently monitored in the

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environment. B[*a*]P is generated through the burning of fossils fuels or wood, vehicle exhaust emission, heat and power generation, industrial processes or oil contamination and is always found environmentally as a mixture with other PAHs as in cigarette smoke, charcoal-cooked food and industrial waste by-products [7,8].

Human environmental exposure to B[*a*]P mainly occurs through cigarette smoking and the ingestion of contaminated food and water [7], with significant concentrations found in Western diet such as in fried and grilled meats. Both environmental and dietary factors are associated with an increased risk of different organ cancers [9], autoimmune [10] and inflammatory diseases [11]. The daily intake of B[*a*]P in the average human diet has been estimated to range from 120 to 2800 ng/day [12] and concentration of B[*a*]P in full flavoured cigarettes has also been reported to be about 10 ng per cigarette [13,14], which is equivalent to an intake of about 200 ng/day for a pack-a-day cigarette smoker [15]. B[*a*]P is transported across the cell membrane by lipoproteins [16] and B[*a*]P's presence activates the Aryl hydrocarbon receptor (AhR), which in turn binds with AhR nuclear translocator (ARNT) and induces the expression of genes involved in B[*a*]P metabolism as well as other genes that are not related to B[*a*]P metabolism. Several other genes such as the cytochrome P450 genes *CYP1A1*, *CYP1A2*, and *CYP1B1*, and glutathione-S-transferase (*GST*) and UDP-glucuronosyltransferase (*UGT-1*) are also induced via AhR-mediated pathways. Indeed, the relative expression of phase I and phase II enzymes determines the ratio of activation and detoxification of B[*a*]P. B[*a*]P is often first oxidized at the bay region by phase I *CYP1A1* into B[*a*]P-7,8-epoxide, which through hydration by microsomal epoxide hydrolase (*mEH*) is metabolized to B[*a*]P-7,8-dihydrodiol (BPD). BPD serves as a substrate for a second CYP dependent oxidation reaction and generates the metabolite B[*a*]P-7,8-dihydrodiol-9,10-epoxide (BPDE) [17]. BPDE contains an epoxide ring within the bay region making it highly reactive with DNA in a time dependent manner.

The genotoxicity of most direct acting genotoxins is dependent on the dose and length of exposure whereby genotoxic effects are observed at or above a critical dose. These doses are referred to as threshold doses, below which no observable genotoxic effect is seen. Nowadays a threshold level is commonly used to determine or establish a safe limit of a chemical for human use but only when their mechanism of action is established and accepted by risk assessment agencies. This threshold limit is derived from a combination of dose-dependent responses, mathematical and statistical models, to cover any variability, and risk assessment procedures. Typically a threshold level is a value between a no observable effect level (NOEL) and lowest observable effect level (LOEL) [18] and can be confirmed by mathematical modelling [19]. The nature of the chemical, its interaction with the non-DNA targets, and cell protective mechanisms (detoxification, DNA repair, etc.) are the mechanistic factors that influence dose-dependent responses [19]. Therefore, it is important to consider these factors while assessing if thresholds exist for any genotoxins. These considerations become even more complex if the genotoxin involved is a pro-carcinogen that requires metabolic activation to initiate genotoxicity. As compared to direct acting agents, little is known about the threshold doses of pro-carcinogenic agents and the critical factors controlling dose response relationships for genotoxicity.

Considering this, the aim of this study was to identify the dose responses at the low dose range of B[*a*]P and determine whether there are differential genotoxicity and mutagenicity of B[*a*]P in different human cell lines based on their metabolic capacity of phase I enzymes. The cell lines used in the present study were human lymphoblastoid cell lines AHH-1, MCL-5, TK6 and primary hepatoblastoma cell line HepG2, known to have differential expression of phase I CYP450 enzymes. AHH-1 cell line only expresses CYP1A1, MCL-5 cells express all five CYP450 enzymes (detailed information for each cell line in Material and Method section). TK6 cells were used as a control as they are devoid of CYP450 enzymes. Therefore, all of these cell lines serve as a good tool to investigate the role of P450 enzymes in assessing the genotoxicity of chemicals that require metabolic activation. The stable expression system also furthers understanding of the process from metabolic activation of test compounds to the appearance of toxicological consequences entirely in the same intact cells. The study utilised the *in vitro* cytokinesis blocked micronucleus (CBMN) assay and hypoxanthine (guanine) phosphoribosyltransferase (H(G)PRT) assay in human cell lines to examine the induction of chromosomal damage and the frequency and spectra of point mutations, respectively. CYP450 enzymes levels and activity were also investigated in parallel through gene expression analysis and reporter assays.

2. Materials and methods

2.1. Reagents

HAT supplement (2×10^{-4} M hypoxanthine, 8×10^{-7} M aminopterin, and 3.5×10^{-5} M thymidine), HT supplement (2×10^{-4} M hypoxanthine and 3.5×10^{-5} M thymidine) were purchased from Invitrogen (Paisley, UK), 6-thioguanine, B[*a*]P, trypsin, hygromycin B, dimethyl sulfoxide (DMSO) and phosphate buffered saline (PBS) were purchased from Sigma (Gillingham, UK). N-Cyclohexyl-*N*-dodecylurea (NCND) was purchased from Calbiochem (EMD Chemicals Inc. US). Cytochalasin B was from Merck (Darmstadt, Germany). All powdered reagents were reconstituted according to manufacturer's instructions. All chemical dilutions were freshly prepared from stock solutions and for each experiment new stock was prepared.

2.2. Cell culture

The present study utilised human male lymphoblastoid cell lines AHH-1, MCL-5 (ATCC, Middlesex, UK), TK6 and human hepatoma cell line HepG2 (ECACC, Salisbury, UK). The cell cycle doubling time for TK6 was 18 h, for AHH-1 and MCL-5 22–24 h, and 26–28 h for HepG2. TK6 was isolated from WIL2, heterozygous at thymidine kinase (TK) locus and has a stable and wild-type *p53* gene but minimal or no expression of CYP450 enzymes.

AHH-1 is a TK+/− with native CYP1A1 activity which was used for oxidative xenobiotic metabolism and was devoid of microsomal epoxide hydrolase [20]. It has +/− *p53* gene.

MCL-5 cell line was a derivative of AHH-1 TK+/− that has been transfected with 4 cDNAs of the human CYP450 s (carried on plasmids). These cDNAs include *CYPA12, CYP2A6, CYP3A4* and *CYP2E1* and microsomal epoxide hydrolase (*mEH*). MCL-5 has been shown to be more sensitive than AHH-1 TK+/− to the mutagenic properties of many pro-mutagens and pro-carcinogens and direct acting agents [21]. The plasmids were maintained in these cells through hygromycin (B) resistant genes. Both AHH-1 and MCL-5 harbour a heterozygous transition (C > T) p53 mutation at the codon 281/282 interface within exon 8, it retains the ability to undergo DNA damage–induced apoptosis and has been reported to express *p53* which can be phosphorylated [22].

HepG2 cells were isolated by Aden et al. [23] from a primary hepatoblastome of an 11-year-old Argentine boy [23]. HepG2 have epithelial like morphology which resembles liver parenchymal cells and retain many of the specialized functions in culture such as secretion of major plasma proteins [24]. This cell line is a useful model of human liver cells and expresses many functional phase I and phase II xenobiotic metabolic enzymes [25].

All media used were purchased from GIBCO® (Life technologies, Paisley, UK) unless otherwise stated.