A common carcinogen benzo[a]pyrene causes p53 overexpression in mouse cervix via DNA damage

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A B S T R A C T
Benzo[a]pyrene (BaP) is cytotoxic and/or genotoxic to lung, stomach and skin tissue in the body. However, the effect of BaP on cervical tissue remains unclear. The present study detected DNA damage and the expression of the p53 gene in BaP-induced cervical tissue in female mice. Animals were intraperitoneally injected and orally gavaged with BaP at the doses of 2.5, 5, and 10 mg/kg twice a week for 14 weeks. The single-cell gel electrophoresis (SCGE) assay was used to detect the DNA damage. Immunohistochemistry (IHC) and in situ hybridization (ISH) were used to detect the expression of p53 protein and p53 mRNA, respectively. The results showed that BaP induced a significant and dose-dependent increase of the number of cells with DNA damaged and the tail length as well as Comet tail moment in cervical tissue. The expression level of p53 protein and mRNA was increased. The results demonstrate that BaP may show toxic effect on the cervix by increasing DNA damage and the expression of the p53 gene.

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1. Introduction

Benzo[a]pyrene (BaP) is a representative compound in the case of polycyclic aromatic hydrocarbons (PAHs), which shows cytotoxicity and/or genotoxicity in lung, stomach and skin of the body. Epidemiological studies indicated that cigarette smoking is one of the cofactors and doubles the risk of cervical cancer. BaP is an important component of tobacco smoke. However, the effect of BaP on cervical tissue remains unclear. BaP is a known carcinogen. It has been proposed that the carcinogenic properties of BaP are from the increased amount of carcinogenic BaP metabolites and reactive oxygen species (ROS), which are both generated by cytochrome P4501A1 (CYP 1A1) during metabolic activation of BaP [1–5]. Reactive oxygen species including superoxide, H2O2, •OH and semiquinone radicals caused by BaP and the ultimate carcinogenic BaP-diolepoxides, chemically attack DNA to produce adducts [6,7]. If the DNA adducts are not correctly repaired by the DNA repair system [8–10], they can lead to further DNA damage and mutation.

The p53 gene, mapped to chromosome 17p13, encodes a 53-kDa nuclear phosphoprotein (p53). Normally, p53 functions as a tumor suppressor gene by blocking the replication of cells that have sustained DNA damage and by occasionally triggering programmed cell death through apoptosis [11–13]. PAHs, including BaP, have been found to cause p53 mutations, leading to lung in tumorogenesis [9,14].

Cervical cancer is the rapid, uncontrolled growth of severely abnormal cells in the cervix. It is widely accepted that specific human papillomavirus (HPV) types are the central etiologic agent of cervical carcinogenesis [15]. Epidemiological studies indicate that environmental and host-related cofactors act in conjunction with HPV to promote malignant progression of cervical lesions [16]. It has been proposed that cigarette smoking is one of the cofactors and doubles the risk of cervical cancer. Additionally, BaP, a potent inducer of carcinogenesis, has been detected in the cervical mucus of women [16–20]. Some reports have indicated that alterations in p53 expression are often observed in precancerous lesions and carcinomas of the uterine cervix [11–13,15]. However, the variation of p53 expression induced by BaP in cervical tissue is still unclear.

The aim of this study was to investigate the changes of BaP on DNA damage and p53 expression in cervical tissue of CD-1 mice. In addition, we evaluated whether a correlation exists between the
expression of p53 protein and DNA damage in cervix induced by BaP.

2. Materials and methods

2.1. Chemicals

Benzo(a)pyrene was purchased from Sigma (St. Louis, MO, USA). Collagenase 1A, RPMI-1640 medium and the fetal calf serum (FCS) were purchased from Pronutra (Madison, USA). Agarose with normal or low melting point were purchased from Bio-Rad (Heracles, CA, USA). The p53 monoclonal antibody and LSAB Kit K6800 were purchased from DAKO (Denmark). In situ hybridization detection kit was purchased from Santa Cruz Biotechnology, Co., Ltd. (USA).

