Diet as a Modifier of Benzo(a)pyrene Metabolism and Benzo(a)pyrene—Induced Colon Tumors in Apc\textsuperscript{Min} mice

Deacquinita L. HARRIS\textsuperscript{1}, Mohammad S. NIAZ\textsuperscript{1}, Jason D. MORROW\textsuperscript{2\dagger}, Mary K. WASHINGTON\textsuperscript{3} and Aramandla RAMESH\textsuperscript{1}

\textsuperscript{1}Department of Cancer Biology, Meharry Medical College, 1005 D.B. Todd Blvd., Nashville, TN 37208, U.S.A.
\textsuperscript{2}Division of Clinical Pharmacology, Departments of Medicine and Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232-6602, U.S.A.
\textsuperscript{3}Department of Pathology, Vanderbilt University, Nashville, TN 37232, U.S.A.

(Received 30 January 2009; accepted 3 March 2009)

Abstract—Every year 56,000 deaths are attributed to colorectal cancer in the United States, and consumption of well-done red meat and saturated fats, rich in polycyclic aromatic hydrocarbons (PAHs) may be a causative factor. The objective of this study was to investigate whether the formation of colon tumors in adult Apc\textsuperscript{Min} mice was influenced by the ingestion of saturated fat containing benzo(a)pyrene [B(a)P], a PAH compound. Treatment consisted of 100 µg B(a)P/kg body wt., dissolved in olive, cod, and coconut oil (representatives of monounsaturated, polyunsaturated and saturated fats, respectively) administered to 6-week-old male Apc\textsuperscript{Min} mice daily via oral gavage for 60 days. At the end of exposure, mice were sacrificed; blood samples were collected; colons were retrieved and preserved in 10% formalin for observation for gross pathological changes. A portion of these tissues and plasma were subjected to a liquid-liquid extraction method and analyzed by a reverse phase HPLC for B(a)P/metabolites. The B(a)P metabolite concentrations in plasma, and colon of mice that received B(a)P through saturated fat were greater when compared to their control, mono- and polyunsaturated counterparts (\(P < 0.05\)). The concentration of reactive metabolites such as B(a)P 7,8-diol, B(a)P 3,6-dione, and B(a)P 6,12-dione were high in plasma and colon of mice that ingested B(a)P through saturated fat relative to B(a)P alone and unsaturated fat counterparts (\(P < 0.05\)). An increased prevalence of adenomas in colon of mice that ingested B(a)P through saturated dietary fat compared to unsaturated fat and controls (\(P < 0.05\)) was noticed. Our studies therefore strongly suggest B(a)P’s involvement in colon carcinogenesis and its potentiation by dietary fat.

Keywords: benzo(a)pyrene, polycyclic aromatic hydrocarbons, colon cancer, adenomas, polyps, tumors, dietary fat, metabolism
INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers in the Western world. In the United States alone, nearly 150,000 new cases of CRC are reported every year and 50,000 deaths are attributed to this cancer (NCI, 2008). Of the familial forms of CRC, familial adenomatous polyposis (FAP; associated with germ line mutations in genes such as adenomatous polyposis coli (Apc) and hereditary non-polyposis colon cancer (HNPCC; associated with DNA mismatch repair enzymes) are the most common. Familial adenomatous polyposis cancer (FAP) is inherited as an autosomal dominant disorder. Individuals with FAP develop large numbers of adenomas in their colon and rectum during their late teens or early twenties. The adenomas are capable of progressing to carcinomas, becoming invasive and producing metastases. The gene responsible for FAP has been identified and designated as Apc (Joslyn et al., 1991; Kinzler and Vogelstein, 1996). In 90% of the colon cancer cases, there is no familial history of colon cancer. Sporadic gene damage seems to play an important role in the development of tumors in colon. It has been postulated that dietary factors might contribute to the sporadic gene mutations and therefore are involved in the induction of sporadic colon carcinomas (World Cancer Fund and the American Institute for Cancer Research, 1997).

Epidemiological studies have shown that environmental factors, and especially diet, play an important role in colon cancer susceptibility (Kazerouni et al., 2001). It is estimated that diet contributes to 80% of the known colorectal cancer cases (Bingham, 2000). Therefore, understanding the role of environmental chemicals that contaminate food stuffs or diet during its preparation towards the development of gastrointestinal tract cancers is important. In this context, one group of chemicals, the polycyclic aromatic hydrocarbons (PAHs) have generated the most interest as they are formed in red meat cooked at high temperatures (Sinha et al., 2005a, b; Ramesh and Morrow, 2008).

