Carcinogenesis vol.17 no.12 pp.2741–2745, 1996

Transplacental mutagenicity of cisplatin: H-ras codon 12 and 13 mutations in skin tumors of SENCAR mice

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Cisplatin is an anticancer agent sometimes used in pregnant women. It is also a potent initiator of skin tumors in mice when administered transplacentally. For characterization of the transplacental mutagenicity of cisplatin, tumors initiated in fetal skin by cisplatin or 7,12-dimethylbenz[a]anthracene (DMBA) and promoted by postnatal 12-O-tetradecanoyl-phorbol-13-acetate (TPA) were analyzed for H-ras mutations by ‘cold’ single-strand conformation polymorphism analysis and direct sequencing. The expected high incidence of exon II codon 61 mutations (20/20) was found in transplacental DMBA-initiated tumors, with no exon I change. By contrast, 6/10 cisplatin tumors had seven mutations in codons 12 or 13 of exon I, all at GpG dinucleotides. Four of these were unique codon 13 GGC -> GTC changes, significantly different from the DMBA group and from historical TPA-only controls. The activation of codons 12 and 13 by cisplatin is in accord with the known in vitro preference of cisplatin for GpG sites for intrastrand cross-linking adduct formation. These results provide the first evidence that cisplatin can act transplacentally to cause specific mutations in fetal skin that are not seen in skin tumors caused by treatment of adult skin with this agent. This is evidence for unique molecular fetal carcinogenic pathways and underscores concern about human fetal risk due to maternal cisplatin treatment.

Introduction
cis-Dichlorodiammineplatinum(II) (cisplatin*) is a broad spectrum anti-neoplastic drug that has been widely used by itself and in combination for the treatment of various types of cancers, including testicular, head and neck, urinary bladder and prostate carcinomas and pediatric osteogenic sarcomas (1). Since cisplatin-based chemotherapy is used clinically during pregnancy for the treatment of malignant ovarian and uterine tumors (2,3) and cisplatin can also reach the infant through the mother’s milk (4), the potential transplacental and transnatal effects of maternal cancer chemotherapy remain a significant concern to pediatricians caring for the infants of these patients. It is estimated that annually in the USA 3500 cancers (0.8% of all cancers in women) are diagnosed during pregnancy (5).

The observations that cisplatin causes a variety of mutagenic effects in mammalian cells in culture (6,7) and is a potent initiator of skin tumors in adult mice (8) have raised the possibility of perinatal carcinogenicity in this context. We recently investigated the transplacental carcinogenic effects of cisplatin in two rodent species, i.e. SENCAR mice and F344 rats, and found that tumors were initiated in fetuses of both species (9,10). We further showed that cisplatin crosses the placenta to cause fetal DNA damage in Patas monkeys during pregnancy (11).

A strong correlation exists in some situations between the type of carcinogenic agent used for tumor initiation and the nature of the resultant gene mutations. This has been termed the agent’s ‘mutagenic fingerprint’. In the present study, we investigated the pattern of mutations in the H-ras oncogene to study the mechanism by which cisplatin exerts its transplacental carcinogenic effect on the skin of fetal mice. The H-ras oncogene was chosen because point mutations in codon 61 activate it at high frequency in skin tumors chemically induced in adult mice, especially those induced by direct cutaneous exposure to 7,12-dimethylbenz[a]anthracene (DMBA) (12-14). DMBA also causes H-ras activation in mouse skin tumors initiated transplacentally (15,16). H-ras has been previously shown not to be activated in skin tumors initiated by cisplatin treatment of adult SENCAR mice (17); however, our decision to analyze the gene in comparable tumors initiated transplacentally was based on the concept that molecular pathways for tumorigenesis may change during tissue differentiation. To pursue this possibility, we analyzed skin papillomas, initiated transplacentally by either cisplatin or DMBA and then promoted by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) post-natally, for H-ras mutations.

