Gene-specific Formation and Repair of DNA Monoadducts and Interstrand Cross-links after Therapeutic Exposure to Nitrogen Mustards

Vassilis L. Souliotis,1 Meletios A. Dimopoulos, and Petros P. Sfikakis
National Hellenic Research Foundation, Institute of Biological Research and Biotechnology, Athens 11635 [V. L. S.], and Department of Therapeutics [M. A. D.] and First Department of Propedeutic Medicine [P. P. S.], Athens University Medical School, Athens, Greece

ABSTRACT

Purpose: To investigate the possibility of measuring the gene-specific DNA damage after therapeutic exposure to nitrogen mustards and to examine its relationship with the clinical response.

Experimental Design: The kinetics of gene-specific monoadducts and interstrand cross-link formation/repair were measured in the p53 and N-ras genes. DNA extracted from human peripheral lymphocytes following in vitro exposure to melphalan or therapeutic exposure to melphalan or cyclophosphamide was used.

Results: When lymphocytes were treated in vitro with biologically relevant doses of melphalan, monoadducts accumulated rapidly in both p53 and N-ras genes, reaching maximal levels within 2 h, whereas the highest interstrand cross-link levels were found within 8 h. Thereafter, the adducts were repaired with half-lives of 14.5 ± 0.3 h (p53) or 18.8 ± 1.5 h (N-ras) for monoadducts and 12.4 ± 0.8 h (p53) or 14.1 ± 2.2 h (N-ras) for interstrand cross-links. Moreover, peak levels of monoadducts in both genes were observed 2 h after treatment in peripheral leukocytes from patients with multiple myeloma treated with high-dose i.v. melphalan, supported by autologous stem cell transplantation, whereas interstrand cross-links were maximal within 8 h. Of seven patients examined, the three who showed the least levels of DNA damage did not respond to the high-dose melphalan.

Conclusions: This is the first report showing that it is feasible to measure gene-specific DNA damage in a readily accessible tissue of humans exposed to bifunctional alkylating drugs and to examine, at the level of the individual patient, the relationships between the induction/repair of cytotoxic DNA damage and clinical response or long-term complications.

INTRODUCTION

Nitrogen mustards are bifunctional alkylating agents, and one of the main classes of clinically used anticancer drugs. These drugs react extensively with cellular macromolecules (DNA, RNA, and proteins), thus inducing multiple kinds of molecular lesions. The critical cellular target of these agents is DNA, which is alkylated primarily at the N-7 position of guanine with lesser reaction at the N-3 position of adenine (1). Besides monofunctional binding of the drug to a single site in the DNA molecule (monoadducts), drug-induced cross-linking between bases in the complementary strands of a DNA molecule (DNA interstrand cross-links), as well as DNA-protein cross-links, also occur (2, 3). Nucleotide excision repair and base excision repair play a crucial role in the repair of monoadducts (4, 5). Furthermore, a number of multistep DNA repair pathways including nucleotide excision repair, homologous recombination, and postreplication/translesion repair all contribute to the DNA interstrand cross-link repair (6).

Cyclophosphamide is a nitrogen mustard widely used, in combination with other agents, for the treatment of lymphoma, multiple myeloma, breast cancer, and systemic rheumatic diseases (7, 8). It is a known carcinogen associated with therapy-related leukemia and bladder cancer (9, 10). Cyclophosphamide requires metabolic activation by the cytochrome P-450 enzymes to become an alkylating agent (11). It has been reported that the drug induces 67% phosphorriester monoadducts, 26% N-7-guanine monoadducts and 6.7% N-7-guanine-N-7-guanine diadducts (12).