Though PAHs have been suspected of contributing to colon cancer, more than 100 types of PAHs are known to exist in nature and at least 20 of them are detectable in most dietary items at measurable levels (reviewed in Ramesh et al., 2004). This necessitates using a surrogate PAH compound to gain comprehensive information on the mechanisms involved in development of colorectal cancer by these chemicals. Hence, the focus of this study is limited to benzo(a)pyrene [B(a)P] as this chemical is considered as a prototypical PAH compound, being the most extensively characterized and studied in terms of its toxicity.

For our studies we have used the Apc<sup>Min</sup> mouse, which is widely used in recent years to study the onset and progression of gastrointestinal cancers. Apc<sup>Min</sup> mouse is a mutant mouse with multiple intestinal neoplasias (Min; Su et al., 1992). It has a mutated Apc gene, similar to that in patients with familial adenomatous polyposis. This model is most promising as it mimics the rapid development of adenomatous polyps that affect humans (Radtke and Clevers, 2005). The objective of our study was to assess the influence of dietary fat on B(a)P-induced colon tumors/carcinogenesis using the Apc<sup>Min</sup> mouse model.
MATERIALS AND METHODS

Exposure of mice to benzo(a)pyrene

Six-week-old male Apc^Min mice (Jackson Labs, Bar Harbor, ME) weighing approximately 25 g were used in this study. The animals were housed in groups of 2–3 per cage, maintained on a 12/12 hour light/dark cycle (lights on at 0600 hour) and allowed free access to rodent chow (NIH-31 open formula diet, National Institutes of Health, MD) and water. All animals were allowed a seven-day acclimation period prior to being randomly assigned to a control (n = 5 per each time point) or treatment group (n = 5 per each time point). Treatment consisted of a single dose (100 µg/kg) of B(a)P (97% pure, unlabeled; Sigma Chemical Co., St. Louis, MO) dissolved in olive (Colavita, Linden, New Jersey)-, cod (Premier Research Labs, Round Rock, Texas)- and coconut (Tropical Traditions, Reno, Nevada) oils. These oils are representatives of mono-, poly-, and saturated fats, respectively. Control groups were given only the oils without B(a)P. Mice that received no fat and B(a)P served as negative controls, whereas mice that received no fat, but received B(a)P served as positive controls. The test chemical was administered daily via oral gavage for 60 days.

Tissue sampling, adenoma scoring and histopathological analysis

At the end of 60 days of exposure, mice were sacrificed and colons were harvested from control and experimental mice. The colons were longitudinally opened and gently rinsed with physiological saline to flush the excreta. The size, location and number of adenomas in colon were documented. The colons were Swiss rolled and preserved in 10% formalin for 24 hrs and then transferred to 70% ethanol until processing for observation for gross pathological changes.

Colons of control and B(a)P-treated mice were embedded in paraffin wax, cut in parallel with the mucosal surface, and stained with H&E. A pathologist blind to the treatment groups, enumerated the polyps and evaluated the histopathological features. Adenomas were characterized as displaying low grade dysplasia (typical Apc^Min adenomas, with low nucleus/cytoplasm ratio, elongated nuclei, and maintenance of polarity) or high grade dysplasia, characterized by architectural complexity, higher nuclear/cytoplasm ratio, and loss of polarity; Boivin et al., 2003).

Extraction of tissues and HPLC analysis of B(a)P/metabolites

Five hundred microliters of plasma or five hundred milligrams of colon tissue was homogenized in two volumes of Tris-sucrose-EDTA buffer (0.25 M; pH 7.4). Twenty microliters of sodium dodecyl sulfate (1%) was added to the mixture, vortexed for 1 min and the homogenate was subjected to a liquid-liquid extraction using methanol, deionized water and chloroform. The extracts were processed, and analyzed as detailed in Ramesh et al. (2001).
Table 1. Distribution of polyps in colon of ApoMin mice treated with 100 µg BaP/kg bw. Mice were exposed to benzo(a)pyrene [B(a)P] alone, [B(a)P] in monounsaturated fat (MUSF), polyunsaturated fat (PUSF), and saturated fat (SF). Polyps were enumerated microscopically, and assessed for damage by histopathology as outlined in the “Materials and Methods” section. Asterisks represent statistical significance (p < 0.05) in polyp numbers between each treatment category compared to their respective controls.

