DNA crosslinks, DNA damage and repair in peripheral blood lymphocytes of non-small cell lung cancer patients treated with platinum derivatives

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Abstract. Lung cancer is the leading cause of cancer-related mortality in the world. Chemotherapy has been the mainstay of treatment for advanced non-small cell lung cancer (NSCLC) and platinum-based derivatives have been shown to improve overall survival. The aim of the present study was to investigate the DNA damage [single strand breaks (SSBs) and DNA crosslinks] and DNA repair in peripheral blood lymphocytes in patients with NSCLC treated with platinum derivatives using modified comet assay. Twenty patients in the final (4th) stage of NSCLC and 10 age-corresponding healthy controls participated in the study. Alkaline comet assay was performed according to the appropriate protocol. The DNA base excision repair (BER) activity of the controls was significantly higher compared to that of cancer patients, and the activity of DNA nucleotide excision repair (NER) was almost at the same level both in controls and patients. We observed changes in the amount of SSBs and DNA crosslinks during the course of chemotherapy. We found a significantly higher level of SSBs immediately after administration of chemotherapy. Similarly, we found the highest incidence of DNA crosslinks immediately or 1 day after chemotherapy (compared to measurement before chemotherapy). Moreover, we compared the levels of DNA repair in patients who survived chemotherapy with those in patients who died in the course of chemotherapy: the activity of BER was higher in the case of surviving patients, while the levels of NER were essentially the same. The data arising from the present study confirm the findings of other studies dealing with DNA damage and repair in cancer patients treated with chemotherapy. Moreover, our results indicated that despite the fact that cisplatin-DNA adducts are removed by the NER pathway, BER may also play a role in the clinical status of patients and their survival.

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Introduction

Lung cancer is the leading cause of cancer-related mortality in the world. It is classified into two groups: non-small cell lung cancer (NSCLC) and small cell lung cancer (1). Chemotherapy has been the mainstay of treatment for advanced NSCLC. Of the various types of chemotherapy regimens available, platinum-based derivatives have been shown to improve overall survival (2). Cisplatin was the first platinum agent to be used in the clinic in the early 1970s and proved very effective in the treatment of several types of cancer (3-5). It is classed as an alkylating agent, DNA binds to it by nucleophilic substitution, and it is one of the substances which can cause crosslinking (6). Cisplatin can react with DNA in several ways, the most common is the reaction with guanine leading to the formation of intrastrand crosslinks (65% of DNA modifications). It has been shown that platinum-DNA adducts correlate with the disease response. Thus, the capacity to repair DNA damage caused by platinum derivatives may reflect cellular sensitivity to this agent (7,8).

There have been many studies on patients under treatment for cancer; the majority of investigations examine peripheral blood lymphocytes (PBLs) for increased levels of DNA damage, although other cells (for example, buccal cells) can also serve as surrogates. Collection of PBLs is usually less invasive than that of target tissue, and they are the surrogate cells of choice in studies where target tissue is not readily attainable (9,10). With specific regard to cancer, this approach has been used experimentally to measure the levels of basal DNA damage in PBLs of cancer patients, as well as to assess the susceptibility of cells to the DNA-damaging effects of radiotherapy or chemotherapy (9). DNA repair is growing in popularity as a biomarker in human biomonitoring. It seems

that more useful information has come from phenotypic assays for repair-enzyme activity than from measuring expression of repair genes. Results thus so far tend to be inconsistent. Studies on the effect of occupational or environmental exposure to genotoxins have shown the full range of positive, negative and null effects on repair and the same is true of nutritional studies. Even the generally assumed decline in repair capacity with age has not been confirmed in population surveys with biomarker assays for DNA repair (11).

