Formation of DNA Adducts in the Skin of Psoriasis Patients, in Human Skin in Organ Culture, and in Mouse Skin and Lung Following Topical Application of Coal-Tar and Juniper Tar

Bernadette Schoket, Ph.D., Irén Horkay, M.D., Ágnes Kósa, M.D., László Páldeák, M.D., Alan Hewer, M.I.Biol., Philip L. Grover, Ph.D., and David H. Phillips, Ph.D.
Chester Beatty Laboratories, Institute of Cancer Research, London, United Kingdom (BS, AH, PLG, DHP), and Department of Dermatology, University Medical School, Debrecen, Hungary (IH, AK, LP)

Preparations of coal-tar and juniper tar (cage oil) that are used in the treatment of psoriasis are known to contain numerous potentially carcinogenic polycyclic aromatic hydrocarbons (PAH). Evidence of covalent binding to DNA by components of these mixtures was sought in a) human skin biopsy samples from 12 psoriasis patients receiving therapy with these agents, b) human skin explants maintained in organ culture and treated topically with the tars, and c) the skin and lungs of mice treated with repeated doses of the formulations following the regimen used in the clinic. DNA was isolated from the human and mouse tissues and digested enzymatically to mononucleotides. 32P-Post-labeling analysis revealed the presence of aromatic DNA adducts in the biopsy samples at levels of up to 0.4 fmol total adducts/µg DNA. Treatment of human skin in organ culture produced similar levels of adducts, while treatment with dichranol, a non-mutagenic therapeutic agent, resulted in chromatograms indistinguishable from those from untreated controls. In mouse skin, coal-tar ointment and juniper tar gave similar DNA adduct levels, with a similar time-course of removal: maximum levels (0.5 fmol/µg DNA) at 24 h after the final treatment declined rapidly to 0.05 fmol/µg at 7 d, thereafter declining slowly over the succeeding 25 d. However, while coal-tar ointment produced only very low levels of adducts in mouse lung (<0.03 fmol/µg DNA), juniper tar produced adducts at a high level (0.7 fmol/µg DNA) that were persistent in this tissue. These results provide direct evidence for the formation of potentially carcinogenic DNA damage in human and mouse tissue by components of these therapeutic tar preparations. J Invest Dermatol 94:241–246, 1990

Psoriasis is a proliferative, non-malignant skin disease that affects 2%–3% of the population. Several agents are available for the alleviation of its symptoms, including the topical application of coal and wood tars, sometimes in combination with other therapies [1]. These tars are known to contain carcinogenic PAH and to produce tumors in experimental animals [2]. Indeed, a single dose of a therapeutic preparation of crude coal tar has been found sufficient to initiate tumors in the skin of a sensitive strain of mice [3]. There is also evidence from case-control studies of an increased incidence of skin carcinoma among psoriasis patients who have received tar therapy [4], although other studies have found no appreciable increase [5]. Other treatments include the use of dithranol (anthralin), which, although not carcinogenic, has been shown to possess tumor-promoting activity in animal experiments [6,7].

Although most, if not all, chemical carcinogens are thought to exert their biologic effects through the formation of covalent adducts with cellular DNA, it has only recently become possible to assess the DNA-binding potential of complex mixtures of carcinogens following the development of several sensitive analytical techniques that do not require the use of radiolabeled carcinogens [8]. 32P-Post-labeling analysis is a highly sensitive method for the detection of DNA adducts [9] which is ideally suited to studying adduct formation by complex mixtures of aromatic compounds. The detection of adducts in human DNA has applications for the monitoring of human populations for carcinogen exposure [10,11]. We have previously used this method to demonstrate the DNA damaging ability of some PAH mixtures, namely, crude coal-tar, creosote and bitumen, in mouse skin and lung in vivo [12] and in human skin explants maintained in short-term organ culture [13].

In this study we have analyzed, by 32P-post-labeling, DNA isolated from skin biopsies of psoriasis patients treated with coal-tar ointment and juniper tar (cage oil), and present evidence for the formation of aromatic adducts in DNA by components of these complex mixtures. The adduct profiles bear similarities to those obtained with DNA from explanted human skin treated in culture

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Dr. Schoket’s permanent address: Department of Biochemistry, National Institute of Hygiene, Budapest, Hungary.

