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Urinary excretion of mutagens and covalent DNA damage induced in the bladder and kidney after passive smoking in rats

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Abstract Using ^{32}P-postlabeling assay, we studied the effect of sidestream smoke of cigarettes, so-called passive smoking, on the covalent DNA adduct formation in an animal model. Urine samples of 18 rats, 9 male and 9 female, before smoking resulted in an average of 2.4 adducts per 1×10^7 nucleotides per 24-h urine of a rat in the target plasmid DNA after incubation for 2 h in vitro. Urine samples of 4 out of 6 rats after exposure to sidestream smoke induced additional adducts in the target DNA. The incidence increased to 17.5 adducts per 1×10^7 nucleotides per 24-h urine of a rat. Without exposure to smoke, no increase in the adduct formation was observed. Adduct formations similar to those induced in vitro were detected in the bladder and kidney DNA, but not in the testicular DNA, of the four rats exposed to sidestream smoke. These observations suggest that passive smoking causes covalent DNA damage of the cells in the bladder and kidney by excreting chemicals in urine. Passive smoking as well as active smoking might contribute to the bladder and renal carcinogenic process.

Key words Passive smoking · DNA damage · Bladder · Kidney · ^{32}P-postlabeling assay · Urine

Carcinogens have been identified in cigarette smoke [1]. About 40–80% of all bladder cancer cases have been associated with smoking cigarettes [19]. Smokers have a 2- to 4-fold increased risk over nonsmokers for developing bladder cancers [2, 19]. Sidestream smoke of cigarettes is known to contain higher levels of several carcinogens than mainstream smoke. This is particularly true of the aromatic amines [29]. An epidemiological investigation demonstrated that non-smokers married to heavy smokers and persons whose mothers smoked have an increased risk of lung cancer [4]. Urine of individuals exposed to sidestream smoke has an increased mutagenic activity in the *Salmonella*/microsome assay [3], which is about 90% accurate in detecting a wide variety of carcinogens as mutagens [17].

Although the pathogenesis of bladder cancer remains unclear, *ras* oncogenes have been found in bladder cancers. The mechanism by which *ras* genes have been activated as transforming genes in tumor cells involves a single point mutation [25, 27]. Evidence for the direct involvement of chemical carcinogens in the activation process has come from studies showing that the *ras* activation occurs in a number of chemically induced animal tumors [20, 30, 31], and that the in vitro modification of plasmids containing the normal c-Ha-*ras*-1 protooncogene with ultimate carcinogens generates a transforming oncogene [16]. Not only oncogenes, but also tumor suppressor genes have been found to be altered in bladder cancer cells [13, 24]. Some bladder cancers have genetic alterations including point mutations in the p53 tumor suppressor gene [24].

It is thought that most chemical carcinogens exert their biological activity through the covalent interactions of the reactive intermediates (ultimate carcinogens) with DNA [18]. During cell replication, the covalent DNA chemical products (adducts) can result in misincorporations of nucleotides, which may be within genes that control cell growth and lead to neoplasia [28]. The ^{32}P-postlabeling assay is a procedure for detecting the DNA adducts. DNA samples are digested to mononucleotides, and labeled with ^{32}P at the 5′ position. Then the adducted nucleotides were separated from the normal nucleotides by the thin-layer chromatography [11]. Recent modification of this assay has permitted a highly sensitive detection of DNA adducts (one adduct per 10^9 to 10^{10} nucleotides) [21].