2.2. Animals and housing

Female Crl: CD-1(ICR) mice (4–5 weeks old, 18–22 g) were purchased from the Experimental Animal Center of Xi'an Jiaotong University (Shaanxi Province, China). The mice were kept in a cross-ventilated room (22 ± 2 °C, relative humidity of 50–60%, and a 12 h: 12 h light–dark cycle). Mice received a minimum of 1-week acclimatization period before the beginning of experimental procedures. All the experiments on the mice were performed based on animal ethics guidelines of the Institutional Animal Ethics Committee in Xi'an Jiaotong University.

2.3. Experimental design

BaP was dissolved in sesame oil immediately before oral gavage or intraperitoneal injection. In the experiment to test the effect of BaP on DNA damage, 100 mice were used, 10 mice were analyzed in each subgroup. In the p53 protein and mRNA expression assay, 200 mice were used, 20 mice were analyzed in each subgroup. The animals were randomly divided into two groups, the intraperitoneal injection group and the oral gavage group. The intraperitoneal injection and the oral gavage groups were further divided into five subgroups, control, vehicle, low dose, middle dose and high dose subgroups of intraperitoneal injection and oral gavage. The low, middle and high dose subgroups were administrated with 2.5, 5 and 10 mg/kg body weight of BaP twice a week, respectively. The doses were fixed based on the investigations of BaP genotoxic effect through analyzing DNA damage in other studies [9,21]. The vehicle subgroups received equal volume sesame oil twice a week and the control subgroups were untreated until the termination of the experiment. Some of the mice died due to BaP administration during the experimental period, so the number of mice in each of the groups was lowered to 20 and 10 to maintain equality. The experiment lasted for 14 weeks.

2.4. Isolation of cervical cells

Cellular suspensions from cervixes were prepared by using collagenase 1A (2 ml, 0.1%) and being incubated during 30 min at 37 °C. Thereafter, cervixes were carefully cut free into a nylon (150 μm) filter-funnel placed in a sterile tube. The capsule was carefully cut open and the cells were broken up by adding 1 ml RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). The cellular suspensions were centrifuged at 40 × g for 3 min at room temperature. The resulting pellets were resuspended in 1 ml RPMI-1640 medium. Cell viability, as determined by both trypan blue exclusion technique and by the appearances of the ‘comets’ in the fluorescence microscope [22], was greater than 96%.

2.5. Alkaline single-cell gel electrophoresis (SCGE) assay

For DNA damage study, the procedure of SCGE assay was used. The procedure was conducted according to the protocol of Singh et al. [23] with minor alterations and also in accordance with Tice et al. [24]. In brief, cell pellets were mixed with 60 μl 0.5% low-melting point agarose, transferred to precooled (1.5% normal-melting point agarose) glass slides, and sealed with a coverslip. The slides were allowed to set on a cold plate and the coverslips were then removed. Next the slides were immersed in lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, 1% Triton X, 10 mM ethylsulfate, pH 10.0) and stored them at 4 °C overnight. To prevent possible DNA damage arising from cell manipulation, lysis and all subsequent steps were conducted under red light. After lysis, we placed the slides on a horizontal gel electrophoresis platform and allowed the DNA to unwind at 4 °C in alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na2EDTA, pH ≥ 12.5) for 20 min. Electrophoresis was performed at 25 V and 300 mA for 20 min; then the slides were covered with neutralization buffer (0.4M Trizma base, pH 7.5, 4 °C) for 2 × 8 min and air dried. The coded slides were stained with ethidium bromide (20 μg/ml) and evaluated under a fluorescence microscope (Nikon 027012; Nikon, Tokyo, Japan). Slides were scored and analyzed using an automated analysis system of SCGE assay Software. A total of 600 cells were randomly scored per experimental group for the evaluation of the distribution of DNA damaged cell classes. The extent of DNA damage was quantified by measuring the tail moment of 200 cells.