Reprint requests to: D. H. Phillips, Chester Beatty Laboratories, Institute of Cancer Research, Fulham Road, London SW3 6JB, UK.

Abbreviations:
BP: benzo[a]pyrene
PAH: polycyclic aromatic hydrocarbons
PEI-cellulose: polyethyleneimine-cellulose
and from mouse skin treated in vivo, and attest to the potential carcinogenic hazard of the dermatologic use of coal- and wood-tar products.

MATERIALS AND METHODS

Chemicals  Coal-tar ointment (Loriden T) was manufactured by Pharmaceutical Works Polfa (Jelenia Gora, Poland) and contained 0.2 mg flumethasone pivalate, 15 mg (1.5%) coal-tar, and 10 mg salicylic acid per gram of vehicle. Juniper tar (pix juniperis) was obtained from Adrienne (Marseilles, France). Dithranol was supplied by Fermion (Finland) and formulated as a cream (1% in vialine). Other chemicals, biochemicals, and materials for chromatography were purchased from previously mentioned sources [14].

Animals  Male Parkes mice (4 - 6 weeks old) were obtained from the National Institute for Medical Research (Mill Hill, London). Coal-tar ointment (approximately 45 mg, equivalent to 675 μg coal-tar) was applied with a glass rod to the shaved dorsal skin of the mice once daily for 5 d. Animals (four/group) were killed 1, 4, 7, 14, and 32 d after the last treatment, and the lungs and treated areas of skin were excised and stored frozen for subsequent DNA isolation. Mice treated with juniper tar received approximately 50 mg (50 μl), applied topically with the aid of a micropipette, once daily for 5 d, and groups were killed at the time intervals described above. Untreated control animals were killed on days 1 and 14 of the experiment.

Human Skin Explants  Samples of normal adult human skin were obtained through the Pathology Department of the Royal Marsden Hospital (Sutton, Surrey) from patients undergoing mastectomy or reduction mammoplasty. The preparation of samples for organ culture and the incubation conditions used have been described previously [13,15]. In the present experiments, samples from four individuals were each subdivided into eight 6 - 14-cm² pieces and treated in organ culture as follows: coal-tar ointment, 6 – 7 mg/cm² (equivalent to 90 – 105 μg coal-tar/cm²), spread with a glass rod; juniper tar, 6 – 7 mg/cm², applied with a micropipette; dithranol cream, 5 – 8 mg/cm² (equivalent to 50 – 80 μg dithranol/cm²), applied with a glass rod. Control samples of untreated skin were maintained in identical culture conditions, and duplicate samples of skin from each individual received each treatment. The skin samples were maintained in culture at 37° for 24 h after treatment in an atmosphere of 5% - 10% CO₂ in air and then frozen pending DNA isolation.

Human Skin Biopsy Samples  Samples of skin from psoriasis patients undergoing treatment at the Department of Dermatology of the University Medical School (Debrecen, Hungary) were obtained with informed consent under local anaesthesia. The biopsies (9 – 12 mm²) were taken from the arms of the subjects 24 h after the last of five daily treatments with coal-tar ointment or juniper tar. Of the 12 patients from whom biopsies were taken, two had received coal-tar ointment, four had received juniper tar, and the remaining six had received coal-tar ointment on one arm and juniper tar on the other. In each case one biopsy sample was taken from the treated area that contained psoriatic plaques, and another was taken from an uninvolved, untreated area immediately adjacent to the treated area. For control purposes, small portions of skin samples obtained at surgery (see above) were dissected and frozen prior to isolation of DNA.

DNA Isolation  DNA was isolated from mouse skin and lung and from human skin explants as described previously [12,13,16]. In order to isolate DNA from biopsy material, the samples were first cut into very small pieces on a glass slide with a scalpel blade, then homogenized in 10 mM EDTA (500 μl). A 10% solution of SDS in 10 mM EDTA (0.1 vol) was added followed by proteinase K (25 μl, 10 mg/ml). After incubation at 37° for 1 h, 1 M Tris (pH 7.4, 25 μl) was added and the solution extracted sequentially with phenol, phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1) (500 μl each). DNA was precipitated overnight at −20° after the addition of 5.0 M NaCl (0.1 vol) and ethanol (900 μl). Traces of RNA were removed by redissolving DNA in 1 mM EDTA (300 μl), adding 50 mM Tris (pH 7.4, 36 μl) and incubating with RNase (45 μg) and RNase T1 (22.5 U) for 15 min, extracting once with chloroform:isoamyl alcohol (300 μl), and precipitating the DNA as described above. Yields of 22.6 ± 9.9 μg DNA were obtained.