We performed our measurements (DNA damage and repair) using the comet assay test. The comet assay or single-cell gel electrophoresis (SCGE) assay is a rapid, sensitive and relatively simple method for detecting DNA damage at the level of individual cells (12). This assay is now widely accepted as a standard method for assessing DNA damage in individual cells, and is used in a broad variety of applications including human biomonitoring, genotoxicology, ecological monitoring and as a tool to investigate DNA damage and repair in different cell types in response to a range of DNA-damaging agents (13). The comet assay would be eminently suitable for use in clinical practice, since it is a relatively simple and inexpensive technique which requires only a small quantity of cells, and results can be obtained within a matter of hours. Comet assay is a method specific for investigation of DNA damage, and is recommended for studies on reagents that cause DNA damage. Studies have shown that results generated by the comet assay provide important information about the nature of a particular cancer, and could be used by oncologists in their determination of the best possible course of intervention (9).

Materials and methods

Chemicals and reagents. All chemicals were purchased from Sigma Aldrich.

Cell culture. HeLa cells were obtained from Professor Andrew Collins (University of Oslo, Norway). Cells were cultivated in DMEM supplemented with 10% fetal bovine serum, antibiotics and an antimycotic mixture (all ingredients from PAA Laboratories, Pasching, Austria). Cells were passaged twice a week by trypsinization in the ratio 1:10.

Patients. Blood samples were obtained from 20 patients with NSCLC stage 4. Patient age was between 47 and 74 years. Patients were programmed for chemotherapy according to the following protocol: blood samples were collected from each patient during the 1st and 3rd cycles of chemotherapy; 1 day before starting the cycle, immediately after administration of the chemotherapy, and 1 and 7 days after completion of the chemotherapy cycle. The last control measurement was performed 3 weeks after the final (4th cycle) completed course of chemotherapy. All patients received chemotherapy for the first time. They received platinum-based derivatives (cisplatin or carboplatin) in a combination therapy. Blood samples were also obtained from an age-corresponding control group (10 subjects). The study was conducted according to the guidelines of the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Committee of the Charles University Medical Faculty in Hradec Kralove. Written informed consent was obtained from all subjects.

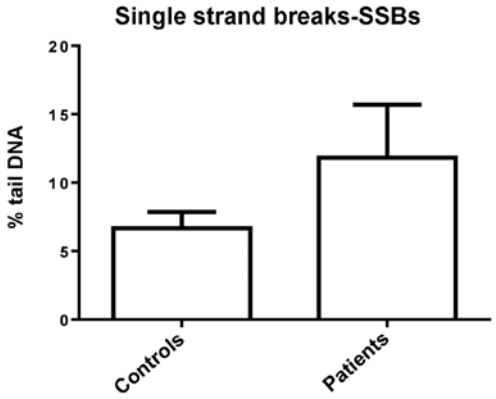

Figure 1. Percentage Tail DNA as a measure of SSBs in cancer patients compared to healthy controls. In healthy controls, percentage Tail DNA in SSBs was 6.679±1.193. In cancer patients, percentage Tail DNA was higher than in controls (11.830±3.865), but not significantly.

Comet assay. Lymphocyte preparation. Peripheral blood lymphocytes were obtained from heparinized blood and separated using LSM (PAA Laboratories) according to the method of Boyum in 1964 (14). Peripheral blood lymphocytes were washed and re-suspended with PBS buffer and adjusted to 1 million cells/ml.

The comet assay modification was used as previously described (15). Briefly, 35 μl of cell suspension (»35,000 cells) was mixed with 85 μl low melting point agarose, spread onto an 85 μl high melting point on normal agarose pre-coated microscope slide and left at 4˚C to allow for solidification. Cells were lysed for 1 h in high salt and detergent solution. They were then exposed for 40 min to alkali to allow for DNA unwinding and cleavage of alkali-labile sites. Electrophoresis was applied for 30 min at 4˚C, after which the slides were neutralized, stained with ethidium bromide and analyzed by fluorescence microscopy. Fifty cells per slide were scored according to % tail DNA by the Lucia G analyzing software (Laboratory Imaging, Prague, Czech Republic).

DNA crosslinks were measured using the modified comet assay, using styrene oxide. Briefly, cells were incubated with styrene oxide to induce DNA breaks prior to comet assay. Incubation of cells with styrene oxide leads to the formation of comets containing ~80-90% of DNA in the tail. When crosslinks are present in the DNA the alkaline unwinding is blocked and the % of DNA in the tail is reduced accordingly (16).