2.6. Immunohistochemistry (IHC)

Immunohistochemistry (IHC) for p53 protein was performed using the following antigen retrieval system and labeled with Streptavidin-biotin system (LSAB Kit K808; DAKO, Denmark). Micrometer sections of formalin-fixed, paraffin-embedded tissues were prepared, air-dried at room temperature overnight, incubated at 55 °C for 300 min in sodium citrate solution in xylene. After antigenic unmasking, they were immersed in 0.1 M citrate buffer (pH 6.0) and microwaved at 750 W power for 10 min. Slides were then treated with 3% H2O2 for 30 min in phosphate-buffered saline (PBS) to block endogenous peroxidase activity. The tissue sections were incubated with the primary p53 antibody (p53 DO 7; Dako, Denmark) for 1:50 dilution for 1 h at room temperature. Staining was achieved using a biotinylated secondary antibody from the LSAB Kit in 1:200 dilution for 20 min, and finally incubated with the streptavidin peroxides from LSAB Kit in 1:50 dilution for 20 min. The reaction products were visualized by immersing the sections in 3, 3′-diaminobenzidine (DAB) solution and counterstained with hematoxylin. Sections of colon carcinoma that were known to be positive for p53 expression were included as positive controls for p53. The negative control consisted of sections that were treated with the same technique with the primary antibody omitted.

The results were viewed using a light microscope (BX 51 microscope, Olympus, Tokyo, Japan). Two histologists who were blinded to the clinical information of the cervical samples independently evaluated the immunolabelling scores. The labeling intensity was graded semi-quantitatively and the HSCORE was calculated using the following algorithm: HSCORE = Σi (1+i), where i = intensity of labeling with a value of 1, 2 or 3 (weak, moderate or strong, respectively) and Pi is the percentage cells that stain at each intensity, varying from 0% to 100%.

2.7. In situ hybridization (ISH)

The levels of p53 mRNA were assayed with in situ hybridization (ISH) on 3 μm sections of cervical tissues using digoxigenin-labeled oligonucleotide probes at 1:100 dilution, according to the Digoxigenin Labeled Probes Detection Kit. The sequence of gene-specific oligonucleotide probes were 5′-CGAGCTCTCTCGACTGAGAAGAAC ATTTC-3′ for p53. Determined positive samples, as well as mRNA expression of β-actin were used as the positive control for probe sensitivity. Specificity of p53 mRNA expression was confirmed by performing in situ hybridization on the determined positive subgroups in the absence of the probes. The scores of hybridization signals were evaluated by two observers in a blinded fashion, according to Pathology Techniques.

2.8. Statistical analyses

In this study, statistical calculations were performed using the computer program SPSS® (version 13.0). In the SCGE assay, arbitrary classes of DNA damage in cell populations were defined according to the percentage of DNA in the tail: 0–20% (class 0), 20–40% (class 1), 40–60% (class 2), 60–80% (class 3) and >80% (class 4). For the frequency distribution of the different degrees of DNA damage between the experimental groups in SCGE assay, significance of the difference was determined by χ² test. For p53 protein expression in IHC assay, statistical differences were calculated using the Mann–Whitney U test. Results were expressed as mean ± S.D., differences were statistically analyzed with a one-way ANOVA. The difference in mRNA expression of p53 gene was compared with rank sum test among groups. In all analyses, statistical significance was defined as p < 0.05 throughout.

3. Results

3.1. Effect of vehicle on DNA damage and the expression of p53 gene in cervical tissue

The results showed that no significant difference was found in the vehicle groups as compared to the controls in DNA damage and the expression of p53 gene. In addition, no significant difference was found between the intraperitoneal injection and the oral gavage groups.

3.2. Effect of BaP on DNA damages in cervical tissue

To evaluate the effects of BaP genotoxicity on cervix, CD-1 mice were intraperitoneally injected and orally gavaged with various doses of BaP. DNA damage was measured using the Comet assay in cervical tissue of mice (Tables 1 and 2, Fig. 1). The data in Tables 1 and 2 showed that treatment of BaP induced a significant and dose-dependent increase of the tail length and the number or the percentage of DNA damaged cells compared to controls (p < 0.01, p < 0.001, respectively). Additionally, similar changes were observed for tail moment when compared to con-
3.3. Effect of BaP on p53 protein expression in cervix

Cervical tissues were immunostained with antibody to p53. The rate of the positive cells and the intensity of immunoreactivity were quantified. The p53 protein is localized in nuclei by p53 antibody staining (Fig. 2). The p53 protein positive cells were mainly localized to epithelial cells, meanwhile some are dispersed among stromal cells. In the control groups, few cells with p53 staining in nuclei were detected (Fig. 2A). BaP treatment significantly increased the rate of p53-positive cells and also enhanced the intensity of p53 immunostaining ($p < 0.001$). Furthermore, the rate of the positive cells and the intensity of immunoreactivity were higher with the dose of BaP (Fig. 2B–D and Table 2).

