Toxicology and Applied Pharmacology 273 (2013) 269–280

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Toxicogenomic outcomes predictive of forestomach carcinogenesis following exposure to benzo(*a*)pyrene: Relevance to human cancer risk☆

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ARTICLE INFO

Article history:
Received 3 April 2013
Revised 17 May 2013
Accepted 21 May 2013
Available online 2 June 2013

Keywords:
Polycyclic aromatic hydrocarbons
Cancer
Microarrays
Human relevance
Antigen processing and presentation
Immunoproteasome

ABSTRACT

Forestomach tumors are observed in mice exposed to environmental carcinogens. However, the relevance of this data to humans is controversial because humans lack a forestomach. We hypothesize that an understanding of early molecular changes after exposure to a carcinogen in the forestomach will provide mode-of-action information to evaluate the applicability of forestomach cancers to human cancer risk assessment. In the present study we exposed mice to benzo(*a*)pyrene (BaP), an environmental carcinogen commonly associated with tumors of the rodent forestomach. Toxicogenomic tools were used to profile gene expression response in the forestomach. Adult Muta™Mouse males were orally exposed to 25, 50, and 75 mg BaP/kg-body-weight/day for 28 consecutive days. The forestomach was collected three days post-exposure. DNA microarrays, real-time RT-qPCR arrays, and protein analyses were employed to characterize responses in the forestomach. Microarray results showed altered expression of 414 genes across all treatment groups (± 1.5 fold; false discovery rate adjusted $P \leq 0.05$). Significant downregulation of genes associated with phase II xenobiotic metabolism and increased expression of genes implicated in antigen processing and presentation, immune response, chemotaxis, and keratinocyte differentiation were observed in treated groups in a dose-dependent manner. A systematic comparison of the differentially expressed genes in the forestomach from the present study to differentially expressed genes identified in human diseases including human gastrointestinal tract cancers using the NextBio Human Disease Atlas showed significant commonalities between the two models. Our results provide molecular evidence supporting the use of the mouse forestomach model to evaluate chemically-induced gastrointestinal carcinogenesis in humans.

Introduction

A review of the Carcinogenic Potency Database (http://toxnet.nlm.nih.gov/cpdb/) and the US National Cancer Institute/National Toxicology Program (http://ntp-server.niehs.nih.gov) databases reveals that exposure to approximately 120 different substances results in the development of cancerous lesions in the rodent forestomach (Proctor et al., 2007). Indeed, the forestomach is a target for carcinogenesis in rodents following oral exposure to various environmental chemicals. However, the human health relevance of this type of cancer is unclear because humans lack a forestomach (Proctor et al., 2007).

Anatomically, the rodent forestomach exhibits similarities to both the human esophagus and stomach. Detailed analyses of cancers of the forestomach reveal that these tumors are initiated by hyperplasia of the forestomach squamous epithelial cells that form preneoplastic lesions (Fukushima et al., 1997). These lesions progress into benign papillomas and metastatic carcinomas over time, which histologically resemble squamous cell carcinomas of the human esophagus (Nyrén and Adami, 2002), stomach (Callery et al., 1985), colon (Landau et al., 2007), and anal canal (Szmulowicz and Wu, 2012). However, forestomach tumors are strictly of squamous cell origin, whereas esophageal cancers can also originate from glandular columnar epithelial cells in the form of adenocarcinomas, suggesting multiple mechanisms in the development of esophageal tumors in humans (Proctor et al., 2007). In addition, more than 95% of human stomach cancers are adenocarcinomas that originate from glandular columnar epithelial cells or poorly differentiated cells (Tsukamoto et al., 2007), and are thus distinct from rodent forestomach tumors (Proctor et al., 2007). Although there appear to be some fundamental differences between the aforementioned tumor types, it seems reasonable to contend that the molecular initiating

Abbreviations: AhR, aryl hydrocarbon receptor; BaP, benzo(*a*)pyrene; BPDE, benzo(*a*)pyrene-7,8-dihydrodiol-9,10-epoxide; BW, body weight; FDR, false discovery rate; GO, Gene Ontology; GSH, glutathione; MHC, major histocompatibility complex; NKT, natural killer T cells; PAH, polycyclic aromatic hydrocarbon; RT-qPCR, quantitative real time PCR.