Materials and methods

Animals and tumors
Animals and tumors have been described in detail previously (9). Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (NIH publication no. 86-23, 1985). Cisplatin was dissolved in 2.5% sterile aqueous sodium chloride solution, DMBA was dissolved in corn oil at 25 mg/ml. Briefly, pregnant SENCAR mice, 6-8 weeks old, received a single i.p. injection of cisplatin (7.5 mg/kg body wt) or DMBA (25 mg/kg body wt) or NaCl on day 17 of gestation. Starting at 4 weeks of age, offspring received topical applications of 2 μg TPA in acetone twice a week for 20 weeks (0.2 ml/application). Animals were killed at 25 weeks of age. Thirty four papillomas (10 cisplatin-initiated, 20 DMBA-initiated and 4 TPA-only-promoted tumors) were fixed in 10% buffered formalin and embedded in paraffin. The blocks were sectioned at 6-10 μm by using disposable blades and ribbons were floated in individual plastic weighing boats in a heated water bath, to avoid cross-contamination between blocks. The first and last slides cut from each block were routinely stained with hematoxylin and eosin for histological evaluation and used as a guide for selective tumor microdissection.

*Abbreviations: Cisplatin, cis-dichlorodiammineplatinum(II); DMBA, 7,12-dimethylbenz[a]anthracene; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; SSCP, single-strand conformation polymorphism.
The denatured tube, 1–5 μl Microcon 30-purified PCR product were added to a mixture the full-length mutation-containing SSCP positive controls. These products to synthesize 

were gel-separated by SSCP from any residual wild-type DNA, eluted and mutation-containing PCR products were made. These short PCR products and 1/100 (0.16 μM) of the usual PCR primer concentrations, short intermediate mutations were synthesized (Table I). By using wild-type DNA as the template activating the most commonly reported codon 12, 13 or 61 mouse directed PCR mutagenesis protocol (20). Antisense 20mer primers containing mutant controls

Positive SSCP mutant controls were constructed by the 'Megaprimer' site-directed PCR mutagenesis protocol (20). Antisense 20mer primers containing the most commonly reported codon 12, 13 or 61 mouse H-ras activating mutations were synthesized (Table I). By using wild-type DNA as the template and 1/100 (0.16 μM) of the usual PCR primer concentrations, short intermediate mutation-containing PCR products were made. These short PCR products were then used as the upstream 'Megaprimers', in conjunction with the usual downstream primers at their standard concentration of 16 μM, to synthesize the full-length mutation-containing SSCP positive controls. These products were gel-separated by SSCP from any residual wild-type DNA, eluted and re-amplified.

'Cold' SSCP analysis

'Cold' SSCP analysis (21) was performed as summarized below. In a 500 μl tube, 1–5 μl Microcon 30-purified PCR product were added to a mixture of 0.4 μl 1 M methylethylamine(II) hydroxide (Johnson Matthey Products, Ward Hill, MA), 40 μl Ficol loading buffer and sufficient 1 × Tris–borate–EDTA (TBE) buffer to make a total volume of 20 μl. For later work the TBE buffer was replaced with water and 6 μl 1 M NaCl. This mixture was heated to 95°C for 2 min and then chilled on ice. The denatured mixture was then subjected to electrophoresis in a 1 mm thick 8 × 10 cm commercially precast non-denaturing 20% polyacrylamide TBE gel (NOVEX Inc.). Gels were run at 300 V (37.5 V/cm, 30–34 mA) at a constant optimal temperature. Optimal conditions were determined empirically using the positive mutant controls: for the exon 19 1 bp product this was 5°C for 3 h; for the exon 1 156 bp product this was 28°C for 2 h at 300 V (or 3 h at 200 V); for exon II this was 5°C for 6 h. Constant temperature was maintained by recirculating 1.25 × TBE buffer from a thermostatically controlled chiller bath through both chambers of the electrophoresis cell, as described previously (21). Duplicate PCR reaction products were run side by side for the SSCP analysis.