In this report, to gain insights into the relationship between passive smoking and the bladder carcinogenic process, we examined the ability of urine, collected from

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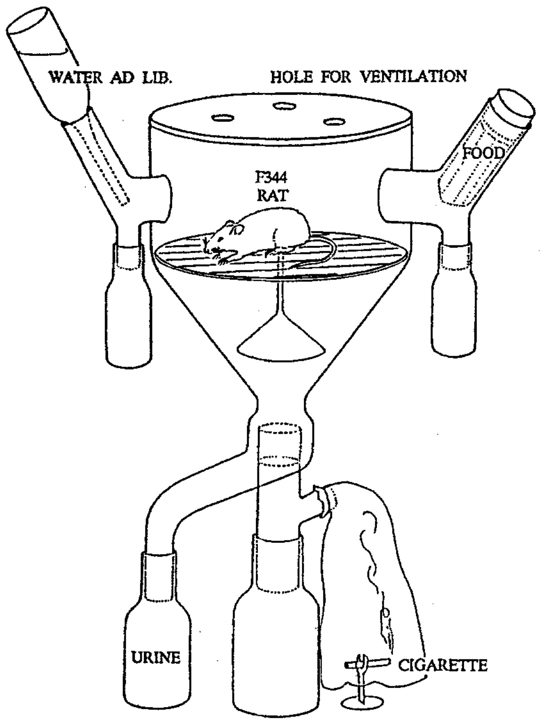

Fig. 1 Rat cage used for passive smoking. Sidestream smoke of a cigarette is led into the cage. Urine collected without contamination of food or feces

rats exposed to sidestream smoke of cigarettes, to chemically modify the plasmid DNAs in vitro. We also investigated whether exposure to sidestream smoke induced DNA adducts in the urinary bladder and kidneys of these rats.

Materials and methods

Animals

Eighteen Fisher 344 rats (9 male, 9 female) aged 7–8 weeks, weighing about 180 g (male) and 120 g (female), were obtained from SLC company, Japan. They were maintained on standard laboratory diet and water ad libitum.

Exposure to cigarette smoke and collection of samples

Two days before the initiation of passive smoking, rats were moved into an individual metabolic cage (5560 cm^3) as shown in Fig. 1 and urine samples were collected for a 24-h period everyday thereafter [14]. The sidestream smoke was collected from a burning cigarette (Sevenstars, Japan Tobacco Inc., Tokyo, Japan) and directed into the cage. Six rats (3 male and 3 female) were exposed to sidestream cigarette smoke for 15 min at 1, 2, 3 and 4 o'clock every afternoon for 5 days and urine samples were collected every 24 h. They were exposed for a total of 5 days and terminated on day 6. As a control 12 rats were caged but not exposed to smoke, and urine samples were collected every 24 h for a total of 5 days, and then terminated on day 6. The urine samples were individually filtered through Whatman No. 1 paper. Chemicals present in urine samples were concentrated by using blue cotton as described by Hayatsu et al. [12], and dissolved in 100 µl of ethanol. The urinary bladder, kidney and testis were removed from each rat and the DNA was extracted as described [6].

Treatment of plasmid DNA

Plasmid pP-1, which has an 8.3-kilobase *Bam*HI fragment of the normal human c-Ha-*ras* protooncogene inserted in a pBR322 vector was provided by the Japanese Cancer Research Resources Bank [27]. Plasmid DNA was extracted by using the Qiagen plasmid kit (Qiagen Inc. Studio City, Calif.) according to the manufacturer's instructions. The pP-1 plasmid DNA (10 µg/in 20 µl of 1 mM Tris-0.1 mM EDTA, pH 7.6) was mixed with 20 µl of the urine extract and incubated at 37°C for 2 h. The reaction mixture was extracted five times with ether. Plasmid DNAs were then precipitated with ethanol and stored at −20°C until assayed.