<table>
<thead>
<tr>
<th>Indices</th>
<th>Benzo(a)pyrene only</th>
<th>Benzo(a)pyrene + MUSF</th>
<th>Benzo(a)pyrene + PUSF</th>
<th>Benzo(a)pyrene + SF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treatment</td>
<td>Control</td>
<td>Treatment</td>
</tr>
<tr>
<td>Total numbers</td>
<td>3 ± 0.6</td>
<td>7 ± 0.6*</td>
<td>4 ± 0.3</td>
<td>8 ± 0.7*</td>
</tr>
<tr>
<td>Polyps &gt; 2.5 mm</td>
<td>3 ± 0.2</td>
<td>5 ± 0.4</td>
<td>3 ± 0.4</td>
<td>5 ± 0.6</td>
</tr>
<tr>
<td>Polyps &gt; 5.0 mm</td>
<td>0</td>
<td>2 ± 0.3</td>
<td>1</td>
<td>3 ± 0.4</td>
</tr>
<tr>
<td>Polyps with severe dysplasia</td>
<td>1</td>
<td>4 ± 0.5</td>
<td>2 ± 0.3</td>
<td>5 ± 0.6</td>
</tr>
</tbody>
</table>
HPLC analysis of B(a)P metabolites

Sample analyses was conducted on a High Performance Liquid Chromatograph, (HPLC; Model 1050, Agilent, Wilmington, DE) equipped with a HP1046 fluorescence detector as detailed in Ramesh et al. (2001). Identification and quantitation of the metabolites will be accomplished by comparing the retention times and peak areas of samples with that of standards (National Cancer Institute Chemical Carcinogen Repository, Midwest Research Institute, Kansas City, MO).

Statistical analyses

Data are expressed as mean ± SEM. Differences in tumor number and B(a)P metabolite concentrations were analyzed by student’s t test or one-way ANOVA. Differences among groups were detected using Fisher’s least significant difference at $P < 0.05$.

RESULTS AND DISCUSSION

Most of the studies using rodents as animal models to investigate the gastrointestinal tract cancers induced by environmental carcinogens such as B(a)P have considered the number of tumors present in the small intestine as primary endpoint and tumor incidences in colon have been overlooked. Oral administration of B(a)P through gavage and diet in doses ranging from 0.1 mg to 30 mg/kg bw in mice and rats over varied periods of time (few weeks to two years) revealed tumor development on tongue, in esophagus and forestomach (Weyand et al., 1995; Culp et al., 1998; Sattar et al., 1999., Kroese et al., 2001).

Data on the incidence of polyps (adenomas) in colon of Apc$^{Min}$ mice is presented in Table 1. The numbers of polyps were more in B(a)P-treated mice compared to controls (vehicle-treated ones) and the difference was statistically significant ($p < 0.05$). Similar trend was observed in mice that received B(a)P.
through unsaturated and saturated fat individually, compared to their respective controls ($p < 0.05$). On the other hand, the differences in tumor numbers between mice that received B(a)P alone and B(a)P in unsaturated fat were not statistically significant. However, the differences in tumor numbers between mice that received B(a)P alone and B(a)P in saturated fat were statistically significant ($p < 0.05$). Similarly the mice colon polyp numbers induced by B(a)P between the two dietary fat categories were statistically significant ($p < 0.05$), with mice that received B(a)P through saturated fat registering more number of polyps than their unsaturated fat counterparts.

Colon polyps from vehicle (olive oil, cod liver oil, and coconut oil) only-treated mice were found to be similar in number, morphology and histology to polyps from the Apc$^{Min}$ mice that received a standard diet. No appreciable differences in polyp size were detected. Likewise, no notable differences in histology were noted. On the other hand, mice that received B(a)P through saturated fat have shown larger size polyps in distal colon, as depicted in Fig. 1. Representative microphotographs of colon that received B(a)P alone, and B(a)P through unsaturated and saturated fats is shown in Fig. 2. Most of the tumors observed in Apc$^{Min}$ mice that received B(a)P only were typical low grade adenomas. While adenomas in mice that received B(a)P through saturated fat
invariably showed high grade dysplasia with invasive adenocarcinomas, only in one case of mouse that received B(a)P through unsaturated fat, invasive adenocarcinoma was seen. As the growth of cancerous tumor requires energy, an excess of energy available from the laboratory rodent diet and the dietary fat used to administer B(a)P together may have served as growth stimulants for tumors (Giovanucci and Goldin, 1997; Guthrie and Carroll, 1999), since an increased number of adenomas were registered for mice that received B(a)P through the saturated fat-based vehicle.