DNA repair, base excision repair (BER) and nucleotide excision repair (NER), were measured using the modified comet assay in an extract of lymphocytes. Nucleoids of HeLa cells containing specific lesions (8-oxoguanine or cyclobutane pyrimidine dimers) in their DNA embedded in agarose were incubated along with the extracts prepared from lymphocytes of the study subjects whose excision repair activity was to be measured. The nature of the DNA lesion in the substrate defines the repair pathway that is measured. Substrate containing 8-oxoguanine is used to measure BER activity, and if the substrate contains bulky adducts or cyclobutane pyrimidine dimers NER is measured (17-20).

Statistical analysis. Statistical significance of the differences between groups was assessed by using GraphPad Prism 5.03 software (GraphPad Software, Inc., San Diego, CA, USA).

Table I. Comparison of values of BER and NER of healthy controls and cancer patients.

% Tail DNA ± SE	Controls	Patients	P-value
BER	17.10±1.170	10.57±2.569	0.0320[a]
NER	12.88±1.131	12.75±3.074	0.9670

BER of healthy controls was higher ([a]P=0.0320) than BER of cancer patients. NER was almost at the same level in both healthy controls and cancer patients (P=0.9670). BER, base excision repair; NER, nucleotide excision repair.

Results

In the present study, we investigated DNA damage and repair in 20 NSCLC patients and in 10 subjects from the control group as well. Patients were in the last stage of cancer and chemotherapy was indicated as a palliative. Thirteen patients completed the entire course of chemotherapy and seven patients died during the chemotherapy.

The degree of DNA damage was estimated by the comet assay and was expressed as the percentage Tail DNA. In the healthy controls, the percentage Tail DNA in single strand breaks (SSBs) was 6.679±1.193. In cancer patients, percentage Tail DNA in SSBs was 11.830±3.865. In the cancer patient group, we found high inter-individual variability, with coefficient of variation 142.46%. Results were not statistically significantly different: P=0.3674 in the Mann-Whitney test (Fig. 1).

We compared values of BER and NER activity in healthy controls and in cancer patients. BER of healthy controls was higher (P=0.0320 in the T-test) than BER of cancer patients. NER was almost at the same level in both healthy controls and cancer patients (P=0.9670 in the T-test). Data are shown in Table I.

We monitored changes in SSBs in PBLs during the entire course of chemotherapy. Samples were collected from each patient during the 1st and 3rd cycles of chemotherapy: 1 day before starting the cycle, immediately after administration of the chemotherapy, and 1 and 7 days after completion of the chemotherapy cycle. The last control measurement was performed 3 weeks after the final (4th cycle) completed course of chemotherapy. In both cycles (1st and 3rd), we found a significantly (Friedman test) higher level of SSBs in measurement immediately after administration of the chemotherapy (compared to the measurement before chemotherapy). The level of SSBs then returned towards baseline values (Fig. 2).

The amount of SSBs increased throughout the course of chemotherapy, but there were no statistically significant differences among SSBs measured before chemotherapy, in the middle of the chemotherapy (3rd cycle) and at the end of the chemotherapy (Fig. 3).

The comet assay protocol, modified for the measurement of crosslinks (using styrene oxide treatment), was used to measure the induction and repair of DNA crosslinks caused by platinum-based derivatives. A significant reduction (Friedman test) in percentage Tail DNA was found during the 1st and 3rd cycle of the chemotherapy, indicating the presence of crosslinks (Fig. 4). The value of Tail DNA increased again after one week although it did not reach the baseline value. The