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0041-008X/$ – see front matter.
http://dx.doi.org/10.1016/j.taap.2013.05.027

events leading to cancers in the rodent forestomach and the human stomach share commonalities. A detailed and systematic characterization of the carcinogenic modes of action in the rodent forestomach will provide an improved context for evaluating the biological relevance of rodent forestomach tumor data for human health risk assessment.

Benzo(*a*)pyrene (BaP), a known human carcinogen (IARC, 2012), is a polycyclic aromatic hydrocarbon (PAH) that is produced during the incomplete combustion of organic materials from various sources including wood and tobacco smoke, vehicle exhaust, residential heating, electric power, and cooking. Human exposure to BaP occurs primarily through oral consumption of BaP-containing foods (Hettemer-Frey and Travis, 1991). BaP requires metabolic activation by members of the cytochrome P450 family of enzymes, which can generate several DNA-reactive metabolites including BaP-7,8-dihydrodiol-9,10-epoxide (BPDE). These metabolites are capable of forming covalent adducts with proteins and DNA. If left unrepaired, DNA damage can cause mutations leading to impaired gene function. We and others have demonstrated the formation of DNA adducts following exposure to BaP in a variety of tissues, including the gastrointestinal tract (Lemieux et al., 2011), which is a primary site of contact following oral gavage, and also in distant organs, such as the lungs and liver (Halappanavar et al., 2011; Labib et al., 2012; Malik et al., 2012). In experimental animals, exposure to BaP *via* feed or gavage predominantly and consistently leads to the development of squamous cell papillomas in the forestomach that primarily originate from the epithelial cell lining (Culp et al., 1998; Wester et al., 2012). Although genotoxicity is the primary mode of action of BaP in virtually all tissues, we previously used toxicogenomics to demonstrate that the nature and extent of molecular responses at the gene and protein level are somewhat different between tissues (Halappanavar et al., 2011; Labib et al., 2012; Malik et al., 2012; Yauk et al., 2011). For example, a comparison of gene expression profiles in mouse lung and liver following acute BaP exposure (*i.e.* 150 or 300 mg/kg-body-weight/day by oral gavage for 3 days) revealed broad commonalities, including the activation of pathways involved in oxidative stress, xenobiotic metabolism, AhR signaling, and the DNA damage response (Halappanavar et al., 2011). However, significant inhibition of B-cell receptor signaling was a predominant perturbation uniquely found in the lungs of BaP-exposed mice (Halappanavar et al., 2011). In addition, the magnitude of the overall transcriptional response (fold changes and the total number of genes) is much greater in the lungs than in the liver. We also recently demonstrated that DNA adduct levels are higher in the lungs of BaP treated mice as compared with the liver, but that mutation frequencies in these two tissues are quite similar (Labib et al., 2012). In alignment with our findings, Uno et al. (2004) showed tissue-specific roles of *Cyp1a1* using *Cyp1a1* knockout mice. These authors showed that *Cyp1a1* expression in the intestine plays an important role in detoxification, whereas *Cyp1a1* activation in the liver is associated with DNA damage-induced liver carcinogenesis (Uno et al., 2004). These studies collectively demonstrate that although genotoxicity is a primary component of the mode of action of BaP-mediated carcinogenesis, differences in the underlying molecular responses to BaP likely contribute to the observed tissue-specificity during tumorigenesis.