Gels were stained with ethidium bromide (0.5 μg/ml for 25 min) or SYBR Green II at 1:1000 (Molecular Probes, Eugene, OR) or silver (Silver Stain Plus; BioRad Laboratories, Hercules, CA, or SilverXpress; NOVEX Inc.). If the non-wild-type SSCP bands appeared to comprise at least 25% of the yield (as estimated by the relative intensity of the stained bands), the PCR product was sequenced, as described below, without further amplification. We punched out non-wild-type SSCP bands comprising <25% of the total DNA with a 1 ml micropipette tip. The plug eluate was used directly as a template for 15–25 additional cycles of PCR amplification, using the same conditions as in the original PCR. Re-amplification from silver stained gels was as described previously (22). SSCP analysis was repeated, as described above, to compare the re-amplified and original PCR products for evidence of relative enrichment of the mutant bands. PCR products showing desired enrichment were then subjected to dideoxy sequencing, as described below.

All of the DMBA-initiated tumors were negative for mutations in exon I by SSCP analysis of the 91 bp product and all were positive for exon II

**Extraction of DNA**

DNA was extracted from the tumor-rich portions of the paraffin sections by standard octane extraction and proteinase K digestion procedures (18). A disposable scalpel blade was used to scrape the tumor area into 200 μl octane in a 500 μl tube. Following octane extraction, with vortexing for 1 h at room temperature, the tissue was pelleted and the octane removed. Washing the pellet with acetone (not ethanol, as originally described) and air drying removed traces of the octane. The tissue pellet was digested with 200 μl 0.2 mg/ml proteinase K (in proteinase K buffer (as described) at 37°C for 6 h. The digest was vortexed for 1 h to complete tissue disruption. The proteinase K was inactivated by heating the tubes to 95°C for 10 min. Undigested cellular debris was pelleted for 5 min in a microfuge. The supernate was washed three times in a Microcon 100 concentrator (Amicon, Beverly, MA) with 400 μl ddH₂O and the retenate was recovered in 200 μl ddH₂O.

**PCR amplification**

The PCR primers used for amplifying portions of H-ras exons I (91 and 156 bp products) and II (130 bp product) are shown in Table I. At least one primer for the 156 and 130 bp products was intron-based to avoid H-ras pseudogene amplification. Hot-start (19) was performed prior to the standard PCR For the exon I 91 bp product, this was 48–50 PCR cycles of denaturation (94°C, 45 s) and annealing (47°C, 45 s); for the exon I 156 bp product, denaturation (94°C, 60 s), annealing (60°C, 60 s) and extension (72°C, 60 s); for the exon II 130 bp product, denaturation (94°C, 45 s), annealing (43°C, 45 s) and extension (72°C, 45 s). A 7 min terminal extension step at 72°C followed amplification. PCR products were analyzed on 10% polyacrylamide mini gels (NOVEX Inc., San Diego, CA).

**Construction of positive single-strand conformation polymorphism (SSCP) mutant controls**

Positive SSCP mutant controls were constructed by the 'Megaprimer' site-directed PCR mutagenesis protocol (20). Antisense 20mer primers containing the most commonly reported codon 12, 13 or 61 mouse H-ras activating mutations were synthesized (Table I). By using wild-type DNA as the template and 1/100 (0.16 μM) of the usual PCR primer concentrations, short intermediate mutation-containing PCR products were made. These short PCR products were then used as the upstream 'Megaprimers', in conjunction with the usual downstream primers at their standard concentration of 16 μM, to synthesize the full-length mutation-containing SSCP positive controls. These products were gel-separated by SSCP from any residual wild-type DNA, eluted and re-amplified.