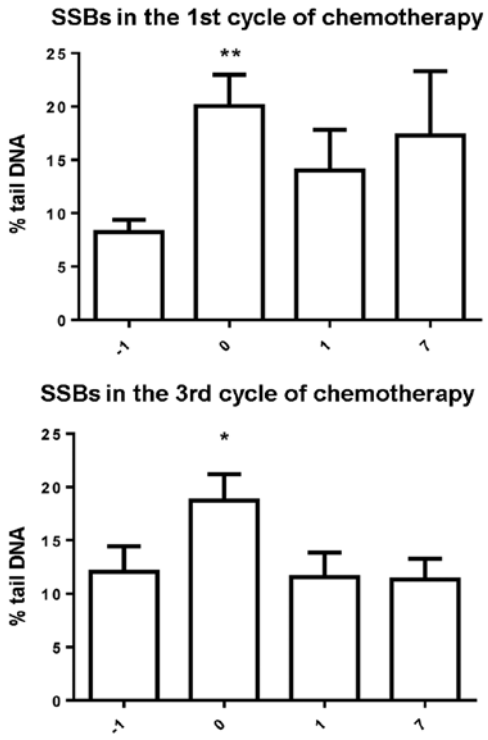

Figure 2. Percentage Tail DNA as a measure of SSBs in peripheral blood lymphocytes of cancer patients during the course of 1st and 3rd cycle of the chemotherapy. In each cycle, -1 represents measurement before starting chemotherapy; 0 represents measurement immediately after the administration of chemotherapy; 1 and 7 represent measurement 1 and 7 days after administration of the chemotherapy. We found changes in SSBs level during the cycle; significantly higher level of SSBs (*P<0.05, **P<0.001) was observed in measurements immediately after the administration of the chemotherapy (compared to measurements before the chemotherapy).

Figure 3. Percentage Tail DNA as a measure of SSB level in peripheral blood lymphocytes of cancer patients in the entire course of chemotherapy. Three measurements were performed: before chemotherapy, in the middle of the chemotherapy (3rd cycle) and at the end of the chemotherapy. The level of SSBs increased throughout the course of chemotherapy, but not significantly.

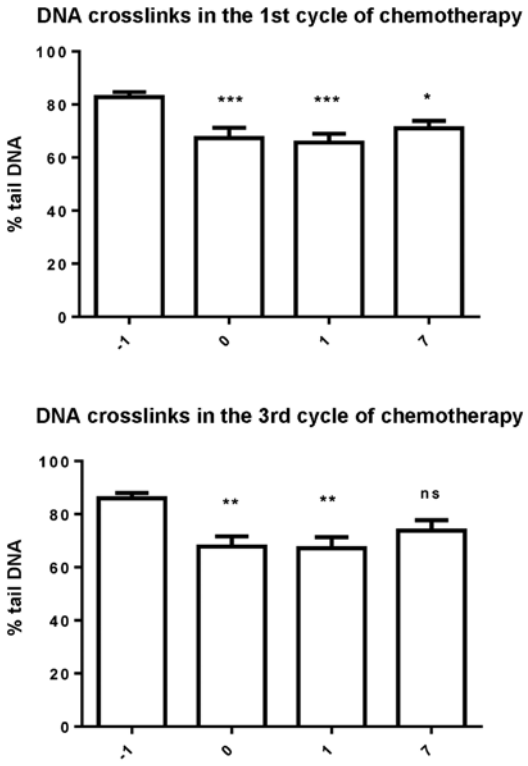

Figure 4. Percentage Tail DNA as a measure of induction and repair of DNA crosslinks in the course of the 1st and 3rd chemotherapy cycle in peripheral blood lymphocytes of cancer patients treated with platinum derivatives. In each cycle: -1 represents measurement before starting the chemotherapy, 0 represents measurement immediately after the administration of the chemotherapy, 1 and 7 represent measurement 1 and 7 days after administration of the chemotherapy. Significantly lower levels of percentage Tail DNA (indicating presence of DNA crosslinks) (*P<0.05, **P<0.001, ***P<0.0001) were noted in measurements immediately after administration of the chemotherapy, and 1 and 7 days after the chemotherapy (compared to the measurements before the chemotherapy).

Table II. Comparison of values of BER and NER of cancer patients in the course of chemotherapy.

% Tail DNA ± SE	Before chemotherapy	At the end of chemotherapy	P-value
BER	12.03±2.355	19.62±4.191	0.0505
NER	13±3.015	22.01±5.551	0.2061

In both cases, we found a non-significant increase (paired T-test in the case of BER and Wilcoxon matched pairs signed-rank test for NER) of repair at the end of the chemotherapy compared to the status before the chemotherapy. BER, base excision repair; NER, nucleotide excision repair.