3.4. Effect of BaP on p53 mRNA expression in uterine cervix

In contrast to the localization of p53 protein in the cell nuclei by immunostaining, the accumulation of p53 mRNA in the cell cytoplasm can be detected by ISH. The cells with p53 mRNA were localized in both epithelial and stromal region of the cervical tissue. Compared with the control groups (Fig. 3A), BaP treatment significantly ($p < 0.05$) elevated the number of cells with p53 expression and enhanced the expression on levels of p53 mRNA in positive cells with the dose increased (Fig. 3B–D and Table 3). The result from the p53 mRNA transcription confirms the effect of BaP treatment on the cells in uterine cervix detected by p53 protein immunostaining.
Fig. 1. Effects of BaP on DNA damage of cervical tissue. (A) The tail moment were measured in cervix treated with different doses of BaP using Comet assay. The results are shown as the mean ± S.D. of triplicate slides. The asterisk denotes the significant difference from the control group (p<0.05, p<0.001). (B) Photographs are images of Comet cells in BaP treated and control groups (C, control; V, vehicle control; L, 2.5 mg/kg; M, 5 mg/kg; H, 10 mg/kg, respectively). In control and vehicle groups, there are minimal DNA damage and lack of DNA fragment migration away from the nucleoid. In the treated group by 2.5 mg/kg and 5.0 mg/kg of BaP, there is some DNA fragment migration from the core, forming the comet tail. In the treated group by 10 mg/kg of BaP, there is high degree of DNA damage and significant DNA fragment migration from the core.

Fig. 2. Effect of BaP on p53 protein expression in cervix by immunohistochemical staining. Positive cells are indicated by a yellow coloration of the nuclei. Labeled cells were observed mainly in intra-epithelia and dispersed in stroma. (A) p53 immunostaining in the control and vehicle control group: the percentage cells was 5%; the intensity of positive cell staining was estimated as score 0. (B) p53 immunostaining in cervical tissue in 2.5 mg/kg BaP treated mice: the percentage cells were 55%; the intensity of positive cell staining was estimated as score 1. (C) p53 immunostaining in cervical tissue in 5.0 mg/kg of BaP treated mice: the percentage cells were 78%; the intensity of positive cell staining was estimated as score 2. (D) p53 immunostaining in cervical tissue from 10 mg/kg BaP treated mice: the percentage cells were 85%; the intensity of positive cell staining was estimated as score 3. The original magnification was 400×.
3.5. The influence of DNA damage in p53 expression

To understand whether the p53 overexpression is indeed associated DNA damage, the correlation between p53 protein and the tail length levels were compared in different groups. The data given in Table 2 showed a highly significant positive correlation between p53 expression and DNA damaged levels \( r = 0.940, p = 0.00001 \) among the study animals upon statistical analysis.

4. Discussion

Previously, it has been reported that the cytotoxicity and genotoxicity of BaP or other PAHs can be detected in skin, liver or lung [25–28]. The effects of BaP on cervical tissue however remain unclear. In this study, we analyzed the effects of BaP on DNA damage and p53 expression in the mouse cervix. The result demonstrated that BaP induced DNA damage and increased p53 expression in the cervical tissue.

In the present study, both DNA damage and the expression of the p53 gene were very low in the vehicle groups, which indicate no effect of sesame oil. This result is similar to the reports from other labs [29,30]. However, the results showed that DNA damage and the expression of p53 gene were both affected in cervix by BaP treatment.