32P-Post-Labeling Assay  Samples of DNA (4 μg) were digested with micrococcal nuclease and spleen phosphodiesterase and then with nuclease P1 essentially as described previously [9,17] with minor modifications [13]. The DNA digest was then 32P-labeled with carrier-free [γ-32P]ATP (50 μCi, 1.85 MBq; synthesized in the laboratory [18] using 32P-orthophosphate obtained from Amersham International, Amsberham, Bucks) and polynucleotide kinase, and the reaction was terminated by the addition of apyrase [9]. Resolution of 32P-labeled adducts was then carried out by chromatography on polyethyleneimine-cellulose (PEI-cellulose) tlc sheets using the solvents described previously [14]. The presence of radiolabeled adducts on the chromatograms was detected by autoradiography at −70°C using intensifying screens.

Quantitation of DNA Adducts  For each batch of [γ-32P]ATP the specific activity of the laboratory-synthesized [γ-32P]ATP was determined by measuring the kinase-catalyzed incorporation of radioactivity into a known amount of dAp [17]. Values fell within the range 460-1750 Ci/mmol. The areas of the chromatograms containing 32P-labeled adducts were excised and the levels of radioactivity were determined by Cerenkov counting. Background levels, obtained by counting areas of the chromatograms not containing adducts, were subtracted. The levels of adducts present in a DNA sample were then calculated from the radioactivity present in the adduct spots and the specific activity of the [γ-32P]ATP used and expressed as fmol adducts/μg DNA (1 fmol/μg DNA is equivalent to 33 adducts/10° nucleotides).

RESULTS

Adducts in Mouse Skin and Lung  Treatment of mouse skin with coal-tar ointment or juniper tar resulted in the formation of aromatic DNA adducts in both the epidermis and the lungs of the animals (Fig 1). Radioactive spots were detected in both tissues lying principally in a diagonal band stretching from close to the

Figure 1. Autoradiographs of PEI-cellulose tlc maps of 32P-labeled digests of DNA from mouse skin and lung. Mice were treated topically with coal-tar ointment or juniper tar for 5 d as described in the text and killed on day 6. 24 h after the final treatment. Control animals were untreated. DNA was isolated, digested enzymically, subjected to 32P-post-labeling, and chromatographed as described in the text. The origin is located at the bottom left-hand corner of each chromatogram and was excised before autoradiography, which was carried out at −70° for 3 d. The maps shown are of DNA from (a) skin of control mice, (b) skin of mice treated with coal-tar ointment, (c) skin of mice treated with juniper tar, (d) lungs of control mice, (e) lungs of mice treated with coal-tar ointment, (f) lungs of mice treated with juniper tar.
origin to near the center of the thin-layer chromatograms. Although the adducts were not completely resolved into discrete spots, some major spots were evident within the radioactive bands. The patterns seen thus indicate the formation of a complex mixture of adducts by a variety of components. Radioactive areas were either absent or present at very low intensity in DNA from control animals (Fig 1a,d). All DNA samples showed the presence of an area of radioactivity, located midway from the vertical edges of the chromatograms and approximately one-quarter of the distance from the lower edge, which we previously referred to as ‘spot X’ and is seen in all DNA samples subjected to 32P-post-labeling using nuclease P1 digestion [10,14].

Where, as here, adducts formed by unknown compounds are detected, some uncertainty inevitably exists in their quantitation. The use of nuclease P1, enhancement of sensitivity limits detection of adducts to those types that are resistant to 3′-dephosphorylation by this enzyme [19], and adducts with small carcinogen moieties (e.g., alkyl substituents) require different chromatographic conditions from those used here [20]. Thus, while the conditions used here are well suited to the detection of adducts formed by PAH, the class of compounds chiefly responsible for the biologic activity of the tars [2], adducts formed by some other types of compounds present in the mixtures, may go undetected or may be underestimated.