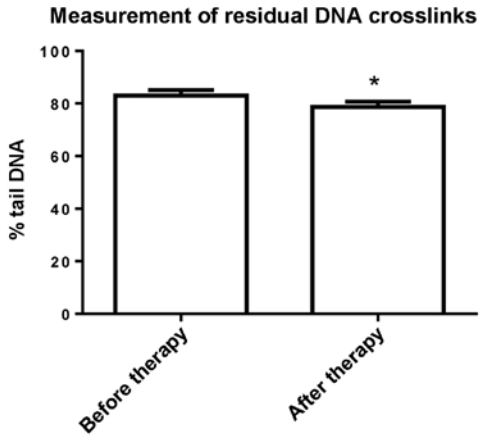

Figure 5. Detection of residual DNA crosslinks in peripheral blood lymphocytes of cancer patients treated with platinum derivatives. Percentage of Tail DNA in the measurement at the end of the chemotherapy was significantly lower than in the measurement before the chemotherapy (*P=0.0280).

response in patients showed a similar trend, although there appeared to be high inter-individual variability. Percentage of Tail DNA in the measurement at the end of the chemotherapy was significantly lower than in the measurement before the chemotherapy, indicating that some DNA crosslinks persist even after the chemotherapy (P=0.0280 in the paired T-test) (Fig. 5).

We performed tests for DNA repair, specifically tests for BER and tests for NER, to determine whether is there any relationship between DNA repair and patient survival in the last stage of NSCLC. Using the paired T-test we compared BER and NER in 13 patients who finished the entire course of chemotherapy. In both cases, we found a non-significant increase of repair at the end of the chemotherapy compared to the status before the chemotherapy (Table II). Moreover, we compared input values of DNA repair (BER and NER) in patients who finished the whole course of chemotherapy with those who died during the chemotherapy. We found similar results as for comparison of repair capacity between patients and controls, thus input values of BER were non-significantly higher than input values of patients who finished chemotherapy (P=0.0787 in the Mann-Whitney test) and input values of NER were almost at the same level in both groups of patients (P=0.8930 in the Mann-Whitney test) (Fig. 6).

We found negative correlation between input values of NER of cancer patients and the level of DNA crosslinks on the 1st day after administration of chemotherapy in the 3rd cycle of chemotherapy. We found a negative correlation between NER measured before the 3rd cycle and persistent DNA crosslinks (measurement at the end of the chemotherapy).

Discussion

In the present study, we monitored the induction and repair of DNA breaks and interstrand crosslinks in the DNA of peripheral lymphocytes isolated from patients with non-small cell lung carcinoma (NSCLC) during chemotherapy with platinum-based derivatives. The level of single strand breaks (SSBs) in cancer patients was compared to SSBs in controls, and also the repair capacity [base excision repair (BER) and nucleotide excision repair (NER)] of these patients was compared to that of control subjects. We found that the number of SSBs was higher in cancer patients relative to controls, but not significantly. Thus, our findings are in accordance with published studies

