Differential Effect of Gestation Stage on Benzo(a)pyrene-induced Micronucleus Formation and/or Covalent DNA Modifications in Mice

Mian-Ying Wang and Lee-Jane W. Lu

Department of Preventive Medicine and Community Health, The University of Texas Medical Branch, Galveston, Texas 77550

ABSTRACT

Benzo(a)pyrene (BP), an environmental carcinogen, binds ubiquitously to the DNA of maternal and fetal tissues (Lu et al., Cancer Res., 46: 3046–3054, 1986). These studies further investigated the effect of gestation age on the induction of genetic damage by BP. Timed-pregnant ICR mice were treated with one dose of BP on various days of gestation and sacrificed 24–120 h after treatment. At the molecular level, BP covalently bound to the DNA of adult bone marrow and fetal liver of mice at all gestation ages. Compared to the nonpregnant mice (adduct level = 15 adducts/10^6 bases), the adduct levels in the pregnant adult bone marrow were decreased up to 50% during early gestation (days 3–9) and then increased steadily to a 4-fold excess over nonpregnant values during late gestation (days 15–18). In the fetal liver, adduct levels exhibited little variation (3–4 adducts/10^6 bases) between days 11 and 15 of gestation and then increased sharply to 14 adducts/10^6 bases after day 16. At the cellular level, a higher percentage of polychromatic RBCs from adult and fetal mice after BP treatment contained micronuclei (MN) than controls (solvent or untreated). Bone marrow from pregnant mice exhibited greater increases in the formation of MN during early gestation (days 3–9) relative to late gestation (days 15–18), compared to the nonpregnant mice. In the fetuses, the amounts of MN formed were higher than those found in the adult nonpregnant or maternal mice, but these amounts decreased with gestation progression. Thus, MN induction with gestation progression differed from DNA adduction in adults and fetuses. In addition, the dose and time responses of MN formation also differed from those of covalent DNA modifications, when analyzed in the bone marrow of pregnant mice treated on gestation day 5. Collectively, our results showed that pregnancy and development modulate different types of genetic damage in different ways. Fetal tissues may be more sensitive than maternal tissues to genetic damage. Factors in addition to DNA adduct formation may be responsible for MN induction.

INTRODUCTION

Transplacental exposure of fetuses to carcinogens is known to induce tumors in the offspring of experimental animals and humans (1, 2). However, prenatal tissues exhibit variations in sensitivity to carcinogens, compared to the adult tissues (3, 4). In the adults, limited experimental evidence indicates that the gravid females may also be at altered risk for carcinogenesis. In particular, differential sensitivity has been demonstrated for the reproductive organs (5–8).

The mechanisms responsible for the observed differential response of pregnant and fetal mice, compared to adult mice, to carcinogens are not well defined. Rapid growth rate, changing metabolic competence, immunosuppression, and the degree of cell differentiation in the fetus may be contributing factors. A great majority of carcinogens depend on metabolic activation to covalently bind to DNA bases (9). During fetal development, enzymes necessary for the metabolic activation/deactivation of carcinogens either are not sufficiently matured or develop at differential rates (10). In the adult mice, the major functional changes in the female endocrine system during pregnancy affect maternal metabolism. Therefore, a given dose of a carcinogen may yield different amounts of reactive metabolites and, consequently, cause different levels of genetic damage in an organ. In line with this hypothesis is our previous observation that the extent of covalent DNA modifications in a particular tissue by a carcinogen can indeed be altered by development and pregnancy (11, 12). The consequence of a variable extent of genetic damage per se to the altered susceptibility of pregnant and fetal mice to carcinogenesis has not been explored.

Chromosome aberrations have been frequently observed in neoplastic tissues (13) and in cells after carcinogen exposure (14). Several forms of cytogenetic changes, including MN,3 have been used extensively to evaluate the potential genotoxicity/carcinogenicity of chemicals (14, 15). MN are thought to be formed as a result of chromosome breaks (16). The ability to induce MN formation appears to correlate well with the carcinogenicity of chemicals and MN are easily quantitated (17). Several studies have shown that prenatal tissues can be more sensitive to chromosome damage than adult tissues (18–20). In this report, (a) the relationship between gestation age and genetic damage at the molecular and cellular levels, (b) the role of DNA adducts in MN formation, and (c) the relative sensitivity of adult and fetal tissues to the formation of genetic damage are investigated.

MATERIALS AND METHODS

Chemicals. BP and trioctanoin (same batch was used for all experiments) were obtained from Sigma Chemical Co. (St. Louis, MO). All other materials used were exactly as previously described (21–23). Carrier-free [32P]phosphate was obtained from ICN Radiochemicals Inc. (Irvine, CA). PEI-cellulose TLC plates were prepared in our laboratory.