Figure 2 shows the levels of adducts present in mouse skin and lung at various times after the animals received five daily treatments of coal-tar ointment or juniper tar. The levels of total detected adducts formed by coal-tar ointment in skin declined rapidly from a level of 0.5 fmol/μg DNA 1 d after the final treatment to one-tenth of that level after 7 d, after which a gradual removal of the remaining adducts was observed, until no detectable level of adducts was observed after 32 d (Fig 2a). Only very low levels of adducts were detected in lung DNA. In the skin of mice treated with juniper tar, a similar time course of adduct formation and removal was observed as was seen with the coal-tar ointment treated mice, the initial level of adducts at 24 h being approximately 0.37 fmol/μg DNA (Fig 2b). However, the levels in lung DNA were in marked contrast to the results obtained with coal-tar ointment. A higher level of adducts was found in the lung (initially 0.64 fmol/μg DNA) than in the skin, and the adducts were found to persist at or about this level for the entire duration of the experiment (Fig 2b).

**Adducts in Cultured Human Skin** Examples of the chromatograms of 32P-labeled digests of DNA from human skin explants treated with coal-tar ointment or juniper tar are shown in Fig 3. A diagonal area of radioactivity was seen in the analyses of samples from treated pieces of skin, indicating the presence of adducts. faint areas of radioactivity were seen with DNA from pieces of skin that were maintained in culture as untreated controls (Fig 3a,d), similar to those observed in a previous study [13]. In the case of the coal-tar ointment-treated skin, the adduct profile had the appearance of a radioactive band composed of many different adducts, although as with the mouse DNA profiles (Fig 1), there was some evidence for the existence of discrete major components within the band (Fig 3b,e). With juniper tar-treated samples, the area of radioactivity on the chromatograms was larger than with coal-tar ointment, indicating the formation of a greater diversity of DNA adducts than in the latter case. In two experiments, DNA samples from pieces of skin that had been treated with dithranol were found to have chromatograms identical to those obtained with DNA from untreated portions of skin from the same individuals (data not shown).

In order to quantitate the levels of adducts in the DNA samples, areas of the chromatograms containing adducts were assayed for radioactivity without including areas very close to the origin or the horizontal and vertical axes, because these areas contained relatively high backgrounds. Figure 4 shows the levels of adducts determined for samples of skin derived from each of four individuals. It cannot be stated unequivocally whether the adducts are due directly to components of the applied preparations or to enhanced formation of adducts by endogenous components, but the first possibility would seem from previous work with pure PAH [14,21] to be much the more likely. The levels of background radioactivity in untreated samples were generally less than the equivalent of 0.15 fmol/μg DNA but varied slightly between individuals. Treatment with coal-tar ointment led to a slight increase in adduct levels above those of controls, while juniper tar produced adduct levels significantly higher than in controls, particularly in the case of sample 1 (mean...
value 0.9 fmol/µg DNA; see Figs 4 and 3). In the two experiments where skin samples were treated with dithranol (samples 3 and 4, Fig 4), the amount of radioactivity on the chromatograms was reproducibly similar to the levels observed in the untreated control samples.

**Adducts in Human Skin Biopsies** Analysis of DNA from biopsies of the skin of psoriasis patients treated with coal-tar ointment or juniper tar produced the chromatograms depicted in Fig 5. A diagonal band of radioactivity, similar to that obtained with mouse skin DNA (Fig 1) and DNA from cultured human skin explants (Fig 3), was observed in each case, together with discrete spots of greater mobility visible in the upper-right quadrant of the chromatograms. Also, the existence of major adduct spots within the diagonal band was more evident in some samples than in the in vitro samples, suggesting a greater degree of selectivity in DNA binding. Biopsy samples taken from areas of skin immediately adjacent to the treated areas gave adduct patterns identical to those obtained from treated areas, with similar levels of adducts (see below), indicating the spread of the components of the medications responsible for DNA binding into the adjacent areas. These samples were therefore considered as duplicates of the treated samples.

In order to provide control samples of skin for the psoriasis patient skin samples, DNA was extracted from small pieces of skin obtained at surgery from non-psoriasis patients and analyzed by 32P-post-labeling. These experiments resulted in chromatograms very similar to those shown in Fig 3a,d, which demonstrates that the levels of radioactivity seen in the incubated control samples are not a significant consequence of their having been maintained in short-term organ culture.