The nitrogen mustard melphalan does not require metabolic activation to become an alkylating agent and forms ~38% N-7-guanine monoadducts, 20% N-3-adenine monoadducts, 20% N-7-guanine-N-7-guanine diadducts and 13% N-3-adenine-N-7-guanine diadducts (13). Melphalan can induce cancer: it gives rise to a variety of tumors in mice and rats; and there is some evidence that it can cause leukemia in humans (14). The role of melphalan today is essentially reserved for the management of multiple myeloma. Oral melphalan at low doses, in combination with prednisone, is still being considered as an acceptable primary treatment for elderly patients with multiple myeloma. Melphalan i.v. at high doses (200 mg/m²), with autologous stem cell support, is the standard high-dose treatment for many patients with multiple myeloma. Despite its remarkable activity, complete responses are noted in up to 40% of patients, long-term disease-free survival is seen in [l]10% of patients, and the majority of patients experience disease recurrence (15).

Evidence has accumulated that the biological role of DNA lesions must be evaluated in terms of the fine structure of DNA repair (16). Selective DNA damage and repair appear to affect...
cellular sensitivity to DNA-damaging agents, cancer susceptibility, aging, and other biological endpoints (17). It was shown previously that in Chinese hamster ovary cells there was heterogeneity of mechlorethamine-induced DNA damage, with active genes being damaged more than the bulk genomic DNA (18). Also, repair of mechlorethamine amage was faster in the active genes than in the genome as a whole or in noncoding sequences (18). Bartosova et al. (19) found that the genespecific repair activity of UV irradiation-induced cyclobutane pyrimidine dimers in CD4+ lymphocytes can reflect the proliferative state of the cells as well as the transcriptional state of the gene. Finally, similar relative rates and extent of cisplatin-induced interstrand cross-links repair were observed in active or silent genes, in human G0 or proliferating CD4+ lymphocytes (20).

This is the first report on the kinetics of gene-specific monoadducts and DNA interstrand cross-link formation and repair in a readily accessible tissue of cancer patients after chemotherapeutic exposure to melphalan or cyclophosphamide. Results of in vitro studies with lymphocytes from healthy volunteers after melphalan treatment are also given.

**MATERIALS AND METHODS**

**Patients.** Blood samples were collected after informed consent from seven consecutive patients with multiple myeloma and receiving (200 mg/m²; Table 1) and one polyarteritis nodosa patient treated with cyclophosphamide (1 g/m²). Among the seven multiple myeloma patients, only one had been previously exposed to standard dose, oral melphalan. This was patient 2, who had been exposed to melphalan and prednisone for 12 months as primary treatment, 5 years before the high-dose melphalan, and had achieved a partial response at that time. The remaining six patients have not received alkylating agents with the exception of venous blood were collected just before and various times after completion of drug administration. To avoid the decrease in DNA yield from blood samples stored at −70°C, 0.67 ml of 0.48 g/100 ml citric acid, 1.32 g/100 ml sodium citrate, 1.47 g/100 ml glucose solution per 4 ml of blood was added. The sample was immediately transferred to −70°C freezer and used for the extraction of DNA from whole blood.

**Peripheral Blood Mononuclear Cell Cultures.** Peripheral blood mononuclear cells were isolated from freshly drawn peripheral blood from healthy volunteers using standard methods (21). Peripheral blood mononuclear cells (3 × 10⁶ cells/ml) were treated with 5, 10, or 25 μg/ml melphalan for 1 h at 37°C in RPMI supplemented with 10% FCS. In all experiments, cell viability, assessed by trypan blue exclusion, was >95%. After incubation, the cell suspensions were cooled in ice and washed twice. For time course experiments, cells were resuspended in drug-free medium and incubated at 37°C for 2, 4, 6, 24, and 48 h. Cells were harvested and stored at −70°C. All experiments were performed in duplicates.

**Measurement of N-Alkylpurine Formation in the p53 and N-ras Genes.** Isolation of genomic DNA was performed as described by Sunters et al. (22) with minor modifications. After DNA isolation, genomic DNA was digested to completion with HindIII or EcoRI for the analysis of p53 or N-ras alkylation, respectively. Then, DNA samples, dissolved in sterile deionized H₂O, were heated at 70°C for 30 min to depurinate N-alkylated bases. Apurinic sites were converted to single-strand breaks by the addition of freshly prepared NaOH for 30 min at 37°C. The DNA samples were size-fractionated using a 0.6% agarose gel in Tris-borate EDTA at 2 V/cm for 16 h. The gel was transferred onto Hybond N⁺ (Amersham Pharmacia Biotech) as described by Southern (23).