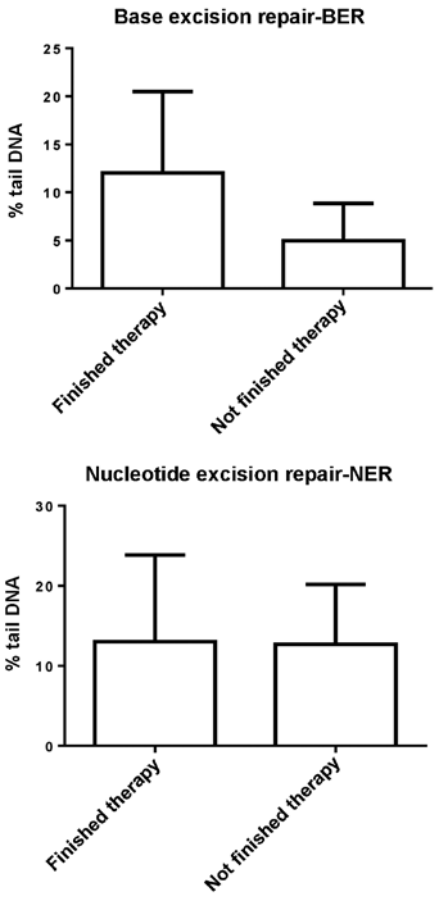

Figure 6. We compared input values of BER and NER in patients who finished the whole course of chemotherapy to those in patients who died during chemotherapy. Input values of BER were non-significantly higher than input values of patients who finished chemotherapy (P=0.0787). Input values of NER were almost at the same level in both groups of patients (P=0.8930).

showing that the level of basal DNA damage in peripheral blood lymphocytes (PBLs) of patients suffering from a variety of cancers is higher than that of controls (9,21-27).

DNA BER in PBLs of healthy controls was higher (P=0.0320) compared to that of cancer patients, and DNA NER was almost at the same level in both controls and patients. Our results may indicate a link between the process of carcinogenesis and BER, but not between carcinogenesis and NER. Functional assays performed in blood leukocytes of cancer patients and matched controls show that specific BER pathways are less efficient in cancer patients, which suggests that BER capability may represent a risk factor (28). Another study which examined whether reduced DNA repair is associated with lung cancer suggested that reduced activity of various DNA repair mechanisms causes lung cancer predisposition (29).

We observed changes in DNA damage (SSBs) in the course of chemotherapy. In both measured cycles, we found a significantly higher level of SSBs immediately after administration of chemotherapy (compared to measurement before chemotherapy). The level of SSBs increased throughout the entire course of chemotherapy, but non-significantly. Other studies have confirmed an increase of SSBs in PBLs of cancer patients after different combined chemotherapies; in some studies the increase was significant (30,31), but it was non-significant elsewhere (32).

We also measured the induction and repair of DNA crosslinks caused by platinum-based derivatives. In both cycles (1st and 3rd), we found the highest degree of crosslinking immediately or 1 day after chemotherapy. Seven days after chemotherapy the level of crosslinks gradually decreases and returns to pre-chemotherapy values. We found that some crosslinks persist in the DNA even after the entire 4 cycles of chemotherapy and that there is great inter-individual variability among patients as well. Similar studies demonstrating the formation and repair of DNA crosslinks have also demonstrated great inter-individual variability among patients but, in general, the trend for repair of DNA crosslinks can be monitored (3,33).

DNA repair (BER and NER) was monitored throughout the whole course of chemotherapy. In patients who survived the chemotherapy, DNA repair (both BER and NER) increased during the course of chemotherapy. This increase (at least in the case of BER) was on the border of statistical significance. Moreover, we compared levels of DNA repair in patients who survived chemotherapy with those in patients who died in the course of chemotherapy. We found similar results as in the comparison of cancer patients with healthy controls: values of BER were higher in the case of surviving patients, while levels of NER were essentially the same. The removal of cisplatin-DNA adducts is mediated by the NER pathway, in which ERCC1 is one of the key enzymes. The present study confirmed this, since we found negative correlation between NER and the level of DNA crosslinks in the course of chemotherapy. Other studies showed that high expression of ERCC1 had an adverse effect on survival following the administration of cisplatin-based chemotherapy in patients with NSCLC (34). There is a hypothesis that lung cancer patients with lower ERCC1 levels and, thus, lower NER DNA repair capacity, may have an enhanced response and survival with cisplatin-based chemotherapy. In an experimental model, elevated DNA repair capacity was associated with resistance to cisplatin in lung cancer cell lines (35). However, no correlation between NER, formation and repair of DNA crosslinks, or survival of patients was found.

Results from the present study confirm those of other studies dealing with DNA damage and repair in cancer patients treated with chemotherapy. Moreover, our results indicated that despite the fact that cisplatin-DNA adducts are removed by the NER pathway, BER also plays a role in the clinical status of patients and their survival. For confirmation of these conclusions, a study on a larger number of patients is required.

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