Animals and Treatment. Male and female ICR mice (25–30 g) were obtained from Harlan Sprague Dawley Co. (Houston, TX). They were housed in polycarbonate cages with corn cob bedding (The Andersons Co., Maumee, OH) in rooms with temperature control (20–22°C) and a 12-h dark/light cycle. The mice were fed Purina 5008 laboratory rodent chow and provided with tap water ad libitum. Mating was performed by placing one male in a cage with three females. Females were checked for vaginal plugs twice daily, once at 8:00 a.m. and once at 5:00 p.m. Only mice with positive vaginal plugs were used for experiments. The day that a positive plug was found was designated as day 0 of pregnancy. The gestation period for ICR mice is approximately 19 days. The body weight (in g, mean ± SE) of pregnant mice on days 5, 11, 17, and 18 of gestation was estimated to be 28.0 ± 1.3, 34.9 ± 0.4, 50.8 ± 8.0, and 53.2 ± 5.3, respectively. Nonpregnant mice weighed 25.6 ± 1.1. Timed-pregnant mice (3–12/group) were exposed to one dose of BP p.o. on various days of gestation and killed by cervical dislocation 24–120 h after treatment. Control mice (pregnant or nonpregnant) received trioctanoin (10 ml/kg) or no treatment. Fetal tissues were dissected immediately and pooled from the same litter for evaluation of MN formation and DNA isolation.

A portion of the fetal liver, in fetal calf serum, was pressed through a 26-gauge needle. Maternal femurs were flushed with fetal calf serum. Liver cell suspensions or bone marrow were then centrifuged. A portion

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2To whom requests for reprints should be addressed, at Department of Preventive Medicine and Community Health, The University of Texas Medical Branch, 24 Keiller, F-19, Galveston, TX 77550.

3The abbreviations used are: MN, micronuclei; BP, benzo(a)pyrene; PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes;RAL, relative adduct labeling;PEI-cellulose,polyethyleneimine-cellulose; TLC, thin layer chromatography; ANOVA, analysis of variance.
of the pellet from either pooled fetal liver cells or bone marrow of each mouse was used for slide preparation of MN. The remaining pellets were frozen and later used for DNA adduct analysis. Pellets from two or three mice from each treatment were pooled in order to obtain sufficient amounts of DNA for adduct analysis.

**32P-Postlabeling Analysis of DNA Adducts.** DNA was isolated by a procedure involving protein and RNA digestion and solvent extraction (24, 25). Bone marrow pooled from three mice yielded ~40 μg DNA. Three mouse fetal livers yielded ~40–100 μg DNA. DNA adducts were routinely analyzed by the P, version of the 32P-postlabeling assay (26). Briefly, 10 μg of DNA were digested first with 0.8 unit of micrococcal nuclease and 8.0 μg spleen phosphodiesterase and then with 8.0 μg nuclease P, The entire digest was labeled with 200 μCi [γ-32P]-ATP (~3000 Ci/mmol) and 4.3 units of T4 polynucleotide kinase and applied to PEI-cellulose TLC for adduct purification, separation, and quantification. [γ-32P]-ATP was synthesized in our laboratory as described (26).

**Mapping of 32P-labeled Adducts.** The chromatographic techniques and conditions have been described (12). The solvent used for adduct purification was 2.8 m sodium phosphate, pH 6.0 (D1; for definition see Ref. 12). For adduct separation, 3.6 m lithium formate, 8.5 m urea, pH 3.6 (D3), was used for the first direction, followed by 0.8 m LiCl, 0.5 m Tris-HCl, 8.5 m urea, pH 8.0 (D4), at a right angle to the previous direction. Additional chromatography in 1.0 m sodium phosphate, pH 6.8 (D5), was performed in the latter direction to 7 cm onto a Whatman 1 wick, for removal of radioactive background material from the chromatograms.

**Estimation of Extent of DNA Adduction.** The count rate (cpm) of each detectable adduct spot was evaluated as previously described (26). RAL was calculated according to the formula:

$$\text{RAL} = \frac{\text{cpm in adducted nucleotides}}{\text{cpm in total nucleotides}}$$

$$\text{RAL} \times 10^8 = \text{No. of adducts in } 10^8 \text{ bases}$$

**MN Test.** Slides were fixed in methanol for 10–15 min and stained with Wright’s Giemsa stain (27, 28). Five hundred to 1000 PCE from each slide were scored. Two or three slides were prepared from femurs with Wright’s Giemsa stain (27, 28). Five hundred to 1000 PCE from each slide were scored. Two or three slides were prepared from femurs with Wright’s Giemsa stain (27, 28). Five hundred to 1000 PCE from each slide were scored. Two or three slides were prepared from femurs with Wright’s Giemsa stain (27, 28). Five hundred to 1000 PCE from each slide were scored. Two or three slides were prepared from femurs with Wright’s Giemsa stain (27, 28).