Hybond N⁺ membranes were hybridized to a gene-specific labeled probe according to manufacturer's instructions. The p53-specific probe was a 610-bp fragment of the human p53 gene, encompassing exons 7 and 8 and intron 7, and was amplified as described previously (24). The following primer pair was used: forward, 5'-AGG-TTG-GCT-CTG-ACT-GTA-C-3'; reverse, 5'-ATT-GTC-CTG-CTG-TAC-CTC-3'. The N-ras-specific probe was a 112-bp fragment of the human N-ras gene and was amplified as described previously (25). The following primer pair was used: forward, 5'-GTT-ATA-GAT-GGT-GAA-ACC-TG-3'; reverse, 5'-ATA-CAC-AGA-GGA-AGC-CTT-CG-3'.

After exposure, films were scanned using a Molecular Dynamics Personal Densitometer, and band volumes were de-

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Table 1 Characteristics of multiple myeloma patients examined and individual amounts of each type of DNA adducts over time, as represented by the AUC for DNA adducts during the whole experiment (0–48 h), in the p53 and N-ras genes

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Months to HDT</th>
<th>Disease status</th>
<th>Response to HDT</th>
<th>Duration of response</th>
<th>Monoadducts p53-N-ras</th>
<th>Interstrand cross-links p53-N-ras</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>61</td>
<td>11</td>
<td>First response</td>
<td>CR</td>
<td>14+</td>
<td>246.1–262.8</td>
<td>17.1–17.5</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>60</td>
<td>20</td>
<td>Primary refractory</td>
<td>PR</td>
<td>10+</td>
<td>335.9–350.7</td>
<td>36.7–41.8</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>58</td>
<td>62</td>
<td>Resistant relapsed</td>
<td>No response</td>
<td>N/A</td>
<td>148.8–28.5</td>
<td>9.1–0</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>59</td>
<td>8</td>
<td>First response</td>
<td>Major PR</td>
<td>7+</td>
<td>248.2–265.1</td>
<td>24.4–31.1</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>67</td>
<td>10</td>
<td>First response</td>
<td>CR</td>
<td>6+</td>
<td>356.9–377.4</td>
<td>47.4–55.2</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>48</td>
<td>5</td>
<td>Primary refractory</td>
<td>No response</td>
<td>N/A</td>
<td>138.1–269.4</td>
<td>11.6–26.3</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>54</td>
<td>17</td>
<td>Primary refractory</td>
<td>No response</td>
<td>N/A</td>
<td>125.2–29.8</td>
<td>8.8–0</td>
</tr>
</tbody>
</table>

*a* Months to HDT, months from initial treatment of myeloma to the time of HDT.

*b* HDT, high-dose therapy; N/A, not applicable; CR, complete response; PR, partial response.
alkylation, respectively. In this case, alkylations were not determined with lysis buffer was 10.4 (22). DNA samples were digested to cross-links, cells were treated as above, except that the pH of the linear range of the film. DNA samples were converted to strand breaks, and DNA was denatured before gel electrophoresis and Southern blotting (22). The frequency of DNA interstrand cross-links was derived directly from the denatured samples; the frequency of DNA monoadducts was derived by subtracting that value from the value obtained from the analysis of N-alkylpurines.

**Statistical Methods.** The group t test was used to determine differences in the mean values between groups of samples. Statistical significance of a test was assumed when P < 0.05.

**RESULTS**

**Measurement of Monoadduct Formation and Repair in the p53 and N-ras Genes of Human Lymphocytes after in vitro Melphalan Treatment.** Human lymphocytes were treated with 5, 10, or 25 μg/ml melphalan for 1 h at 37°C and lysed immediately (for zero time points), or after reincubation in drug-free medium for the desired period (0–48 h). The drug doses used here were similar to the plasma levels of melphalan in multiple myeloma patients after chemotherapeutic treatment (26). Genomic DNA was prepared and N-alkylpurines were converted to strand breaks before Southern analysis. The blotted DNA was hybridized with probes specific for the human p53 or N-ras gene. Similar results were obtained by studying the N-ras gene (data not shown). Cyclophosphamide was not used in such experiments in vitro because this drug requires metabolic activation by the cytochrome P-450 enzymes to become an alkylating agent (11).