^{32}P-postlabeling assay

The DNA adduct formation was analyzed by the nuclease P_1 enhanced version of the ^{32}P-postlabeling assay [21]. Ten µg of DNAs were digested to 3′-phosphorylated mononucleotides by incubation at 37°C for 3 h with 3 units of micrococcal nuclease (Sigma, St. Louis, Mo.) and 0.03 unit of spleen phosphodiesterase (Boehringer Mannheim Yamanouchi, Tokyo, Japan) in 10 µl of 20 mM sodium succinate, 10 mM $CaCl_2$, pH 6.0. Then 2 µl of nuclease P_1 (4 µg/µl) (Sigma), 3 µl of 300 mM sodium acetate, pH 5.3 and 2 µl of 1 mM $ZnCl_2$ were added. After incubation at 37°C for 45 min, 3 µl of 0.5 M Tris base was added. Adducted nucleotides, which were not digested by nuclease P_1, were then [5′-^{32}P] phosphorylated at 37°C for 1 h using 5 units of polynucleotide kinase (Takara Shuzo, Kyoto, Japan) and 120 µCi of [γ-^{32}P] ATP with a specific activity of 7000 Ci/mmol (ICN Biomedicals, Inc., Costa Mesa, Calif.). Excess ATP was removed using 0.04 unit of potato apyrase (Sigma). Each sample was freed from normal nucleotides by chromatography on polyethyleneimine(PEI)-cellulose sheet (Macherey-Nagel, Postfach, Germany) in 2.3 M sodium phosphate, pH 6.0. The adducted nucleotides remaining at the origin were transferred to a fresh PEI-sheet and two dimensional thin layer chromatography was carried out with 4.5 M lithium formate-8.5 M urea, pH 3.5, in the first dimension and 0.8 M lithium chloride-0.5 M Tris-8.5 M urea, pH 8.0, in the second dimension. Areas of radioactivity on the chromatograms were located by autoradiography at −80°C for 72–96 h. These areas of the sheet were cut out with scissors and ^{32}P activities were determined by Cerenkov counting. Adduct levels were calculated according to:

$$\text{relative adduct labeling value (RAL)} = \frac{\text{cpm in adducted nucleotides}}{\text{cpm in total nucleotides}}$$

as described by Reddy et al. [21].

Results

DNA adducts induced in the plasmid DNA by urine

We analyzed whether urine elicited adducts in the plasmid DNA in vitro by using the ^{32}P-postlabeling assay. Each sample was assayed at least twice with two independent batches of plasmid DNA preparations. Under the conditions described, the plasmid DNA amplified in *E. coli* showed a few radioactive spots without any treatment. The average RAL value was $(0.13 \pm 0.044) \times 10^{-7}$ ($n = 4$).

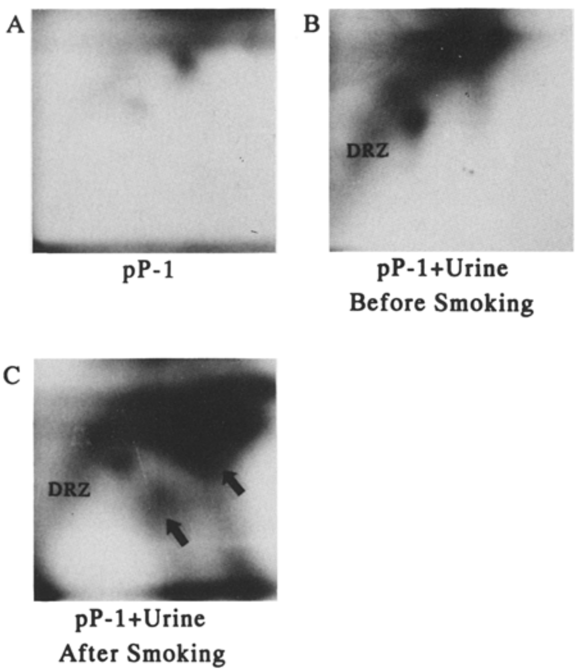

Fig. 2A–C Representative chromatograms of covalent DNA lesions induced by urine in vitro. Plasmid pP-1 DNAs were: (A) untreated; (B) treated with extracts of urine before passive smoking; (C) treated with extracts of urine after passive smoking. All were analyzed by the ^{32}P-postlabeling method. The diagonal radioactive zone (DRZ) was detected in the specimens treated with urine. Arrows indicate the passive smoking-associated DNA lesions