**Cold' SSCP analysis**

'Cold' SSCP analysis (21) was performed as summarized below. In a 500 μl tube, 1–5 μl Microcon 30-purified PCR product were added to a mixture of 0.4 μl 1 M methylethylamine(II) hydroxide (Johnson Matthey Products, Ward Hill, MA), 40 μl Ficol loading buffer and sufficient 1 × Tris–borate–EDTA (TBE) buffer to make a total volume of 20 μl. For later work the TBE buffer was replaced with water and 6 μl 1 M NaCl. This mixture was heated to 95°C for 2 min and then chilled on ice. The denatured...
Fig. 1. SSCP pattern of re-amplification-enriched exon I mutants (negative image of SYBR Green II stained gel). Lanes 1–7, cisplatin tumors; lane 8, TPA-only tumor; lane 9, DMBA tumor with wild-type sequence; lane 10, wild-type DNA. Wild-type (lane 10, -WT) and mutant (lanes 1–8, asterisks *) bands are indicated. The sequence of each was: lanes 1, 2, 4 and 5, codon 13 GTC; lanes 3, 6 and 8, codon 12, GAA; lane 7, codon 13, CGC; lanes 9 and 10: wild-type codon 12 GGA, codon 13 GGC. Traces of codon 12 GAA in lanes 1 and 7 are from gel surface contaminants of the punch-out process. These bands were not present in the original PCR product. Lanes 2 and 3 are the separate mutant alleles from cisplatin tumor 3 (Table II). ds indicates re-annealed double-stranded DNA.

Two were in codon 12 (GGA → GAA, Gly → Glu) and five were in codon 13 (GGC → GTC, Gly → Val in four cases; GGC → CGC, Gly → Arg in one case). One tumor had changes in both codons, but on separate alleles, giving six SSCP bands and a separate mutation in the band re-amplification product of each aberrant band pair. Three additional tumors in the cisplatin group presented codon 61 mutations. One was → CGA, Gin → Arg, as in the TPA controls, one was → CTA, Gin → Leu, as in the DMBA-initiated tumors, and one was → AAA, Gin → Lys.

Discussion

The occurrence of H-ras mutations at a high incidence in skin tumors initiated transplacentally by cisplatin is in marked contrast to the absence of such mutations in skin tumors initiated in adult mice by the same agent. This is molecular support for a conclusion, long held on the basis of dose–response and pathological characteristics, that fetal tissues have unique responses to carcinogenic insults (9,10). These results constitute strong evidence that cisplatin uniquely and specifically initiates mutations in codon 13, and possibly codon 12, of H-ras in the skin of fetal mice in a manner that is quite distinct from that of DMBA. The failure of cisplatin exposure of adult mice to lead to H-ras mutations in skin tumors, compared with the prevalence of such mutations following transplacental exposure, may be due to one or more of the following reasons: the cisplatin adducts may not form in the H-ras gene at the same rate in target adult skin cells; the adducts may be repaired faster (perhaps due to a different H-ras transcription rate) or with a higher fidelity in adult skin cells; the adducts may be bypassed more frequently or handled as a different type of misinformation during DNA replication in adult skin cells; there may be a preferred selection for a cisplatin-damaged gene other than H-ras in adult cells.

All seven of the exon I mutations found in this study were at GpG dinucleotides, in good agreement with reports on the in vitro mutational spectrum of cisplatin (23–25). GpG, ApG and GpNpG purine nucleotide pairs were the reported preferred targets (in that order) for the cisplatin intranuclear cross-linking adduct formation in vitro (26–28). The resulting mutations clustered around sites containing a 5'-AGG-3', 5'-AGA-3' or 5'-GAG-3' sequence, but also occurred outside the site of the actual cross-linked purines. Codons 12 and 13 (GGA and...
of those two prior TPA studies found predominantly codon 61 promoted mouse skin tumors (32,32). The combined results of prokaryotes compared with eukaryotes (30).

G GGC) of the mouse H-ras gene were therefore potential targets for cisplatin in vivo.