Since metabolic processing of chemical by the body is the prime driving force behind toxicity or carcinogenesis, we have analyzed the colon tissues (target of B(a)P-induced tumors) and plasma (the transport medium) for B(a)P and its metabolites. Also, we were interested in finding the extent to which, the dietary fat modulates the B(a)P metabolism. The results for distribution of B(a)P metabolites in plasma, and colon of Apc<sup>Min</sup> mice that received 100 µg BaP/kg body wt. via oral gavage. Values represent mean ±SE (n = 5 for each sample). Asterisks denote statistical significance; "*" indicates statistical significance (p < 0.05) in metabolite concentrations between plasma and colon in each experimental group; "***" indicates statistical significance (p < 0.05) in metabolite concentrations between each fat category containing B(a)P, compared to B(a)P alone. The acronyms are expanded as follows: MUSF, monounsaturated fat; PUSF, polyunsaturated fat; SF, saturated fat.

![Fig. 3. Effect of the type of dietary fat on total benzo(a)pyrene [B(a)P] metabolite concentrations in plasma, and colon of Apc<sup>Min</sup> mice that received 100 µg BaP/kg body wt. via oral gavage. Values represent mean ±SE (n = 5 for each sample). Asterisks denote statistical significance; "*" indicates statistical significance (p < 0.05) in metabolite concentrations between plasma and colon in each experimental group; "***" indicates statistical significance (p < 0.05) in metabolite concentrations between each fat category containing B(a)P, compared to B(a)P alone. The acronyms are expanded as follows: MUSF, monounsaturated fat; PUSF, polyunsaturated fat; SF, saturated fat.](image-url)
In the present study, the absorption of B(a)P from the gastrointestinal tract of mice appear to be enhanced when it is administered via vehicles possessing lipophilic and hydrophobic properties (dietary fats) compared to controls as demonstrated by Vetter et al. (1985) for benzo(a)pyrene; Walker et al. (2007) and Harris et al. (2008) for FLA. Association of B(a)P with chylomicrons and uptake of B(a)P by chylomicrons in the enterocytes (Kararli, 1995) may have an influence on the kinetics of disposition of this chemical and its toxicity as shown for several PAHs (Lipniak and Brandys, 1993).

The lipophilic nature of B(a)P (Librando et al., 2003) probably facilitates its absorption through the gastrointestinal tract. Benzo(a)pyrene in saturated fat is more likely to stay in the body for a longer period of time at high doses. It is likely that B(a)P absorbed through saturated fat i.e., a fat that is mostly made of medium-chain triglycerides, is transported to the liver via portal venous system. In contrast to this, B(a)P absorbed through monounsaturated and polyunsaturated fats that are made of long-chain triglycerides, may have been incorporated into chylomicrons for transport through the lymphatic system or peripheral circulation. That lipid type governs the kinetics of disposition of ingested PAH compounds is also supported by published reports on the association between the biotransformation enzymes and PAHs incorporated in dietary lipids. A positive correlation between the content of polyunsaturated fatty acids in the diet and hepatic and intestinal drug metabolizing enzyme (DME) activities subsequent to PAH administration through diet has been reported (Gower and Willis, 1986; Gower et al., 1986). In this context, it is worth mentioning that in addition to liver,
DMEs have also been detected in several rodent extrahepatic organs and tissues (Kaminsky and Zhang, 2003; Halberg et al., 2008). Since the activities of both constitutive and inducible toxic chemical DMEs in rodents depend on the diet (Stott et al., 2004) this raises the likelihood that the disposition of B(a)P in body is dependent on the nature of the co-administered lipid as well.