**RESULTS**

**Effects of Gestation State on Genetic Damage**

In these studies, the same dose of BP (80 mg/kg) was administered once to timed-pregnant mice on various days of gestation. Adduct levels and MN were examined 24 h after treatment.

**Covalent DNA Modifications.** Using the P, version of the 32P-postlabeling method (26), BP was found to bind to the DNA of fetal liver and bone marrow of pregnant and nonpregnant mice. One major BP-DNA adduct (Spot 1) was detected in bone marrow (Fig. 1, A–C) and fetal liver (12). This spot has previously been determined to be the 3',5'-bisphosphate of 10β-(deoxyguanosin-7-N²-y1)-7β,8α,9α-trihydroxy-7,8,9,10-tetrahydro-BP (12). Spot 4 is derived from BP but remains to be identified (for more detail see Ref. 12). Control samples (Fig. 1D) did not contain the numbered spot.

**Fig. 1.** BP-DNA adducts in adult bone marrow, as shown by autoradiography of two-dimensional PEI-cellulose TLCs of 32P-labeled DNA adducts. P, condition was used for labeling. See “Materials and Methods” for chromatographic conditions. Spot 1 represents 32P-labeled bisphosphate of 10β-(deoxyguanosin-7-N²-y1)-7β,8α,9α-trihydroxy-7,8,9,10-tetrahydro-BP. Film exposure was for 12 h at ~80°C. Adduct patterns were derived from tissues of mice 24 h after treatment with BP (80 mg/kg, p.o.) on the gestation day indicated. OR, origin at the lower left hand corner of the chromatogram; D3 and D4, chromatographic directions.

**Fig. 2.** Effects of gestation age at the time of BP treatment on DNA adduct levels in adult bone marrow and fetal liver. Timed-pregnant mice were treated with one dose of BP (80 mg/kg, p.o.) on the gestation day indicated. Mice were sacrificed 24 h later. Adduct levels were obtained under the P, condition of the 32P-postlabeling method. The results are the mean ± SE of 3–6 litters or 2–4 groups of pooled bone marrow (three mice/group). The result from day 18 bone marrow was from one group of three mice. For ease of presentation, the nonpregnant mice were designated as day –1 of gestation. At least three 32P-postlabeling analyses were performed for each DNA preparation. M, maternal tissues; F, fetal tissues.

**Fig. 2.** Effects of gestation age at the time of BP treatment on DNA adduct levels in adult bone marrow and fetal liver. Timed-pregnant mice were treated with one dose of BP (80 mg/kg, p.o.) on the gestation day indicated. Mice were sacrificed 24 h later. Adduct levels were obtained under the P, condition of the 32P-postlabeling method. The results are the mean ± SE of 3–6 litters or 2–4 groups of pooled bone marrow (three mice/group). The result from day 18 bone marrow was from one group of three mice. For ease of presentation, the nonpregnant mice were designated as day –1 of gestation. Bone marrow from two or three mice had to be pooled to obtain sufficient amounts of DNA for adduct analysis. The adduct levels were estimated to range from 7 to 62 adducts/10⁸ bases (RAL = 0.7–6.2 x 10⁻⁶). Compared to the nonpregnant mice (RAL = 1.55 ± 0.37 x 10⁻⁶), the pregnant mice had 50% fewer adducts on days 3–9 (P < 0.05) and similar adduct levels on days 11–13 but up to 4-fold higher adducts after day 13 (P < 0.05). In the fetal liver, BP binding to DNA can be detected soon after organogenesis (RAL = 2.8 ± 0.8 x 10⁻⁶, day 11).
DNA adduct levels varied insignificantly between days 11 and 15 of gestation ($P > 0.2$ by ANOVA and Scheffe’s test) but increased to $14 \times 10^{-8}$ after day 17 ($P < 0.005$, day 17 compared with days 11–15), a value similar to that of adult bone marrow of nonpregnant mice.

