Differences in Repair of DNA Cross-links between Lymphocytes and Epithelial Tumor Cells from Colon Cancer Patients Measured In vitro with the Comet Assay

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Abstract

Purpose: The more common approach to comet assay studies with cancer patients involves indirect measurement of the effect of antineoplastic drug or radiation regimen by assessing DNA damage in surrogate cells, such as peripheral blood lymphocytes of cancer patients, to predict how tumor cells may be affected. The aim of the present study was to compare the capability of different cells isolated from a series of 23 colon cancer patients to repair the damage induced by a cancer drug.

Experimental Design: We adapted the in vitro comet repair assay for nucleotide excision repair to measure the ability of lymphocytes and normal and tumor epithelial colon cells to remove DNA cross-links induced by oxaliplatin. The excision repair rate was measured quantitatively by the tail parameters: tail DNA, tail length, extent tail moment, and olive tail moment.

Results: Kruskal-Wallis analysis revealed significant differences in recognition and excision activity between different cell types ($P < 0.001$) for all the comet parameters studied. Hence, colon cells showed higher recognition and excision activity than lymphocytes and tumor cells displayed the highest repair capability. We found no significant correlation between the repair activity of tumor colon cells and lymphocytes in any of the comet parameters considered.

Conclusions: Our data support the view that lymphocyte repair activity is not predictive of the repair ability of the tumor and that lymphocytes cannot act as surrogate cells.

The response of the cell to DNA damage and its ability to maintain genomic stability by DNA repair are key molecular events driving cancer initiation and progression. To date, there is considerable evidence that individual variation in the ability to limit DNA damage from endogenous and exogenous carcinogens contributes to cancer risk (1, 2). In addition, high efficiency of DNA repair can be considered one of the main obstacles of cancer therapy based on the resistance of cancer cells to therapeutic drugs and radiation (3, 4). Therefore, differences in the sensitivity of cells to mutagens and their ability to repair damage could predict individual tumor sensitivity to radiation and to chemotherapy.

Comet assay is now widely accepted as a useful tool for investigating DNA damage and repair in different cell types in response to a range of DNA-damaging agents (5). However, a few comet assay studies have used tumor cells isolated from the biopsy sample of cancer patients. Instead, the more common approach involves indirect measurement of the effect of antineoplastic drug or radiation regimen by assessing DNA damage in surrogate cells, such as peripheral blood lymphocytes, from cancer patients (reviewed in ref. 6).

Despite the advantages of the use of peripheral blood lymphocytes as surrogate cells, because they are easily obtained in a noninvasive way and can be seen as reflecting the overall state of the organism (7), they are not a cancer target tissue. As such, it is not clear that the damage detected in these cells mimics the damage in tumor cells or in any other target tissues (5, 6). There are a range of confounding factors, such as smoking, diet, and exercise, which influence the basal and residual level of DNA damage of peripheral blood lymphocytes, resulting in wide intra- and inter-individual variability (8). Nevertheless, previous comet assay studies have shown that they can be...
used as surrogate cells when the target tissue is not readily attainable (reviewed in ref. 9). With specific regard to cancer, many studies have used peripheral blood lymphocytes of cancer patients who are undergoing chemotherapy or radiotherapy to predict how tumor cells may be affected (10–12). These studies found that DNA damage is increased and/or DNA repair capacity is decreased in peripheral blood lymphocytes from cancer patients compared with control group.

Collins et al. (13) developed an in vitro comet assay method to measure the base excision repair capacity of an extract of relevant cells. Recently, this assay was modified for measuring nucleotide excision repair (NER) activity (14, 15). The repair enzymes present in cell extracts recognize the damage in the DNA of the substrate cells and incise the DNA. One of the critical proteins recognizing DNA damage is XPA. In fact, without it, no stable preincision complex can form nor can NER occur (16, 17). The strand breaks produced are detected by the conventional comet assay, and the increase in tail DNA (TDNA) reflects the repair activity of the cell extract. Up to now, the studies done have tested lymphocyte extract activity to repair oxidative damage (13), DNA adducts (14), or cyclobutane pyrimidine dimers (15), but it is unclear if in vitro repair activity in lymphocyte extracts reflects repair capacity in other tissues of the individual (7). Additionally, whether the extract could repair other kinds of damage, such as DNA cross-links induced by cancer drugs, is not assessed.