Since the B(a)P metabolite profiles and proportions will reveal the balance between metabolic activation and detoxification processes as a result of B(a)P exposure, we focused on whether administration of B(a)P through dietary lipid alters the metabolite composition. The B(a)P metabolites identified were as follows: B(a)P 4,5-diol; B(a)P 7,8-diol; B(a)P 9,10-diol; B(a)P 3,6-dione, B(a)P 6,12-dione; 3(OH) B(a)P and 9(OH) B(a)P. The former five are reactive metabolites and the latter two are detoxification products. The percentage composition of metabolite types in plasma, and colon tissues following B(a)P administration in the 3 dietary lipids is presented in Fig. 4.

The percentage composition of individual metabolite types among the total metabolites varied with the lipid type in which B(a)P was administered. The relatively high proportions of B(a)P diols and quinones in plasma and colon of mice that received B(a)P through saturated fat compared to polyunsaturated and monounsaturated fats suggest that B(a)P administered through saturated fat is likely to generate more reactive metabolites. This assumption is also strengthened by the low concentrations of 3-, and 9-hydroxy B(a)P metabolites, which are the major detoxification products of B(a)P in plasma and tissues of mice that ingested saturated fat relative to unsaturated fat.

Our findings on dietary lipid-induced B(a)P metabolism and its implications can be interpreted effectively in the context of mechanisms of chemical carcinogenesis. Harvey (1996) summarized four mechanisms for carcinogenesis of a great majority of PAHs, including B(a)P. They are the diol-epoxide mechanism, the radical-cation mechanism, the quinone mechanism, and the benzylic oxidation mechanisms. The diol-epoxide mechanism involves metabolic activation by CYP enzymes to reactive epoxide and diol-epoxide intermediates that interact with DNA leading to mutations and ultimately to cancer (Shimada et al., 1999, 2001). Oxidative metabolism of diols such as BaP 7,8-diol can also be catalyzed by: prostaglandin H synthase (Marnett et al., 1978; Eling and Curtis, 1992), a myeloperoxidase system (Mallett et al., 1991), lipoxygenases (Hughes et al., 1989) or cyclooxygenase-2 (Wiese et al., 2001). The intermediate diols can also undergo a detoxification process by conjugating with glucuronic acid or glutathione, leading to conjugated metabolites, which can be excreted by renal or biliary routes. The radical-cation mechanism involves one electron oxidation to generate radical-cation intermediates that may attack DNA resulting in depurination (Cavaliere and Rogan, 1995).

The quinone mechanism involves enzymatic dehydrogenation of dihydrodiol metabolites to yield quinone intermediates that may combine directly with DNA or enter into a redox cycle with O2 to generate reactive oxygen species, such as hydroxyl radicals and superoxide anion, capable of attacking DNA. Formation of small amounts of quinone metabolites may result in generation of high ratios of
reactive oxygen species (Flowers-Geary et al., 1992; Penning et al., 1996). The diol-epoxide and quinone mechanisms are widely accepted as important pathways in the generation of reactive metabolites. The detection of B(a)P reactive metabolites such as B(a)P 7,8-diol 9,10-epoxide, B(a)P 3,6-dione, and B(a)P 6,12-dione in colon samples of all experimental groups involved in this study allows for the likely possibility that both epoxide and quinone pathways participate in B(a)P-induced colon carcinogenesis.

In summary, from an environmental health and risk assessment standpoint, our findings evoke interest because using a mouse model, specific for colon cancer, we have demonstrated that subchronic exposure to B(a)P through oral route induces tumors in the colon. The development of such tumors appears to be accelerated by intake of dietary fat.

Studies are in progress in our laboratory to evaluate the pathway-specific colon carcinogenesis by B(a)P. Our future studies are aimed at investigating the expression of genes that are responsible for B(a)P biotransformation, DNA damage caused by genotoxic and epigenetic mechanisms, the role of oncogenes and DNA repair enzymes in colon tumor formation.

Acknowledgments—This research was supported by the National Institutes of Health (NIH) grants 1S11ES014156-01A1-Project#3, 1RO3CA130112-01 (AR), 5T32HL007735-12 (DLH), and G12 RR03032 (Meharry Medical College). The authors also thank Prof. S. Tanabe, Prof. A. Subramanian and the organizing committee of GCOE, Ehime University for travel and per diem support to one of the authors (AR) to present the findings at the Interdisciplinary Symposium on Environmental Sciences.

REFERENCES


Benzo(a)pyrene and Colon Carcinogenesis in Mice 237


D. L. Harris, M. S. Niaz, J. D. Morrow, M. K. Washington and A. Ramesh (e-mail: aramesh@mmc.edu)