**MN Formation.** Fig. 3 illustrates the frequencies of MN formation in relation to gestation progression in the PCE of maternal bone marrow and fetal liver of mice treated with BP or trioctanoin. The spontaneous incidence of MN in the control adult mice receiving trioctanoin varied with the stage of gestation. In the adult mice, the frequencies were higher on gestation days 3–9 than in the late gestation or nonpregnant mice ($P < 0.05$ by ANOVA and Scheffe’s test). Similar patterns of spontaneous incidence of MN were also found in the adult mice that did not receive any treatment (results not shown). In the fetuses, these frequencies did not vary significantly with gestation progression. The basal levels of MN in the pregnant mice after day 9, in the nonpregnant mice, and in the fetal liver were in line with data reported in the literature (19, 29). No data were available regarding pregnant mice of early gestation for comparison.

Based on the dose of BP (80 mg/kg) given, fetal liver and maternal bone marrow from mice of all gestation ages had higher levels of MN than the nonpregnant mice ($P < 0.05$). Fetal liver was more sensitive to MN induction on day 11 of gestation than on days 13–18 ($P < 0.06$). Fetal liver also had more MN than maternal bone marrow of the corresponding gestation age ($P < 0.05$). Compared to the nonpregnant mice, the maternal bone marrow had 3–5 times more MN on days 3–9 of gestation ($P < 0.005$) and 2 times more in the late gestation (after day 15, $P < 0.05$) after BP treatment. Maternal sensitivity to MN induction between days 11 and 13 was very similar to that of nonpregnant mice. Thus, based on the dose given, the pregnant mice were more sensitive than the nonpregnant mice to MN induction at all gestation ages examined. This increased sensitivity was much more pronounced prior to day 9 than after day 9.

To determine if BP treatment induced differential suppression of adult bone marrow during gestation, the ratios of PCE/NCE in bone marrow were evaluated. The ratios of PCE/NCE in control pregnant or nonpregnant mice that received either trioctanoin or no treatment were similar, $1.0 \pm 0.1$. Fig. 4 shows the results of BP treatment in both pregnant (days 0–18) and nonpregnant (day −1) mice. BP treatment induced similar small decreases in these ratios in both pregnant and nonpregnant mice. However, a statistical significance can only be demonstrated for mice treated on day 5 and the controls ($P < 0.01$).

**Dose Response**

In these experiments, timed-pregnant mice on day 5 of gestation were treated with different doses of BP and sacrificed 24 h after treatment. Fig. 5 illustrates the dose response for DNA adduction and MN formation in maternal bone marrow. The amount of adducts increased linearly ($P < 0.005$, by linear trend analysis) with the dose. An 8-fold increase in dose led to a 7.5-fold increase in adduct level. In contrast, the induction of MN as a function of dose exhibited a maximum, which was estimated to occur around 120–160 mg/kg. The MN value at the peak of induction was ~5-fold more than that of nonpregnant mice. At 320 mg/kg, the number of MN was ~2-fold more than that of nonpregnant mice.

**Time Response**

In these studies, timed-pregnant mice on day 5 of gestation were given 160 mg/kg BP and sacrificed 24–120 h after treatment. Fig. 6 shows the persistence of BP-DNA adducts and MN. Within 5 days after treatment, adducts were still detectable in bone marrow and the levels were ~14.5-fold less than that of the 24-h time point. On the other hand, the amount of MN 120 h after treatment was similar to that of the trioctanoin-treated control samples. Thus, adducts appeared to be somewhat more persistent than MN.
Relationship between MN Induction and DNA Adduction

To investigate the role of BP-induced primary genetic lesions in genetic damage at the cellular level, the amount of MN formed was correlated with adduct level. Results shown in Fig. 7 were derived by plotting MN/RAL as a function of gestation age at the time of BP treatment (Fig. 7A), doses administered (Fig. 7B), or time after treatment (Fig. 7C). The MN values used for calculating MN/RAL have been subtracted for spontaneous MN values in the trioctanoin-treated control samples.

Based on the adduct levels measured, the amount of MN formed in the PCE of fetal or adult mice decreased as the gestation age at the time of BP exposure advanced (Fig. 7A). In the fetuses, the MN/RAL values decreased as gestation progressed ($P < 0.01$ by linear trend test). Fetal PCE were more sensitive to MN induction than the maternal PCE of the same gestation age ($P < 0.003$ by paired-wise comparison). The differences, however, became progressively smaller as the gestation approached term. Among the adult PCE, the MN/RAL ratios clustered at two distinct levels. These ratios were higher during days 3–9 of gestation than during days 11–18 or in the nonpregnant mice ($P < 0.002$, $t$ test). This indicated that the correlation of MN frequencies with adduct formation was different in the early as compared to the late stages of gestation. Thus, the amounts of MN produced per adduct level varied with the stage of gestation.

