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Preclinical report

Preferential repair of the N-*ras* gene in K 562 cells after exposure to cisplatin

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Cisplatin is one of the most active and widely used anticancer drugs; however, its clinical efficiacy is often limited by the development of resistance. Since several studies indicated that *ras* oncogenes may modulate the cellular response to cisplatin or radiation, we investigated the gene-specific repair of the N-*ras* gene in the human K 562 cell line after exposure to cisplatin using a novel non-radioactive polymerase chain reaction inhibition assay. This assay is based on the fact that DNA lesions can block the Taq polymerase and thereby result in a decreased amplification of a damaged segment compared to the amplification of the same segment in a non-damaged template. The overall genomic repair rate was measured by atomic absorption spectroscopy. Immediately after cisplatin exposure no amplification segment was observed. However, a complete restoration of the N-*ras* gene (2.4 kb) was seen after 8 h post-treatment incubation. In contrast, only 60% of the overall genome was repaired at this time. Our results clearly indicate that cisplatin-induced DNA lesions are more efficiently removed from transcribed regions within the DNA, suggesting that the efficiency of DNA repair in a given gene may be correlated to its transcriptional activity. Since *ras* oncogenes control several cellular signal transduction pathways, known to be involved in DNA damage response, preferential repair of the N-*ras* gene could therefore be an important step to prevent inactivation of cellular defense mechanisms after exposure to genotoxic agents. [© 1999 Lippincott Williams & Wilkins.]

Key words: Cisplatin, gene specific repair, N-*ras*.

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Introduction

Cisplatin [*cis*-diamminedichloroplatinum(II)] is an active antitumor drug widely used in the treatment of human cancers.[1] However, its clinical efficacy is often limited by the development of resistance. Factors generally implicated in the expression of cisplatin resistance include alterations in transport of drug and amino acids, modified intracellular drug inactivation, altered oncogenes, differential DNA damage, and tolerance of DNA damage and/or its repair (for review, see Perez[2]). It is generally accepted that the antineoplastic activty of cisplatin is due to its bifunctional interactions with DNA,[3] resulting in the formation of four major Pt-DNA adducts which can be detected and quantitated immuno-chemically.[4] The mechanism by which cisplatin kills cells is not fully known, although the major intrastrand d(GpG) and d(ApG) cross-links block replication and transcription. Meanwhile, it is established that there is a considerable intragenomic heterogeneity of DNA repair. Using a quantitative Southern assay, Bohr *et al.*[5–9] provided the first evidence that DNA lesions are removed more efficiently from actively transcribed genes than from non-transcribed regions of the genome. In the meantime, polymerase chain reaction (PCR) inhibition assays have been developed to measure DNA repair even in small DNA fragments.[10–12] Several studies have demonstrated that cellular resistance to cisplatin or radiation can be enhanced by *ras* oncogenes.[13–15] *ras* proto-oncogenes encode 21 kDa inner membrane proteins (p21). Active p21 is known to be involved in cell proliferation/transformation and drug resistance by playing a central role in signal transduction pathways, and has been identified in 20–25% of patients with acute myeloid leukemia.[16] Since *ras* oncogenes are highly conserved in the mammalian genome, it has been suggested that an actively transcribed *ras* gene

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has significant potential to influence cellular behavior and therefore may be critical for cell survival after exposure to genotoxic agents.[17] This prompted us to investigate whether the N-*ras* oncogene is preferentially repaired after exposure to cisplatin as compared with the overall DNA repair rate.