In the present study, we modified the in vitro comet repair assay for NER (15). This was to measure the DNA repair capacity of lymphocytes and normal and tumor epithelial colon cell extracts isolated from the biopsy samples of 23 colon cancer patients and to compare the ability of different cells from the same individual to remove DNA cross-links induced by a cancer drug.

Materials and Methods

Patients and samples. We recruited 23 patients diagnosed with colorectal cancer in our hospital during 2008 and who underwent surgical resection of the tumor. None of the patients received any treatment before resection of the tumor. The study was approved by the Research Ethics Board of our hospital and informed consent was obtained from all participants. Tissue samples, tumor and normal colon mucosa, taken at least 3 cm from the outer tumor margin, were obtained immediately after surgery and processed for the purification of the epithelial fractions as described below. Blood samples (10 mL) were also obtained from all the patients and processed for lymphocyte isolation.

DNA damage treatment for cell substrate preparation. SW480 colon cancer cell line was cultured at 37°C in DMEM (Life Technologies) supplemented with 10% heat-inactivated FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, 100 mg/mL streptomycin, and 0.25 μg/mL fungizone in humidified atmosphere of 5% CO2, 95% air. Near-confluent cultures of cells were incubated for 2 h with 100 μg/mL oxaliplatin to induce DNA cross-links repaired by NER. The formation of DNA cross-links with the treatment administered was verified by comet assay following the procedures described in detail elsewhere (ref. 18; Fig. 1).

After the treatment, the cells were detached from the dish by gentle trypsinization and used as substrate damage cells in the in vitro comet assay.

Isolation of the lymphocytes and epithelial cells. Lymphocytes from patients were isolated from whole blood by standard procedures (Lymphoprep, ATOM, Axis-Shield PoC AS). Following the protocol of Langie et al. (14), the cells were washed in PBS, divided into aliquots, and centrifuged and the pellets were snap-frozen in liquid nitrogen and stored at -80°C.

Fresh samples of normal and tumor cells from patients were disaggregated in a Medimachine instrument (DAKO Cytomation). Cell viability was evaluated by trypan blue exclusion: all (>95%) of the cells in this fraction were viable. Epithelial cells were immunomagnetically purified using superparamagnetic polystyrene beads coated with the BerEP4 antibody (specific for two glycopolyptide membrane antigens expressed in most normal and neoplastic human epithelial tissues; Dynabeds Epithelial Enrich; 1.5 × 10⁶ beads in PBS/0.1% bovine serum albumin/0.6% sodium citrate; Dynal Biotech). Before storage at -80°C, the cells were snap-frozen as pellets in liquid nitrogen.

Nuclear extract preparation of lymphocytes and epithelial cells. To obtain the enzyme extract from relevant cells, we modified the protocol described by Gaviao et al. (15). Just before an assay, the frozen pellets from the lymphocytes and the normal and tumor epithelial cells were resuspended in 65 μL extraction buffer (45 mmol/L HEPES, 0.4 mol/L KCl, 1 mmol/L EDTA, 0.1 mmol/L DTT, 10% glycerol, adjusted to pH 7.8 with 5 mol/L KOH), to which Triton-X 100 was added to 0.25% just before use. After vortex-mixing and leaving on ice for 5 min, the lysates were centrifuged at ≈10,000 × g for 5 min to remove the cell debris. The supernatant was diluted in 4 volumes of reaction buffer (40 mmol/L HEPES, 0.1 mol/L KCl, 0.5 mol/L EDTA, 0.2 mg/mL bovine serum albumin, 2 mmol/L MgCl₂, adjusted to pH 8.0 using 5 mol/L KOH). Extracts were kept on ice until the reaction.