A dose-dependent rapid accumulation of monoadducts, reaching maximal levels within 2 h was found in both genes studied (Figs. 2A and 3A). Thereafter, adduct levels declined slowly to ~10 or 20% of maximal levels after 48 h in the p53 or N-ras gene, respectively. As shown in Figs. 2B and 3B, the semilogarithmic plots of adduct concentration versus time obtained (2–48 h) are linear, indicating that repair is first-order in monoadducts with an apparent t1/2 of 14.5 ± 0.3 h (mean ± SD) in the p53 gene and 18.8 ± 1.5 h (mean ± SD) in the N-ras gene. These results show consistent patterns of repair of monoadducts formed in both p53 and N-ras genes of peripheral blood lymphocytes after melphalan treatment in vitro. Furthermore, similar slopes are observed after different doses of melphalan, suggesting no change in the system available for repair of monoadducts during the whole experiment (Figs. 2B and 3B). Finally, no monoadducts could be detected before the drug treatment.

Measurement of DNA Interstrand Cross-link Formation and Repair in the p53 and N-ras Genes of Human Lymphocytes after in vitro Melphalan Treatment. The extent of DNA interstrand cross-linking in the p53 and N-ras genes was also measured in human lymphocytes treated with 5, 10, or 25 μg/ml melphalan. In this case, alkylations are not converted to strand breaks, and DNA is denatured before gel electrophoresis and Southern blotting. Under the conditions used, gene fragments containing DNA interstrand cross-links reanneal in the gel and migrate as double-stranded DNA. Un-cross-linked material remains denatured and serves as a marker for the migration of cross-linked DNA. The disappearance of the DNA interstrand cross-links, as measured by the assay used in the present study, reflects the excision of one of the cross-linked bases by a glycosylase or endonuclease ("unhooking" step) and not necessarily the complete removal of the damage (27). If inhibition of strand separation is the critical cytotoxic lesion, then the "unhooking" step may be the critical first step in the repair process. The resultant monoadduct may then be observed as persisting damage in the assay that measures total damage produced by the nitrogen mustards.

**Fig. 1** Detection of melphalan-induced DNA adducts in the p53 gene of in vitro treated control human lymphocytes. In A, cells were treated with 25 μg/ml melphalan for 1 h, washed free of drug, and then incubated in drug-free medium for the indicated times. DNA was prepared and digested with HindIII, and N-alkylpurines were converted to strand breaks before Southern analysis. The blotted DNA was hybridized with a probe specific for the human p53 gene. First and second lanes, control DNA. B, as in A, except that alkylations were not converted to strand breaks and DNA was denatured before gel electrophoresis and Southern blotting. First lane, control, nondenatured, double-stranded DNA; second and third lanes, control, denatured, single-stranded DNA.

**Table 1**

<table>
<thead>
<tr>
<th>melphalan</th>
<th>-</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>time (h)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>24</td>
<td>48</td>
</tr>
</tbody>
</table>