Materials and methods

The K 562 cell line, derived from a patient with chronic myeloid leukemia in blast crisis,[18] was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco, Munich, Germany). Logarithmically growing cells were exposed to cisplatin (50 μg/ml) for 1 h resulting in a 99% reduction of cell growth as judged by colony forming assay. After drug exposure cells were washed and harvested immediately or after a 1, 8 or 24 h incubation period in drug-free medium, but in the presence of bromodeoxyuridine (5 μM) and fluorodeoxyuridine (1 μM) to density label the DNA replicated after damage. To negate any possible variations in growth state, cells grown on two or three different occasions were pooled. Cellular DNA was then isolated using a combined phenol-chloroform technique[19] and resuspended in TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). To fractionate parental and replicate DNA, aliquots of the DNA solution were centrifugated in CsCl gradients (n=1.3870, d=1.565 g/cm^3) in the presence of ethidium bromide (0.5 μl/ml of a 10 mg/ml stock). After isolation of parental DNA and extraction of ethidium bromide with isoamyl ethanol, DNA was resuspended in TE buffer and digested to completion with *Eco*RI (Boehringer, Mannheim, Germany) using 10 U/μg DNA for 48 h at 37°C in the supplier's recommended buffer resulting in a 9.7 kb restriction fragment.[20] After digestion the total platinum content in cellular DNA at each time point was assessed by AAS using a Perkin-Elmer spectrometer as described previously.[21] The oligonucleotide primers and the detection probe for a 2.4 kb segment of the N-*ras* gene, shown below, were prepared by Pharmacia (Freiburg, Germany).

Sense start primer:
5′-AGT ACA AAC TGG TGG TGG-3′
Antisense stop primer:
5′-TAG AGG TTA TCC GCA-3′
Detection oligonucleotide (digoxigenated):
5′-CCT TCG CCT GTC CTC ATG TAT TGG TCT CTC-3′

All amplification reactions were performed according to the methodology described by Kalinowski *et al.*[22] This assay is based on the fact that DNA lesions can block the Taq polymerase and thereby result in a decreased amplification of a damaged segment compared with the amplification of the same segment in a non-damaged template. A typical 100 μl PCR reaction contained 50 mM KCl, 10 mM Tris (pH 9.0), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.1% Triton-X, 200 μM of each dNTP, 1 μM of each primer, 2.5 U of Taq polymerase (Boehringer) and 0.5 μg digested DNA. All samples underwent initial denaturation at 94°C for 5 min, followed by 25 cycles of template denaturation at 94°C for 1 min, primer-template annealing at 49°C for 1 min and primer extension at 72°C for 2 min. At the end of either 10 or 23 cycles, the extension reaction was continued for an additional 7 min. Following amplification, the amplification product was analyzed by electrophoresis in a 1% vertical agarose gel in the presence of ethidium bromide. DNA was then transferred to a nylon membrane (Boehringer) in 0.4 M NaOH. Membranes were blocked with caseine, prehybridized in 3 M tetramethylammonium chloride (pH 8.0) followed by hybridization with the digoxigenated detection oligonucleotide (10 pmol/ml tetramethylammonium chloride) at 54°C overnight. The remaining oligonucleotide was removed and membranes were then subjected to the AMPPD detection assay for digoxigenin-labeled oligonucleotides (Boehringer) according to the suppliers instructions (anti-digoxigenin immuno assay). Finally, membranes were exposed to a Cromex-4-film (Kodak, Dusseldorf, Germany) overnight at room temperature. The experimental procedure is summerized in Figure 1.

Results

The formation and removal of total cisplatin lesions in the genomic DNA of K 562 cells after exposure to 50 μg/ml cisplatin for 1 h, as judged by atomic absorption spectroscopy, is shown in Figure 2. The repair kinetics appeared to be biphasic; approximately 60% of the cisplatin lesions induced were removed after 8 h, whereas after 24 h between 20 and 25% of adducts still remained unrepaired. The gene-specific repair of cisplatin induced DNA lesions in the N-*ras* gene was measured using a PCR inhibition assay. Assuming that cisplatin adducts may block Taq polymerase, any decreased amplification of a damaged DNA template (compared with the same amount of non-damaged template) is a measure for adducts remaining unrepaired. Thus, repair of cisplatin-induced DNA damage was assessed by observing whether reculture of the cisplatin-exposed cells

restored the template activity of the subsequently extracted genomic DNA. A typical experiment is shown in Figure 3. Immediately after cisplatin exposure no amplification segment was observed; however, a complete restoration of N-*ras* gene segment amplification (2.4 kb) was seen after a postincubation period of 8 h indicating that all cisplatin-induced DNA adducts were repaired.