The present study employed genomic and bioinformatic tools to identify early molecular initiating events that contribute to tumorigenesis in the forestomach, with the overarching objective of exploring the biological relevance of forestomach tumors for human health risk assessment. More specifically, we employed global gene expression profiling of forestomach tissue from mice exposed to BaP at doses that are known to induce tumors in the mouse forestomach (Culp et al., 1998). Adult male Muta™Mouse were exposed to 25, 50, and 75 mg/kg-bw/day of BaP for 28 consecutive days *via* oral gavage, and the top dose has previously been shown to cause squamous cell carcinomas, papillomas, and hyperplasia in the forestomach (Hakura et al., 1998). Mice were sacrificed 3 days after the last exposure. Global transcription response was analyzed in detail in order to identify the expression changes in biological pathways associated with cancer formation in the forestomach. BaP-induced gene expression profiles were compared with transcriptomic profiles of human diseases (*e.g.*, human gastrointestinal tract cancer) using the NextBio Human Disease Atlas.

Methods

Animal treatment. Mouse exposures and sample collection procedures are described in detail previously (Labib et al., 2012; Lemieux et al., 2011; Malik et al., 2012). The Muta™Mouse contains around 29 +/− 4 copies of λgt10*lac*Z shuttle vector, a non-transcribed insert, stably integrated in the mouse genome (Shwed et al., 2010), thus permitting *in vivo lacZ* mutant frequency analysis. The transgenic insert, which contains *lacZ*, is employed as an *in vivo* mutation target. Briefly, 25-week old male Muta™Mouse were individually housed in plastic film isolators, provided with water and food (2012 Teklad Global standard rodent diet) *ad libitum*, and were subjected to a 12 h light/12 h dark cycle. Mice were divided into four experimental groups consisting of 5 animals each: 0 (control), 25 (low dose), 50 (medium dose) and 75 (high dose) mg/kg-bw/day of BaP (Sigma-Aldrich, Oakville, ON, Canada) dissolved in olive oil. Mice were exposed daily by oral gavage for 28 consecutive days. The control group received only olive oil, the vehicle control. We acknowledge that the doses used in the present study are high compared to the expected daily intake of BaP *via* food in the United States (Hattemer-Frey and Travis, 1991). However, these high doses permit a toxicogenomic investigation of toxicological mechanisms underlying BaP-induced effects at much early post-exposure time points. The animals were sacrificed by cardiac puncture under isofluorane anesthesia on day 3 following the last exposure. The forestomach was excised, flash-frozen in liquid nitrogen, and stored at −80 °C. Care and maintenance of the mice in this experiment were approved by the Health Canada Animal Care Committee.

Tissue RNA extraction and purification. Total RNA was isolated from a random section of the forestomach tissue as described in Labib et al. (2012). In brief, a small random section of the mouse forestomach was homogenized immediately in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) using the Retsch Mixer MM 400. The RNA was isolated using chloroform and precipitated using isopropyl alcohol. The RNA was subsequently purified using RNeasy Mini Plus kits (Qiagen, Mississauga, ON, Canada). All RNA samples showed an $A_{260/280}$ ratio between 2.0 and 2.2, and an $A_{260/230}$ ratio between 1.7 and 2.3. The integrity of the RNA samples was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada). All samples had an RNA integrity number above 6.6 and were all used for microarray analysis.

Microarray hybridization and analysis. Total RNA (200 ng) from each individual mouse forestomach sample in each treatment group and Universal Mouse Reference RNA (UMRR, Stratagene, Mississauga, ON, Canada) was used to synthesize cDNA and cyanine-labeled cRNA using the Agilent Linear Amplification Kit (Agilent Technologies Inc., Mississauga, ON, Canada). Cyanine-labeled cRNA was *in vitro* transcribed using T7 RNA polymerase and purified using RNeasy Mini Kits (Qiagen, Mississauga, ON, Canada); experimental samples were labeled with Cyanine-5 and the UMRR was labeled with Cyanine-3. 300 ng of labeled cRNA from each experimental sample was hybridized with the same amount of labeled reference RNA to Agilent Sureprint G3 Mouse GE 8x60K microarrays (Agilent Technologies Inc., Mississauga, ON, Canada) at 65 °C overnight (17 h) in the Agilent SureHyb hybridization chamber. The arrays were washed and scanned on an Agilent G2505B Scanner according to the manufacturer's recommendations. Data were extracted using Feature Extraction 10.7.3.1 (Agilent Technologies, Inc., Mississauga, ON, Canada).