Fig. 2A shows a representative chromatogram. When the extracts of rat urine collected before exposure to cigarette smoke were assayed, they induced a diagonal radioactive zone of additional spots (Fig. 2B). The average RAL value for the diagonal radioactive zone was $(2.4 \pm 1.3) \times 10^{-7}$ ($n = 12$) per 24-h urine of a rat and significantly higher than that for the plasmid DNA without any treatment (Wilcoxon rank sum test, $P < 0.01$). In addition to this zone, two distinct zones appeared when the plasmid DNAs were treated with urine extracts from 4 out of 6 rats after passive smoking (Fig. 2C arrows). The average RAL value for the urine extracts of these four rats (3 male, 1 female) exposed to cigarette smoke was $(17.5 \pm 8.33) \times 10^{-7}$ ($n = 4$) per 24-h urine of a rat (Fig. 2C). When including 2 rats without the two distinct zones, the RAL value was $(12.9 \pm 9.47) \times 10^{-7}$ ($n = 6$) per 24-h urine of a rat and significantly higher than that for the urine extracts of rats before exposure to cigarette smoke (Wilcoxon rank sum test, $P < 0.01$) or that for the plasmid DNA without any treatment (Wilcoxon rank sum test, $P < 0.05$).

DNA adducts in bladders, kidneys and testes of rats

To assess whether urine of rats exposed to cigarette smoke induces DNA adducts in vivo, we made DNA preparations from bladders, kidneys and testes of the rats and analyzed them by the ^{32}P-postlabeling assay. We examined the testis as a control not exposed to urine. Each sample was assayed twice. Fig. 3 shows representative chromatograms for the bladder and kidney DNAs from 2 rats. The DNAs from rats without smoking showed few radioactive spots (Fig. 3 left). There were far less than those observed in the plasmid DNA in vitro, suggesting that the latter was mainly due to the use of *E. coli.* Qualitatively similar results were obtained with the other eleven control rats examined. In 4 rats urine after passive smoking induced the distinct radioactive zones in the plasmid DNA samples (Fig. 2C). In the bladder DNA from these 4 rats, one distinct radioactive zone and several small spots were noted after the 5-day-inhalation of cigarette smoke (Fig. 3A right). In each rat the kidney DNA also showed a distinct radioactive zone (Fig. 3B right). No additional radioactive spots were noted in the testicular DNAs, suggesting that the presence of urine contributed to the DNA adduct formation observed (data not shown). In the remaining 2 rats, no obvious radioactive zone appeared in the examined tissues despite exposure to cigarette smoke (data not shown).

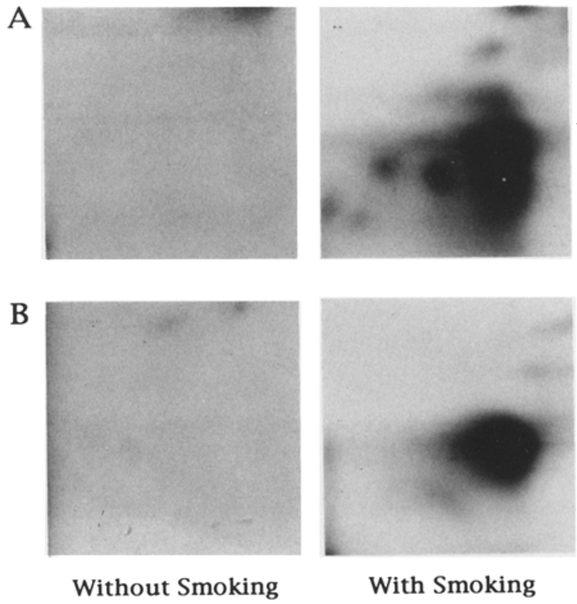

Fig. 3A, B Representative chromatograms of covalent DNA lesions found in rat tissues. DNAs were extracted from the bladder (A) and kidney (B) of rats unexpose and expose to passive smoking, and analyzed by the ^{32}P-postlabeling method