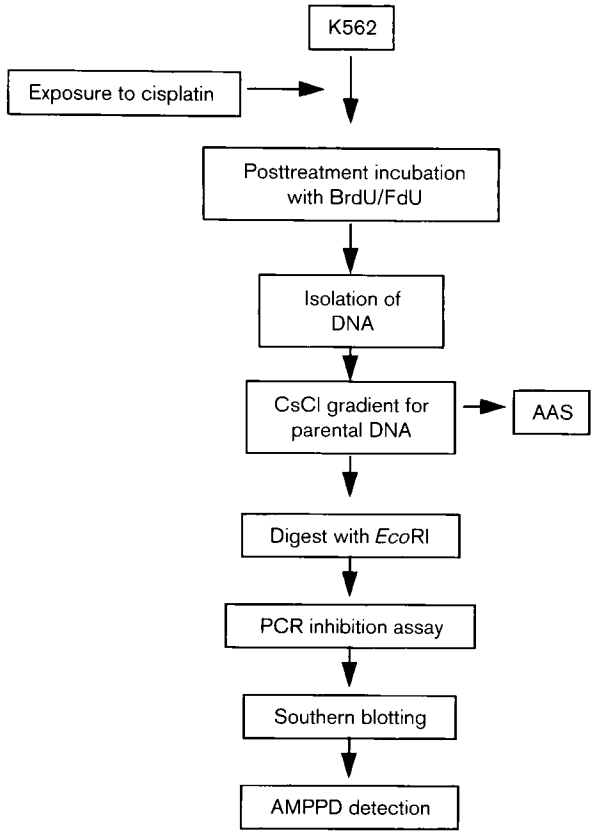

Figure 1. Flow diagram of the assay for measuring cisplatin adducts in the N-*ras* oncogene.

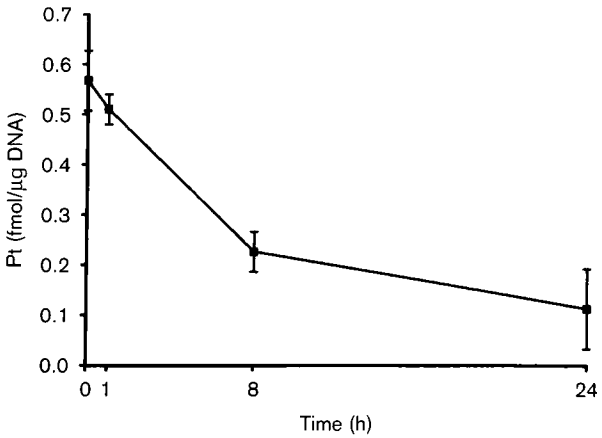

Figure 2. Decline of total DNA platination in K 562 cells following exposure to cisplatin.

Discussion

Using a PCR inhibition assay, we have attempted to investigate whether the N-*ras* oncogene is preferentially repaired in K 562 cells after exposure to cisplatin since this cell line is known to express an actively transcribed N-*ras* oncogene.[23] Our data clearly indicate that cisplatin-DNA adducts are completely removed from the N-*ras* gene after 8 h, whereas only 60% of the overall genome is repaired at this time. These results confirm earlier studies published by Bohr *et al.*[6,8] who also demonstrated a preferential repair of cisplatin adducts in the dihydrofolate reductase and c-*myc* gene using human and mammalian cell lines. Most recently, Guo *et al.*[24] studied gene-specific DNA repair of UV-induced cyclobutane pyrimidine dimers within the albumine and H-*ras* gene of non-dividing rat hepatocytes using the T4 enonuclease V methodology. In this study they clearly demonstrated that even in non-proliferating cells UV-induced DNA damage was preferentially repaired in the actively transcribed albumin gene when compared with DNA repair in the overall genome. In contrast, no specific repair was observed for the H-*ras* gene. However, this lower repair rate seen in the 14 kb *Bam*HI fragment investigated (containing the H-*ras* gene) was most probably indicative of the absence of repair in the non-transcribed region of this fragment since the H-*ras* gene (exons 1-2) made up only 2.4 kb of the 14 kb fragment.[24] Thus, the PCR inhibition assay employed in our study may therefore be advantageous since much smaller DNA segments can easily be examined.[12,25] Our observation that cisplatin-induced DNA lesions are more efficiently removed from transcribed regions within the DNA adds weight to the proposal that the efficiency of DNA repair of a gene may be correlated to its transcriptional activity. Since genes only constitute a small proportion of the overall genome (less than 1%), any changes in gene-specific repair will not be detected using DNA repair assays at the level of the overall genome. Thus, accumulation of DNA damage in certain genomic regions, caused by localized repair heterogenity, may be more important than overall levels of DNA damage or repair.