A large body of evidence has implicated that HPV is implicated in the pathogenesis of cervical cancer, but the potential etiological role of PAHs in cervical pre-cancer and cancer is not well understood. PAHs are widespread environmental contaminants formed as by products of combustion [31]. BaP is a representative promutagen of this family of chemicals. BaP treatment can lead to toxicity to cells from ROS or other radicals which result in the production of DNA damage [32–34]. Our previous reports produced further evidence that BaP induces excessive ROS after acute or chronic treatment in cervical tissue [35,36]. Hence, in the present study, the results of the SCGE assay revealed that DNA damage in cells from mouse cervical tissue induced by BaP treatment. If there is no adequate antioxidant protection, the excessive ROS production can initiate lipid peroxidation (LPO), which may cause oxidative DNA damage and further contribute to the initiation or the progression of carcinogenesis [37–39]. The observed increase in DNA damage in cervical tissue correlates with the increase in ROS production and/or decline in antioxidants status in cervical pre-cancer and cancer. Since DNA damage could lead to genetic disorders that occur at different stages of carcinogenesis, induction of such damage by BaP may in the long run promote development of cervical cancer.

BaP, as the model environmental pollutants, has been found to cause p53 mutations, leading to lung tumorigenesis [9,14]. In addition, mutations involving conserved regions of anti-oncogenes occur preferentially in tumors, without HPV infection [40]. Therefore, similar to the p53 gene mutation induced by BaP in lung cancer, there is a significant accumulation of p53 protein or mRNA levels in the cells of cervical tissue treated by BaP.

Generally, normal p53 protein has a very short half-life and the protein level is too low to be stained immunohistochemically [41,42]. By contrast, most mutant p53 proteins have a longer half-life and are easily detected with immunohistochemical methods [41,43]. The over expression of p53 is often considered to indicate aberrant p53 expression and the presence of mutant form that is deficient in regulating the cell cycle and apoptosis [44,45]. In our study, p53 over expression has been detected in normal epithelium, low grade and high grade precancerous lesions induced by BaP. Compared with other groups' results, the mRNA and the protein levels of p53 gene were relatively high in the normal epithelium and precancerous lesions of cervical tissue in our study [11,46,47]. This is probably due to mutated p53 accumulation in the cells induced.
by BaP treatment [48,49]. Recent studies have reported that the p53 expression is a late event in cervical carcinogenesis in humans [12, 50]. However, there are still some paradoxical results in which the p53 expression was shown increased in early stages of cervical lesions [50]. In our study, due to the fact that only premalignant lesions were observed in the cervix of BaP treated mice, we speculate that the increased expression of p53 induced by BaP is involved in the early step in the development of cervical carcinoma.

Additionally, Huang’s lab and Uchiyama’s lab reported that the expression of p53 is more frequent in squamous cervical carcinomas from the epithelium than that in adenocarcinomas from stroma [11,40]. However, Dimitrakakis’s study did not indicate significant differences between the expression of p53 in epithelium and in stroma [47]. In our study, the over-expression of p53 induced by BaP occurs in normal or precancerous lesions of cervical epithelial cells but rarely in stromal cells. This finding is consistent with Huang and Uchiyama’s results. Further, the results suggest that cervical epithelial cells, which are recognized as major targets for BaP are more susceptible to BaP-induced DNA damage [51]. Therefore, p53 gene mutations are mainly based on data for squamous cell carcinoma of the cervical epithelium. And p53 overexpression in adenocarcinomas in stroma has not been documented [12,40].

From our results, in cervical tissue treated by BaP, there is a positive correlation between DNA damage and p53 overexpression. This result confirmed the previous report on the accumulation of p53 expression increased by DNA damage in the nucleus [52–54]. The results suggest that BaP can lead to the formation of DNA single-strand breaks and/or the induction of mutations, both events being able to inactivate tumor suppressor genes such as the p53 gene. Recent studies have shown that a loss of function in the p53 gene results in a broad spectrum of malignancies [55,56]. So in our experiment, premalignant lesions observed might be due to mutational inactivation of the p53 gene in cervical cells exposed to BaP.

In conclusion, our results demonstrate that BaP treatment results in toxic effect on the cervix evident as increases in DNA damage and the expression of the p53 gene. BaP appears to have the potential to cause p53 gene mutations in the cervix of mice.

Conflicts of interest

The authors involved in this study have no conflicts of interest with respect to the work described in this report.

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