The protein concentration of each extract was determined by the Bradford method.

DNA repair evaluation by the in vitro comet assay method. We performed the in vitro comet assay for NER, as described by Gaivao et al. (15), with some modifications. Briefly, superfrosted and commercially precoated microscope slides (Fisher Scientific) were immersed in hot 1% normal-melting-point agarose solution and allowed to dry on a flat surface. Ten microliters of a cell suspension containing 3 × 10⁶ substrate damage cells were mixed with 85 μL of 0.5% low-melting-point agarose in PBS, kept at 37°C in a dry-bath incubator, spread on an agarose precoated slide, and covered with a coverslip. The agarose was solidified at 4°C for 10 min. The cells were lysed for 1 h at 4°C in a freshly prepared lysis buffer (pH 10) containing 2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Trizma base, 10% DMSO, and 1% Triton X-100. After the lysis, the slides were washed for 3 × 5 min with PBS to remove detergents and salts and then immersed for 3 × 5 min with reaction buffer in Coplin jars. Fifty microliters from each cell extract preparation at 0.5 μg/μL protein were added to each gel, covered with a coverslip, and incubated for 30 min in a humid chamber at 37°C. Samples were tested in two independent incubations within each single experiment. Following the incubation, slides were washed for 3 × 5 min with PBS and processed according to the conventional alkaline comet assay (19) to measure the DNA breaks introduced by the initial excision...
events of repair. Consequently, the slides were immediately placed side-by-side on a horizontal electrophoretic unit (Owl Separation Systems), without power for 30 min in freshly prepared alkaline buffer [300 mmol/L NaOH and 1 mmol/L EDTA (pH > 13)] at 4°C, to unwind the DNA of nucleoids embedded in gels. Electrophoresis was carried out for 30 min at 25 V, with the current adjusted to 300 mA and a constant recirculating flow of 100 mL/min. Finally, the slides were rinsed in PBS for 3 x 5 min, fixed in cold 100% ethanol, and subsequently air-dried.

Quantification of DNA repair rates. For the imaging analysis, slides were stained with 50 μL of a 1 μg/mL 4',6-diamidino-2-phenylindole solution and the observations were made at ×20 magnification using the Olympus BX51 fluorescent microscope (Olympus España) connected to a CoHu 4912 CCD camera (CoHu). Fifty comets per duplicate gel were scored and quantified by Komet 5.5 image analysis software (Kinetic Imaging). The excision repair rate was measured quantitatively by the tail parameters: TDNA (percentage of DNA in the comet tail), tail length (distance in micrometer of the comet tail), extent tail moment (ETM; percentage of the tail length multiplied by the TDNA), and olive tail moment (OTM; percentage of DNA in the comet tail multiplied by the distance between the means of the head and tail distributions). Results were expressed as the mean ± SD of the parameters considered. The excision activity measurement for each enzyme extract was normalized by subtracting the level of DNA damage observed for the enzyme buffer only. Therefore, the results indicate solely the DNA excision rate of the protein extract.

Western blotting and antibodies. XPA protein expression changes in lymphocytes and normal and tumor epithelial cells were analyzed by Western blotting. Cell lysates were separated by SDS-PAGE and blotted onto nitrocellulose membranes by the iBlot dry blotting transfer system (Invitrogen). Membranes were blocked using Odyssey blocking buffer (Li-Cor Biosciences) for 1 h at room temperature and incubated with primary antibodies overnight at 4°C. Membranes were then incubated with the appropriate secondary antibodies for 1 h at room temperature and detection (simultaneously at 700 and 800 nm) and quantification were done with the Odyssey Infrared Imaging System (Li-Cor Biosciences). Normalization of gel bands was accomplished by radiometric analysis of two wavelengths, where β-actin was used as loading control. HeLa and H1299 cells were used as positive control.