In an earlier report, Madhani *et al.*[26] provided the first evidence that the active c-*abl* gene was 4-fold more efficiently repaired than the inactive c-*mos* gene in mouse cells. In contrast, Dong *et al.*,[27] using a method based on DNA denaturation/renaturation gel electro-

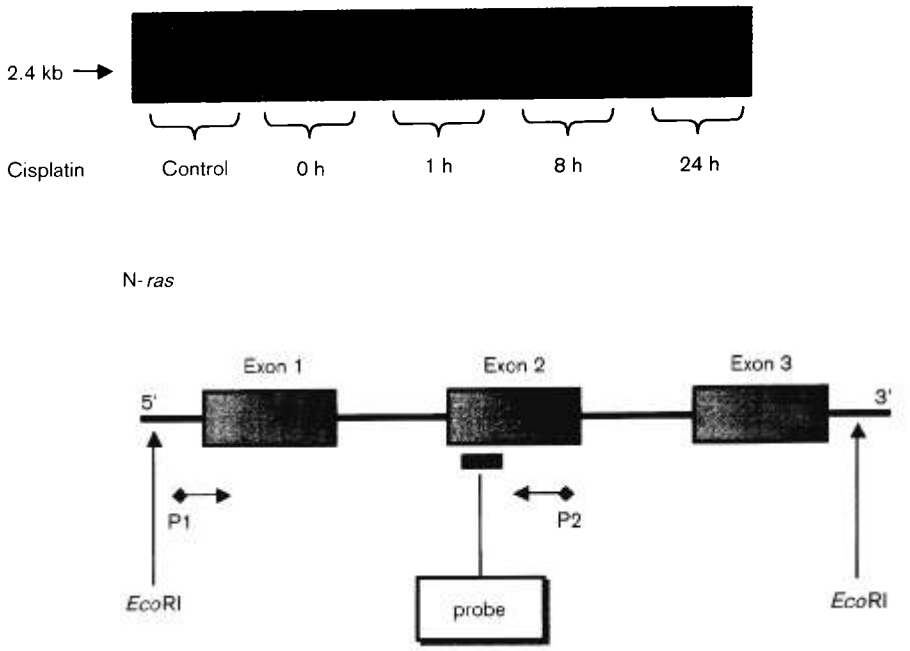

Figure 3. Southern blot demonstrating the repair of cisplatin-induced DNA damage in the N-*ras* oncogene following various postincubation periods. The *upper* part shows the PCR amplified 2.4 kb fragments of the N-*ras* gene. Cisplatin-induced DNA damage is completely repaired after 8 h. The *lower* part shows the genomic map of the N-*ras* gene. Digestion with *Eco*RI resulted in a 9.7 kb DNA segment. The primers used (P1; P2) span a 2.4 kb fragment of the gene containing exons 1 and 2.

phoresis, demonstrated that cisplatin-induced DNA interstrand cross-links in the c-*myc* gene persisted for 12–24 h. These results suggest that DNA repair can be highly variable in oncogenes and may be influenced by factors other than transcriptional activity. Data implicating activated *ras* genes as a mechanism of cisplatin resistance are gradually emerging,[13,15] although the biochemical processes underlying this phenomenon are poorly understood. A recent study provided evidence that the *ras* oncogenes regulate mitogen-activated protein kinases, which are required for *ras*-mediated DNA synthesis[28] after DNA damage. In addition, Milne *et al.*[29] have demonstrated that *ras*-controlled MAPs can also inactivate the tumor suppressor gene p53 which is thought to play a significant role in both cell cycle control and apoptosis,[30] suggesting that *ras* oncogenes may act as 'master genes' in the cellular response after exposure to genotoxic agents. In this context, preferential repair of the N-*ras* gene after exposure to cisplatin would therefore be an important step to prevent inactivation of cellular signal transduction pathways which would result in cell death. Currently, studies in our laboratory are underway to further elucidate the role of *ras* and *p53* in terms of cell cycle control and apoptosis after cisplatin exposure.

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