The adduct levels present in the biopsy samples are shown in Fig 6. Results are the mean values of 3–6 determinations. Six control samples all had levels of radioactivity in the diagonal region of their chromatograms equivalent to less than 0.1 fmol/µg DNA. All but one of the samples from coal-tar ointment-treated skin contained adduct levels greater than 0.1 fmol/µg DNA, the highest value obtained being 0.39 fmol/µg DNA. Similar results were obtained with the juniper tar-treated samples; one sample (from a different individual) had a total adduct level of less than 0.1 fmol/µg DNA, the remaining nine samples falling within the range 0.15–0.36 fmol/µg DNA. Analysis of the adduct levels in DNA from the biopsies that were obtained from each of the six patients who had received coal-tar ointment and juniper tar on different areas of skin did not show any obvious correlation between the levels of adducts obtained with one of the treatments compared to the adduct levels resulting from the alternative treatment.

**DISCUSSION**

Coal-tar preparations have been used for many years to treat psoriasis and other dermatologic complaints. Wood tars, such as cade oil, have also been widely used, and, although largely superseded by coal-tar, they are still used in some instances as an alternative or additional therapy. Furthermore, many non-prescription preparations, such as medicated shampoos, contain coal and wood tars as their active ingredients. In a comprehensive review of treatments for psoriasis [22], it was concluded that none could be considered to be without genetic or carcinogenic risk. Treatment with coal-tar carries a risk of skin cancer, albeit easily treatable, 3–6 times greater than that for psoriasis patients not treated with this agent. A major difficulty in providing conclusive evidence of the carcinogenicity of these therapies towards, for example, psoriasis patients, is the virtual absence of control subjects with more than very mild symptoms who have not received any treatment for their disorder. A further complication is the fact that, as psoriasis is a recurring disease, most patients have received a number of different remedies or combination therapies [1,4].

The purpose of the present experiments was, therefore, to provide some objective evidence of potential carcinogenic risk arising out of exposure of human skin to therapeutic tars. The finding that components of these mixtures produce covalent DNA adducts in human skin is evidence of the occurrence of events characteristic of tumor initiation. Although the doses of tar applied in these experiments were about 75-fold higher for juniper tar than for coal-tar (see Materials and Methods), these doses are comparable to those used in the therapeutic protocols, and, interestingly, they gave rise to similar levels of aromatic adducts in both human and mouse skin DNA. No evidence was found for modification by dithranol of DNA in human skin explants in culture, consistent with the finding that dithranol is non-mutagenic [23]. Although dithranol lacks tumor-initiating activity, it is a well established tumor promoter [6,7]; thus, its use in combination with [24] or subsequent to coal-tar therapy is disquieting and potentially of greater hazard to patients. Indeed, a recommendation for studies to test the proposition that

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**Figure 5.** Autoradiographs of PEI-cellulose tlc maps of 32P-labeled digests of DNA from human skin biopsies from psoriasis patients treated with coal-tar ointment (a,b) or juniper tar (c,d). The patients received daily treatment for 5 consecutive days and the biopsy samples were taken 24 h after the final treatment. DNA was isolated and subjected to 32P-post-labeling analysis as described in the text. Autoradiography of the chromatograms was for 3 d at −70°C.

**Figure 6.** Levels of DNA adducts formed in human skin isolated from psoriasis patients who had been treated with coal-tar ointment or juniper tar, as described in the text. Each point represents the mean of 3–6 determinations.
dithranol may promote the carcinogenic action of known initiators has been made [22]. In essence, this is because treatment with dithranol of patients who have previously received coal-tar or UV therapy means that there is a risk that a two-stage skin carcinogenicity experiment of the type classically carried out in mice [25] is being conducted in man.

Earlier studies on the formation of DNA adducts in human skin explants maintained in short-term organ culture and treated with crude coal-tar, creosote, and bitumen [13] revealed a range of adduct levels similar to that observed here. With some skin samples (e.g., sample 1) adduct levels were significantly higher in the DNA from treated areas than from controls, while with other samples differences between treated and control areas were less significant. These interindividual variations may reflect differences in the metabolic capability of different individuals to activate components of the mixtures to DNA binding intermediates [26]. Variations in the levels of adducts in the biopsy samples may arise as a result of the differential induction of cytochrome P-450 or other drug metabolizing enzymes following the repeated application of the substances [27–29].