If MN were formed as a result of DNA adductions, the MN/RAL values are expected to be constant, irrespective of the dose or the time at which the effects are examined. Data in Fig. 7, B and C, are not in accord with this hypothesis. Under the same physiological conditions, e.g., on day 5 of gestation, the MN/RAL values varied as a function of the dose administered (Fig. 7B) or the time (Fig. 7C) after treatment.

DISCUSSION

This report shows that BP produces both covalent DNA modifications and MN in the PCE of adult bone marrow and fetal liver, in a gestation age-dependent manner. However, the molecular (adducts) and cellular (MN) responses are differentially modulated by the stages of gestation.

Genetic damage is a prerequisite to cancer initiation (30). The covalent DNA modifications (31) and MN formation (17) have both been shown to be useful indicators of the carcinogenicity of chemicals (15). The results from the present studies clearly indicate that DNA adductions and MN formation, while related, can also be dissociated under some experimental conditions, as discussed below.

Under the same dosing condition, an inverse relationship can be demonstrated between DNA adductions (Fig. 2) and MN formation (Fig. 3) in relation to gestation progression. This has been shown for both adults and fetuses.

Based on the dose administered, the pregnant mice contained fewer adducts but more MN in the bone marrow prior to placentation (day 9 in ICR mice) than after placentation. Overall, compared to the nonpregnant mice, the pregnant mice were
BP-INDUCED GENETIC DAMAGE DURING PREGNANCY

The mechanisms responsible for the dissociation of MN induction and DNA adduction over the course of gestation in adult and fetuses is not clear. High levels of adducts observed in pregnant mice in late gestation may block cells at the G2 phase of the cell cycle. Without cell cycle progression, MN cannot be formed. This possibility is unlikely after an 80 mg/kg BP treatment, because the ratios of PCE/NCE (indicator of cytotoxicity) were similar between pregnant (all gestation ages) and nonpregnant mice. A direct effect of pregnancy in enhancing the sensitivity of the cells to MN formation cannot be ruled out. Alternatively, metabolites other than BP-7,8-diol-9,10-epoxide may be responsible for the clastogenic effect. Other studies have suggested that different metabolites of BP are responsible for BP cytotoxic and mutagenic effects (34).

Adduct 1 detected in bone marrow and fetal liver has been previously established to be derived from the 7,8-diol-9,10-epoxide of BP (12). Thus, gestation stages may influence the levels of this adduct by altering the (a) metabolism of BP by the diol-epoxide pathway, (b) repair of BP-DNA adducts, and/or (c) pharmacokinetics of BP absorption and disposition. Pregnancy has been shown to alter the oxidative metabolism of many xenobiotics (35–37). Whether BP metabolism is similarly affected is under investigation. This possibility, if established, might account for the dissociation of adduction and MN formation.

The ontogeny of cytochrome P-450-dependent mixed function oxidase has been a subject of intensive studies. Due to the lack of sufficient amounts of material from fetuses, indirect methods have been used in previous studies to address this question. Blastocyst-stage mouse embryos cultured in vitro metabolized BP to quinine, phenol, and diol derivatives (38) and produced sister chromatid exchanges after BP treatment (39). Aryl hydrocarbon hydroxylase activities were not measurable until late gestation (40). Using the 32P-postlabeling assay, BP binding to fetal liver DNA can be detected soon after organogenesis. BP binding to DNA has been detected in day 7 embryos. Literature data (40–45), together with the results shown here, suggest that basal levels of some mixed function oxidase activities may indeed be present during mouse embryogenesis (41) and fetal development (40, 42, 45). However, the developmental profile of DNA adduct levels observed in ICR mice did not parallel the ontogeny of aryl hydrocarbon hydroxylase activities (40) or of the constitutive or inducible P-450Ia1 mRNA levels (45). The inducibility of P-450Ia1 mRNA increases steadily with gestation progression (40) and of the constitutive or inducible P-450Ia1 mRNA levels (45).

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In summary, we have shown that pregnancy and development...
can affect genetic damage at the cellular as well as molecular levels. The extent of alteration was determined by the type of genetic damage studied and the stage of gestation or organ development in which the effect was examined. In the early days of gestation, sensitivity to MN induction increased relative to late gestation. An inverse effect was observed for DNA adduction. The phenomenon was similar for both fetuses and adults. Together, pregnancy and development represent a period of altered sensitivity to genetic damage and possibly carcinogenesis (46). Fetuses appeared to be more sensitive than adults to the cellular damage induced by BP. Information obtained from this study can be useful in improving the testing protocols used in carcinogenicity studies.

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