**Table 2**

<table>
<thead>
<tr>
<th>melphalan</th>
<th>-</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>time (h)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>24</td>
<td>48</td>
</tr>
</tbody>
</table>
The accumulation of DNA interstrand cross-links was slower than that of the monoadducts, reaching maximal levels within 8 h in both genes studied (Figs. 2C and 3C). Thereafter, adduct levels declined slowly to reach 10 or 20% of maximal levels after 48 h in the p53 or N-ras gene, respectively. As can be seen in Figs. 2D and 3D, the semilogarithmic plots of adduct concentration versus time obtained (8–48 h) are linear, indicating that repair of DNA interstrand cross-links is first-order, with an apparent $t_{1/2}$ of 12.4 ± 0.8 h (mean ± SD) in the p53 gene and 14.1 ± 2.2 h (mean ± SD) in the N-ras gene. Again, these data show consistent patterns of repair of interstrand cross-links formed in both p53 and N-ras genes of peripheral blood lymphocytes after melphalan treatment in vitro. These results are in agreement with those of Hansson et al. (28) who found that the rate constant for the overall removal of DNA interstrand cross-links after melphalan exposure is 12.9 h. Also, Ross et al. (29) found a rapid removal of mechlorethamine-induced DNA interstrand cross-links ($t_{1/2}$: 7 h) in L1210 cells, whereas melphalan-induced cross-links were repaired more slowly. After different doses of melphalan, similar slopes are observed, suggesting no change in the cross-link repair system during the whole experiment (Figs. 2D and 3D). Furthermore, the data presented here indicate that the amounts of DNA interstrand cross-links in lymphocyte DNA were proportional to the melphalan doses, at all time points and in both genes analyzed. Finally, no DNA interstrand cross-links could be detected before the drug treatment.

As can be seen in Table 2, the ratio of interstrand cross-links to monoadducts depends on the time point analyzed and the dose of melphalan used. This ratio, at the end of the melphalan treatment, was close to those found by previous investigators (22). However, at the following time points, the ratio increased because of the protracted formation of DNA interstrand cross-links and the parallel repair of monoadducts, reached maximal levels within 8 h, and decreased thereafter.

**In vivo Formation and Repair of Monoadducts and Interstrand Cross-links in the p53 and N-ras Genes of Human Blood Leukocytes.** Blood samples were collected from seven multiple myeloma patients (patients 1–7; Table 1) receiving single i.v. injections of melphalan at a dose of 200 mg/m² and from one polyarteritis nodosa patient (patient 8) after a single i.v. injection of cyclophosphamide at a dose of 1 g/m². Monoadducts and interstrand cross-links were detected in leukocyte DNA 2–48 h after exposure to these drugs.

The formation and loss of monoadducts in leukocyte DNA of drug-treated individuals are shown in Figs. 4, A and B, and 5, A and B. All eight individuals examined showed a maximum of the adduct levels 2 h after the drug injection in both genes studied. The mean values of peak monoadduct levels were very similar in the two genes analyzed (p53: 13.02 ± 3.68 adducts/10⁶ nucleotides, range 8.37–18.41; N-ras: 12.29 ± 6.27 adducts/10⁶ nucleotides, range 0–18.97). Thereafter, melphalan-induced adduct levels decreased by 34.7 ± 6.6% (range 26.3–41.2%) in the p53 gene and 29.0 ± 4.5% (range 24.1–34.2%)
in the N-ras gene during the first 24 h, whereas adduct levels in the cyclophosphamide-treated individual decreased by 50% after 48 h. No monoadducts could be detected before the drug injection.

The formation and repair of DNA interstrand cross-links in leukocyte DNA of melphalan-treated patients are shown in Figs. 4, C and D, and 5, C and D. All seven individuals examined showed a relatively slow accumulation of DNA interstrand cross-links with a maximum 8 h after the drug injection in both genes. The mean values of peak interstrand cross-link levels were very similar in the two genes analyzed (p53: 1.42 ± 0.62 adducts/10⁶ nucleotides, range 0.80–2.48; N-ras: 1.37 ± 1.05 adducts/10⁶ nucleotides, range 0–2.81). Thereafter, adduct levels decreased by 29.5 ± 9.7% (range 21.0–41.7%) in the p53 gene and 30.0 ± 12.3% (range 17.9–47.8%) in the N-ras gene during the first 24 h. These results, in accordance with the in vitro data, show consistent patterns of repair in both p53 and N-ras genes in blood leukocytes DNA of melphalan-treated patients. After cyclophosphamide administration, DNA interstrand cross-links were detected in leukocytes within 2 h, whereas maximal adduct levels occurred after 24 h. This may not be a real peak level of the interstrand cross-links, because no blood samples were collected from this patient at the 8-h time point. No DNA interstrand cross-links could be detected before the drug injection. Moreover, the ratio of DNA interstrand cross-links to monoadducts after the chemotherapeutic treatment increased rapidly and reached its maximum value after 8 h in patients 1–7 (p53: 0.12 ± 0.02, range 0.08–0.15; N-ras: 0.13 ± 0.02, range 0.11–0.16) or 24 h in patient 8 (p53: 0.26; N-ras: 0.26). Thereafter, the ratio remained constant for up to 24 h in patients 1–7 and up to 48 h in patient 8.