XPA was detected with a mouse monoclonal antibody (ab65963; Abcam), and β-actin was detected with a rabbit polyclonal antibody (ab8227-50; Abcam). Secondary antibodies used were IRDye 800CW donkey (polyclonal) anti-mouse and IRDye 680CW donkey (polyclonal) anti-rabbit (Li-Cor Biosciences).

Statistical analysis. Comet assay measurements were shown after log10 transformation. The differences in the repair rates between samples were contrasted by the Pearson correlation coefficient and Kruskal-Wallis analysis. In all statistical tests, two-tailed P values < 0.05 were considered statistically significant. Statistical analysis used SPSS 13.0 statistical software (SPSS).

Results

Samples from normal and tumor colon tissues, as well as lymphocyte samples, were obtained from 23 colon cancer patients. Clinicopathologic characteristics of the patients included...
were as follows: median age, 67 ± 13 years; gender: male, 14 (60.87%) and female, 9 (39.13%); pathologic stages: 3 (13.04%) patients were in stage I, 10 (43.48%) in stage II, 8 (34.78%) in stage III, and 2 (8.7%) in stage IV; tumor location: right colon 12 (52.17%), left colon-sigma 7 (30.43%), and rectum 4 (17.39%); and tumor grade: well differentiated 3 (13.04%), moderately 19 (82.61%), and poorly differentiated 1 (4.35%). The adjuvant chemotherapy planned for patients in stage III was FOLFOX 6 (infusional fluorouracil, leucovorin, and oxaliplatin dose of 100 mg/m²; ref. 20).

### Table 1. Average mean ± SD of the comet parameters that show the repair activities of protein extracts from lymphocyte and normal and tumor epithelial cells

<table>
<thead>
<tr>
<th></th>
<th>TDNA</th>
<th>ETM</th>
<th>OTM</th>
<th>Tail length</th>
</tr>
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<tbody>
<tr>
<td>Lymphocytes</td>
<td>2.45 ± 0.86</td>
<td>0.65 ± 0.34</td>
<td>0.60 ± 0.29</td>
<td>4.69 ± 2.42</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>10.71 ± 13.00</td>
<td>6.13 ± 11.10</td>
<td>3.78 ± 5.53</td>
<td>17.66 ± 13.16</td>
</tr>
<tr>
<td>Tumor tissue</td>
<td>17.95 ± 16.65</td>
<td>11.80 ± 17.78</td>
<td>6.70 ± 8.64</td>
<td>27.23 ± 20.01</td>
</tr>
</tbody>
</table>

NOTE: Blood cells have the lowest repair rate and tumor epithelial cells have the highest repair rate.

**Repair capability of each cell type.** The repair experiments, carried out with the protein extract obtained from samples of each patient, showed differences in recognition and excision activity between different cell types. Mean ± SD of the comet parameters that measure the repair activities of the lymphocytes and epithelial colon samples are shown in Table 1. The repair assays showed that normal and tumor epithelial cells have much higher recognition and excision activity than lymphocytes. Therefore, epithelial colon cells have higher repair capability than blood cells, as each one of the comet parameters used indicates.

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**Fig. 2.** Representative fluorescence images at ×20 magnification of comets, showing the repair activity of 25 μg protein extracts, during 30 min incubation, from the different samples obtained from one patient. Differences between head and tail sizes indicate the estimated repair capability of the protein extract. Control cells treated only with the enzyme buffer (A), repair activity of the lymphocytes (B), epithelial cells from normal colon tissue (C), and epithelial cells from tumor tissue (D).
Kruskal-Wallis analysis showed not only significant differences between blood cells and epithelial colon cells for all comet parameters used (P < 0.001) but also remarkable differences between normal and tumor epithelial colon cells: TDNA (P = 0.015), ETM (P = 0.029), and OTM (P = 0.04), with tail length (P = 0.056) being the only parameter that did not show significant statistical difference, although it had a clear tendency toward it. This means that there are noteworthy differences in repair capability between samples tested, lymphocytes, and normal and tumor epithelial colon cells.

Characteristic fluorescence images of comets originating due to activity of the protein extracts from lymphocytes and normal and tumor epithelial colon cells from one of the patients are shown in Fig. 2.