Discussion

A strong correlation between habitual smoking and incidence of cancer, including bladder cancer, has been demonstrated [2, 19]. Cigarette smoke is a complex mixture of over 3000 chemicals, among which *N*-nitrosamines are regarded as a major group of carcinogens [1]. Nitrosamines are known as a potent bladder carcinogen

[7]. The presence of benzo(a)pyrene, another bladder carcinogen, in the smoky atmosphere of social meeting rooms and restaurants was reported by Galuskinova [10]. Furthermore, in smoking related bladder cancers 4-aminobiphenyl, another bladder carcinogen, was recently identified as a major adduct [26]. Previously, we have demonstrated excretion of mutagens in urine after passive smoking as well as active smoking by using the salmonella mutagenicity assay (Ames' test) [14]. Here we demonstrated excretion in urine of chemicals that induced DNA adduct formation after passive smoking.

The ^{32}P-postlabeling assay has been used to demonstrate the presence of DNA adducts in the term placentae and other tissues of cigarette smokers [5, 9]. Nuclease P_1 modification of this assay permits a highly sensitive detection of such adducts (one adduct per 10^9 to 10^{10} nucleotides) [21] and adduct levels up to 16 adducts in 10^8 DNA nucleotides were observed in the bladder DNA of current smokers [5]. In this study we investigated whether chemicals present in urine after smoking could elicit adducts in DNA in vitro. The plasmid pP-1 DNA treated with urine of rats before smoking showed a radioactive area. This suggested that urine itself contained a component(s) modifying DNA. Whether it is related to the previous finding by others that the presence of urine increased the incidence of bladder cancer in an animal model is presently unknown [22]. In 4 out of 6 rats exposed to passive smoking, the urine induced additional spots in the plasmid DNA. The adduct levels in these samples were 17.5 in 10^7 DNA nucleotides. In these 4 rats, the chromatograms of bladder DNA after passive smoking showed radioactive areas that were qualitatively similar to the spots induced in the plasmid DNAs by urine of rats after passive smoking. Moreover, similar spots were found in the kidney of the same rats. Thus, the same chemical(s) present in urine seems to have induced the DNA adduct in vivo, although the identity of the chemical(s) remains to be determined. When one considers the volume of a cage (5560 cm^3), the concentration of sidestream smoke in the cage was about 10^4-fold higher than that in an actual room with smokers [3]. If a dose response relationship exists between smoke exposure and adduct levels, and if the metabolic pathways leading to cigarette smoke-associated adducts is common to the rat and human, 1-day-inhalation of the contaminated air by the passive-smoker will result in excretion per 24 h of urine capable of inducing one adduct in about 10^{10} nucleotides.

It has been reported that smoking is associated with a lower sperm count, less motile sperm, and a lower proportion of normally shaped sperm [8, 15, 23]. However, in none of the rats, including 4 rats whose bladder DNAs and kidney DNAs showed radioactive zones after smoking, did the chromatograms of testis DNAs show passive smoking-induced radioactive areas. These data are consistent with a notion that the DNA adducts found in the bladder and kidney DNAs were induced by chemicals excreted in urine. In the two rats exposed to passive smoking, the chromatograms of the plasmid DNA, bladder DNA and kidney DNA did not demonstrate radioactive areas. This indicates that although the genetic background is the same, some rats did not excrete adduct-inducing chemical(s) in urine, and suggests the presence of an intervention mechanism which determines individual susceptibility to the effect of passive smoking. The inability to detect DNA adducts in the bladder DNA exposed to normal urine in vivo is consistent with the presence of such a mechanism. The susceptibility of the testis to smoking-induced DNA damage may be different from other organs. Chronic exposure to passive smoking should be investigated to assess the effect of smoking on sperm.

In summary, this study has demonstrated that exposure to sidestream smoke of a cigarette causes urinary excretion of mutagens and induces covalent DNA damage in the bladder and kidney. Furter studies are required to explore the role of DNA damage induced by passive smoking in bladder and renal carcinogenesis.

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