Furthermore, we observed that among the seven patients with multiple myeloma, the three (patients 3, 6, and 7) who showed the least levels of DNA adducts over time, as represented by the AUC² for DNA adducts during the whole experiment (0–48 h), did not respond to high-dose melphalan (Table 2).

Table 2  Ratios of DNA interstrand cross-links to monoadducts in the p53 and N-ras genes of control human lymphocytes treated in vitro with melphalan

<table>
<thead>
<tr>
<th>Gene</th>
<th>Timea (h)</th>
<th>Melphalan dose (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>p53</td>
<td>0</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.41</td>
</tr>
<tr>
<td>N-ras</td>
<td>0</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.13</td>
</tr>
</tbody>
</table>

a Time period following the end of the melphalan treatment.

The abbreviation used is: AUC, area under the curve.
1). Four patients responded to high-dose melphalan: one patient had primary refractory disease; and three patients had responded to conventional therapy (Table 1). Two of the three latter patients (patients 1 and 4) achieved a complete response, i.e., negative serum and urine immunofixation and absence of clonal plasma cells from the marrow. Patient 5 had the highest number of monoadducts and interstrand cross-links in both genes studied (Table 1).

Finally, marked differences were found between responders (patients 1, 2, 4, and 5) and nonresponders (patients 3, 6, and 7) to chemotherapy in the mean AUC values of both DNA adducts and in both genes analyzed (296.8 ± 57.9 versus 137.4 ± 11.0, respectively, and P = 0.006 for p53 monoadducts; 31.4 ± 13.4 versus 9.8 ± 1.5, respectively, and P = 0.04 for p53 interstrand cross-links; 314.0 ± 58.8 versus 109.2 ± 138.7, respectively, P = 0.04 for N-ras monoadducts; 36.4 ± 16.0 versus 8.8 ± 15.2 adducts/10⁶ nucleotides × h, respectively, P = 0.07 for N-ras interstrand cross-links; Table 1; Fig. 6).

DISCUSSION

The measurement of DNA adducts formed in patients treated with alkylating drugs is of interest, because such adducts may indicate the biological dose received at the level of the individual patient, which may be correlated with therapeutic response or long-term complications (mainly therapy-induced carcinogenesis). The presence of total adducts, monoadducts, and DNA interstrand cross-links as a result of in vitro and in vivo exposure to therapeutic drugs has been reported in the past (26, 28, 30–33). However, the present study is the first dosimetry report of the gene-specific formation and repair of these adducts after in vitro exposure of human lymphocytes to melphalan or after therapeutic exposure of humans to melphalan or cyclophosphamide.

Of the chemotherapeutic alkylating agents known to be carcinogenic in humans, the nitrogen mustards are among the most potent (34). Acute myelogenous (nonlymphocytic) leukemia is the predominant mustard-associated cancer (35). In some case studies as many as 10–20% of long term survivors were found to contract this disease, which is frequently preceded by a preleukemic condition known as myelodysplastic syndrome (MDS). Although not a single gene has yet been identified which is consistently mutated in mustard-associated acute myelogenous (nonlymphocytic) leukemia, mutations in p53 occur in a small fraction of cases, whereas N-ras mutations occur somewhat more frequently (25–50% of patients) (24, 36). Thus, in the present study, the p53 and the N-ras genes were chosen as experimental tools.