**Lymphocytes are not surrogates of tumor cells.** To check whether the results obtained in studies using lymphocyte extracts are representative of those carried out in other cell types from the same cancer patient and, consequently, whether lymphocytes act as surrogate cells of the tumor, we studied the correlation levels between the repair activity observed in lymphocytes and normal and tumor epithelial colon cells in our patient series (Table 2).

Despite the clear difference observed with Kruskal-Wallis analysis in the repair activity of normal and tumor epithelial colon cells, there was a high ratio correlation level between their respective repair capabilities for all the comet parameters studied (Table 2; P < 0.01, where Pearson correlation levels oscillate between 0.57 and 0.62, according to the comet parameter considered). This means that repair capability between normal and tumor epithelial colon cells is analogous and comparable results will be obtained irrespective of the sample tested (Fig. 3A).

On comparing the repair activity of tumor epithelial cells and lymphocytes, we found no significant correlation between them in any of the parameters studied. Nevertheless, TDNA and OTM showed a tendency toward inverse correlation (Fig. 3B). Our data show that the repair capability of lymphocytes and tumor cells is unrelated: in consequence, lymphocyte activity is not predictive of the repair ability of the tumor.

Finally, after analysis and comparison of the repair capability of lymphocytes and normal epithelial colon cells, heterogeneous results were obtained. In this case, the tail moment parameters, ETM and OTM, revealed comparable results: \( r = 0.52; P = 0.036 \) and \( r = 0.532; P = 0.034 \), respectively. However, no significant association was found when we used TDNA and tail length comet parameters, although a remarkable correlation was shown: \( r = 0.311; P = 0.24 \) and \( r = 0.275; P = 0.303 \), in that order (Fig. 3C).

To show any other information indicative of the repair capacity of tissues examined, we also tested XPA protein expression changes in lymphocytes and normal and tumor epithelial cells by Western blotting. Eight patients with different repair capabilities in their lymphocytes and normal and tumor epithelial colon cells with comet assay were tested. Correlation of the comet assay results with XPA protein expression changes were found for two individuals: up-regulation of XPA was observed in samples with high repair capability and down-regulation of XPA in samples with low repair capability. The repair activity results of samples obtained from four representative patients and the corresponding XPA expression ratio are shown in Table 3.

### Discussion

Oxaliplatin is an effective drug in clinical treatment of colorectal cancer. Its therapeutic effect is a consequence of covalent binding to DNA-forming cross-links (21). These are removed by the cell process of the NER pathway, which consists of the following four steps: recognition of the DNA lesion, excision of a 24- to 32-nucleotide stretch containing the lesion by dual excision of the damaged DNA strand on both sides, filling in of the resulting gap by DNA polymerase, and ligation of the nick (22). Therefore, during the process, DNA breaks are produced as intermediates, which can be visualized as DNA migration by the comet assay. In the present study, we adapted the in vitro comet repair assay for NER activity (15) to evaluate the repair capacity of cell extracts to remove cross-links induced by oxaliplatin. Our aims were to measure the DNA repair ability of lymphocytes and normal and tumor epithelial colon extracts of cells isolated from biopsy samples of colon cancer patients and to compare the capacity of different cells from the same individual to remove oxaliplatin damage.