In an attempt to reproduce the therapeutic protocols used in the clinic, mice were treated with five consecutive daily doses of the therapeutic agents and then killed at various times after the final dose. Nevertheless, the present results bear comparison with our earlier experiments in which mice received a single dose of two other complex PAH mixtures, namely, crude coal-tar and creosote [12]. As in the present study, a biphasic pattern of removal of adducts was observed, whereby the levels of adducts fell rapidly in the first 7 d from its maximum value at 24 h. Thereafter, adducts were removed more slowly so that a low level was still detectable 32 d after treatment with crude coal-tar, creosote, and juniper tar, but not with coal-tar ointment. The possible significance of this difference is not known. In the previous study [12] repeated doses of coal-tar, creosote, and bitumen to mouse skin led to an accumulation, over several weeks, of adducts in both the skin and lungs of the animals, the levels in the former tissue being approximately twice that in the latter. When cigarette smoke condensate was applied to mouse skin, higher levels of adducts resulted in lung and heart tissue than in the skin itself [30]. In the present study, however, five daily treatments of mouse skin with coal-tar ointment resulted in only very low levels of adducts in the lung, whereas treatment with juniper tar gave rise to levels of adducts in the lung that were not only twice as high as in skin, but that were also highly persistent. The precise reasons for these differences between mixtures is not known, but they do suggest widely differing pharmacokinetics. Nor is it known whether topical treatment of human skin in vivo with these preparations leads to the formation or persistence of adducts in tissues other than skin, although the detection of PAH in the urine, the urinary mutagenicity, and the chromosomal alterations in peripheral blood lymphocytes of psoriatic patients demonstrate that this probably does occur [31].

It is apparent from the autoradiographs shown in Figs 1, 3, and 5 that DNA adducts are formed in mouse skin and lung, and in human skin in vivo and in vitro by a large number of different components of the tars. The methods of DNA digestion and chromatography employed in this study are suitable for the detection of PAH adducts, and many studies have indicated that it is in the PAH-containing fraction of the mixtures that the carcinogenic activity chiefly resides [2]. The specific compounds responsible for the DNA binding remain to be identified, however. Antibodies raised against benzo[alpha]pyrene(BP)-modified DNA have elicited an antigenic response in DNA from mouse skin that had been treated with coal-tar, but these antibodies are known to cross-react with other PAH-DNA adducts [32]. Current investigations in our laboratory are concerned with improved resolution of the adducts observed on the chromatograms and comparison of their mobilities with those of adducts formed by reference hydrocarbons. Preliminary evidence indicates that material that comigrates with the major BP-DNA adduct is present in these samples (unpublished results). Experiments on other complex mixtures of PAH suggest that the DNA binding potential of BP is lower when present as a component of such mixtures than when applied as the pure compound [32,33]. The combination of 8-methoxypsoralen and ultraviolet light A (320 – 400 nm) (PUVA) is another regimen used in the treatment of psoriasis that can result in the formation of DNA adducts in exposed tissues. The use of monoclonal antibodies raised against psoralen-DNA adducts has recently led to the detection, by indirect immunofluorescence staining, of adducts in skin biopsy samples from three out of five psoriasis patients undergoing PUVA therapy [34].

In summary, the combined evidence of carcinogenic activity in experimental animals, case studies of skin tumor incidence in psoriatic patients, and the detection of PAH-adducts in both animals and human skin treated with pharmaceutical coal-tar and juniper tar is consistent with the hypothesis that these treatments present a genotoxic hazard to humans [22]. In view of this evidence it would seem desirable to reconsider the use of non-prescription formulations containing tar products and, when treating psoriasis patients with dithranol, to take into account possible past treatment with coal-tar and other genotoxic therapies. Alternative widely used treatments for psoriasis, including phototherapy and chemotherapy, cytostatic agents and retinol derivatives, may also be accompanied by side effects such as genotoxicity. Thus, the appropriate individual treatment and consideration of its therapeutic benefits and potential hazards should remain the responsibility of the clinician.

REFERENCES