In all experiments described here, the highest monoadduct levels were found within 2 h, whereas DNA interstrand cross-links accumulated slowly and reached maximal levels within 8 h in both genes analyzed. The relatively long time between monoadduct and DNA interstrand cross-link formation observed in the present study confirms previous reports (37, 38). Kohn (3) proposed a two-step model for the
protracted formation of DNA interstrand cross-links by melphalan. According to this model, rapid binding of the drug to a nucleophilic site in one DNA strand as a monoadduct is followed by a delayed formation of a DNA interstrand cross-link through the binding of a second chloroethyl group to a site in the cDNA strand. Thus, repair of monoadducts before cross-link formation may play an important role in protecting cells from melphalan and cyclophosphamide cytotoxicity and may be a significant factor leading to chemotherapy failure. DNA interstrand cross-links represent only a small fraction of the total adducts formed. However, they are thought to be the main determinant of the toxicity of the nitrogen mustards (28, 39, 40). The exact mechanism by which DNA interstrand cross-links cause cell death is not known. Mu et al. (41) found that DNA interstrand cross-links induce a futile repair synthesis which is not accompanied by damage removal. It is possible that this futile repair cycle and the potentially pre-apoptotic signals resulting from this cycle, rather than the replication block per se, are the main causes of the lethality of cross-link-inducing anticancer drugs. Interestingly, the data from both genes analyzed in the present study show that smaller doses give greater ratios of DNA interstrand cross-links to monoadducts, indicating that smaller doses are more effective in inducing toxic DNA interstrand cross-links (Table 2). This finding has possible implications for the future design of more efficient chemotherapeutic protocols.

Individual differences in the response of tumor cells to antineoplastic agents in vitro as well as correlations between drug sensitivity in vitro and individual responses to therapy have been observed as well (42, 43). However, to perform these assays, invasive surgical treatment is necessary to obtain a sufficient amount of tumor cells, and they are rarely used in clinical practice. Determining DNA adduct formation and repair in peripheral blood leukocytes of patients may provide a noninvasive method for evaluating the effectiveness of some antineoplastic drugs. Previous investigations found a relatively small interindividual variation in Hodgkin’s disease patients treated with procarbazine and lymphoma patients exposed to dacarbazine, because adduct levels in different individuals fell within a range of three (44, 45). Studies on cisplatin adducts have indicated large interpatient variation in adduct formation apparently related to therapy (46, 47). In contrast, measurement of DNA adduct levels after melphalan administration showed a generally low degree of interpatient variation (26). Recently, Spanswick et al. (48) found that similar levels of high-dose melphalan-induced DNA interstrand cross-links were observed in plasma cells from multiple myeloma patients who had not received melphalan therapy (melphalan naive) and those previously treated with melphalan. However, significant differences in DNA interstrand cross-links repair were observed: cells from naive patients showed no repair; whereas those from previously treated patients exhibited between 42 and 100% repair. In the present study, marked differences were found in the

Fig. 5 DNA adducts in the N-ras gene of peripheral leukocytes removed at various times after chemotherapeutic administration of nitrogen mustards. Human blood samples were collected at various times after exposure to melphalan (patients 1–7) or cyclophosphamide (patient 8). Monoadducts (A and B) and interstrand cross-links (C and D) were detected in the N-ras gene. Nucleot, nucleotides. Bars, SD.
individual amounts of each type of DNA adducts over time, as represented by the AUC for DNA adducts during the whole experiment (0–48 h; Table 1; Fig. 6). Among the seven patients with multiple myeloma, the three (patients 3, 6, and 7) who showed the lowest levels of DNA damage over time did not respond to the melphalan therapy (Table 1).

The results reported here show that it is feasible to measure monoadducts and DNA interstrand cross-links in a readily accessible tissue of humans exposed to bifunctional alkylating drugs and indicate a possibility of examining at the level of the individual patient the relationships between the induction/repair of cytotoxic DNA damage and clinical response or long-term complications. In this way, we expect to gain insight into the causes of individual variability in response to chemotherapy and to access the usefulness of DNA damage and repair as predictive molecular markers for the design of more effective and individualized therapeutic protocols.

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**REFERENCES**

Gene-specific Formation and Repair of DNA Monoadducts and Interstrand Cross-links after Therapeutic Exposure to Nitrogen Mustards

Vassilis L. Souliotis, Meletios A. Dimopoulos and Petros P. Sfikakis


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