The comet repair experiments revealed significant differences between different cell types in recognition and excision activity.
to eliminate oxaliplatin injury. Because protein extract from colon epithelial cells showed much higher recognition and excision activity than protein from lymphocyte extract, colon cells have higher repair capability than blood cells. Moreover, as normal epithelial cells have lower repair activity than tumor epithelial cells, cancer cells have the highest repair capability. These differences observed between samples, lymphocytes and colon epithelial cells, are probably because they are distinct cell types from different tissues with different rates of proliferation and metabolic state. These features also explain the differences observed between normal and tumor epithelial colon tissues. In addition, the high repair ability of tumor cells could be due to the enhanced intrinsic repair of chemotherapy damage in cancer tissue (3). Intrinsic resistance is present at the time of diagnosis, and the patient fails to respond to first-line chemotherapy. None of our patient series received any treatment before resection of the tumor, so the increased DNA repair capacity observed would be anticipated as detrimental to oxaliplatin efficacy in an initial response to the therapy. However, in our research, survival analysis was not done because of the short follow-up, median 8 months (range, 7-10 months), and the limited size of our series. Consequently, further studies are needed to clarify whether the in vitro comet assay can be used to predict early resistance to chemotherapy. In our study, we also checked whether the results obtained in studies using lymphocytes with this method are representative of those done in other cell types from the same cancer patient. Thus, we studied the correlation levels between the repair activities observed in different samples obtained from our series of patients. On comparing the repair activity of tumor epithelial colon cells and lymphocytes, we found no significant correlation between them in any of the parameters studied. Therefore, our data show that the repair capability of lymphocytes and tumor cells extracts is unrelated. Consequently, lymphocyte activity is not predictive of the repair ability of the tumor and blood cells cannot act as surrogate cells of the tumor. In the case of the repair capability of extract of lymphocytes and extract of normal epithelial cells, the tail moment parameters, ETM and OTM, revealed comparable results, but no significant correlation was revealed with the TDNA and tail length comet parameters. These heterogeneous results may denote equivalent traits, as they are both healthy cells of the individual. However, at the same time, they are different cell types, blood cells and epithelial colon cells, with distinct rates of proliferation and metabolism state. Despite the clear difference observed in repair activity between normal and tumor epithelial cells, because tumor cells have

![Fig. 3. Diagram of correlations between repair activities measured with OTM and TDNA parameters in protein extracts obtained from lymphocyte samples and normal and tumor epithelial colon cells. Comet assay measurements are expressed after log_{10} transformation. The lines drawn in each panel correspond to 95% confidence intervals for the mean of distribution. We found statistically significant correlation between normal and tumor epithelial repair capability (A), whereas no significant association between repair activity of tumor epithelial cells and lymphocyte samples was revealed (B). In the case of normal epithelial tissue and lymphocyte cells, there was a remarkable correlation in repair rate for TDNA values (r = 0.311; P = 0.24) and a statistically significant association for OTM values (r = 0.532; P = 0.034).](#)
high DNA repair capability for removing the damage, there was a high correlation level between their respective repair activities. This means that repair capability in tumor and normal epithelial cell colonies is analogous and comparable results will be obtained regardless of the sample tested.

Although the *in vitro* comet assay method was developed for specific measurement of theNER capability of a protein extract of relevant cells ([14, 15]), we also tested XPA protein expression changes in the different samples from our patients by Western blotting. XPA is a DNA damage-binding protein required for the first steps of the nucleotide repair process. Several studies found defective repair of cisplatin-induced DNA damage as a result of decreased levels of the critical XPA and other NER protein in testicular cancer cells (23–25). In our study, we did not find good correlation between the comet assay data and XPA expression levels. Nevertheless, it should be noted that our

### Table 3. Repair activity measured with TDNA comet parameter in different samples obtained from four representative patients and the corresponding XPA expression after quantification and normalization with the Odyssey Infrared Imaging System

<table>
<thead>
<tr>
<th>Individual 20</th>
<th>Individual 13</th>
<th>Individual 21</th>
<th>Individual 19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>3.06 0.0023</td>
<td>4.42 0.0174</td>
<td>2.96 ND</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>7.28 0.0212</td>
<td>4.22 0.0232</td>
<td>59.82 0.0322</td>
</tr>
<tr>
<td>Tumor tissue</td>
<td>22.26 0.0101</td>
<td>7.55 0.0302</td>
<td>7.09 0.0020</td>
</tr>
</tbody>
</table>

NOTE: Protein expression results are shown as a ratio between the protein level of XPA and the β-actin housekeeping gene. We found correlation of the comet assay results with XPA protein expression changes in all different samples for individual 21. ND, no expression of XPA detected.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### References

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