GpG adducts are the most common adducts formed by cisplatin in vitro, but ApG adducts are reported to be the more mutagenic. Both resulted in mutations which included all classes of base substitutions, dual alterations, plus and minus frame shifts and short deletions and duplications and show bias for the untranscribed strand. Some prokaryotic system studies show that the 5'-purine is targeted (26), while others find no difference in the mutability of the two positions (reviewed in 25). Both G → A transitions and G → T transversions are found, with varying preponderance depending on the test assay used. Bypass repair/replication may be one mechanism by which the dimer is handled during DNA replication. Excinuclease medium patch repair also removes cisplatin adducts (29). This combination could account for the diversity in the reported mutation spectrum in different in vitro cisplatin mutagenicity assays.

In view of these varied reported findings, the uniformity of the effect of cisplatin in our transplacental model is remarkable. Since the mutations in our study that were clearly assignable to cisplatin were at GpG sites, it appears that ease of adduct formation may have been one critical parameter. The rate and fidelity of adduct repair and translesional replication and the activating potential of the resulting amino acid substitutions were all likely to play an important role in determining the site and nature of the mutation.

The preponderance of G → T changes in codon 13 we found is consistent with those from another study. A single-stranded vector containing a GpG cisplatin adduct placed at codon 13 of the human H-ras gene was introduced into monkey cells. The resulting single base substitutions all occurred at the 3′ G position of the dimer adduct and were predominantly G → T (28). DNA replication in prokaryotic Escherichia coli through a similar single GpG cisplatin adduct, although also resulting in 75% G → T substitutions, occurs mainly at the 5′ G (26), not at the 3′ G, as found in our study and other eukaryotic studies. This may be due to major differences in the mechanism of cisplatin adduct repair and mutagenesis in prokaryotes compared with eukaryotes (30).

The results of the H-ras mutations in our TPA-only control skin tumors agree with two previous studies of TPA-only-promoted mouse skin tumors (32,32). The combined results of those two prior TPA studies found predominantly codon 61 mutations; 7/24 tumors carried CGA and 6/24 had CTA codon 61 changes, 1/24 had a GAA codon 12 change and 1/24 had a CGC codon 13 change; nine TPA tumors lacked H-ras mutations.

The codon 12 mutation found in two cisplatin tumors could arguably be assignable to the TPA treatment, as the same GAA change was noted in one of the four TPA-only controls in this study, but it is equally interpretable as consistent with a direct effect of cisplatin. However, the five mutations seen in codon 13 did not occur in the other groups of this study (although the sole CGC mutation has been seen in one prior TPA-only study) and the frequency is significantly greater (P = 0.0018 versus DMBA/TPA, P = 0.0077 versus the historical TPA controls above). With regard to the three exon II codon 61 changes in the cisplatin tumor series, two of these, CAA → CTA and CAA → CGA, could be related to TPA-only promotion rather than the cisplatin treatment. The CAA → AAA change, however, is rarely reported in TPA-only controls, but neither could it be accounted for by a purine–purine cisplatin dimer.

In summary, it appears that the H-ras gene is the activation target of skin papillomas initiated by cisplatin in the mouse fetus, especially at codon 13. This is in contrast to skin tumors caused by cisplatin in adult mice, in which H-ras is not changed, and to those initiated transplacentally by DMBA, in which codon 61 is the target. These results provide the first evidence that cisplatin can transplacentally initiate a unique and specific spectrum of in vivo mutations and that these are similar to those reported in vitro. The use of cisplatin chemotherapy in pregnant women can unavoidably result in transplacental exposure of the developing human fetus. Our results suggest that further study of human cisplatin exposure and follow-up of exposed progeny are warranted.

Acknowledgements

The authors thank Yih-Hong Shiao for help with selecting PCR primers and Lucy Anderson for manuscript revisions. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services nor does mention of trade names, commercial products or organizations imply endorsement by the US Government.

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