DNA Repair and Cellular Resistance to Alkylating Agents in Chronic Lymphocytic Leukemia

Mark R. Müller, Claudia Buschfort, Jürgen Thomale, Carmen Lensing, Manfred F. Rajewsky, and Siegfried Seeber
Department of Internal Medicine (Cancer Research) [M. R. M., C. L., S. S.] and Institute of Cell Biology (Cancer Research) [J. T., C. B., M. F. R.], West German Cancer Center Essen, University of Essen Medical School, D-45122 Essen, Germany

ABSTRACT
The time course of the formation and persistence of repair-induced DNA lesions such as single-strand breaks (SSBs) were determined in isolated lymphocytes derived from 32 patients with chronic lymphocytic leukemia (CLL) using the single-cell gel electrophoresis (SCGE, "comet") assay. After pulse-exposure to N-ethyl-N-nitrosourea (EtNU), the initial amount of SSBs ($t_0$ SCGE values) and the time periods required to reduce DNA damage by 50% ($t_{50\%}$ SCGE values) were determined in nuclear DNA of individual cells. The $t_0$ SCGE and $t_{50\%}$ SCGE values varied interindividually between CLL specimens by factors of 16.6 and 8.2, respectively. Regarding cell-to-cell variation, no major subpopulations with significantly different DNA repair capacities were observed in cell specimens from a given patient. In addition, a monoclonal antibody-based immunocytological assay was used to determine the elimination kinetics for the cytotoxic alkylation product O6-ethylguanine from nuclear DNA. A strong correlation was observed between the relative times for SSB repair and the elimination of O6-ethylguanine from nuclear DNA. Because SCGE and immunocytological assay measure different steps of DNA repair, this observation suggests coordinated regulation of the respective repair pathways. With regard to chemosensitivity profiles, a "fast" repair phenotype corresponded to enhanced in vitro resistance to EtNU, 1,3-bis(2-chloroethyl)-1-nitrosourea, or chlorambucil. Accelerated SSB repair and pronounced in vitro resistance to chlorambucil, 1,3-bis(2-chloroethyl)-1-nitrosourea, and EtNU were found in lymphocytes from CLL patients nonresponsive to chemotherapy with alkylating agents. Distinct DNA repair processes thus mediate resistance to alkylating agents in CLL lymphocytes.

INTRODUCTION
Drug resistance to chemotherapy represents one of the key problems in cancer chemotherapy and in the treatment of lymphomas including CLL in particular. The capacity to repair cytotoxic DNA lesions induced by alkylating agents has been identified as an important mechanism underlying drug resistance in cell lines derived from primary tumors (1, 2). The repair of DNA monoadducts or cross-links is a complex process involving the coordinated, successive action of many proteins in parallel pathways (1, 2). AT (EC 2.1.1.63) repairs DNA by transferring alkyl groups from the O6-atom of guanine (O6-AlkGua) to one of its own cysteine residues (3–6). Recent reports suggest that DNA repair pathways such as base or nucleotide excision can also contribute to the removal of O6-AlkGua in DNA (7–9). Furthermore, mismatch repair mutants are tolerant to O6-AlkGua, which would otherwise be lethal (8). Enhanced DNA cross-link repair has been attributed to resistance to melphalan in human cell lines (10, 11). Overexpression or loss of various DNA repair proteins, such as 3-methyladenine DNA glycosylase, the ERCCI protein, DNA topoisomerase IIa, or the damage recognition protein XP-A, modulate cellular sensitivity to DNA-damaging agents (12–15). On the other hand, diminished, rather than increased, resistance to alkylating agents has been observed in hamster cells overexpressing 3-methyladenine DNA glycosylase, which catalyzes an early step in DNA base excision repair (16). It is thus probable that deregulation of DNA repair and/or the accumulation of repair intermediates such as SSBs contribute significantly to the cytotoxicity of alkylators in addition to primary drug-DNA reaction products.

Substantial interindividual differences in the expression or activity of DNA repair proteins have been described (17–20). It is not yet known whether this wide interindividual range in DNA repair phenotypes translates into clinical responsiveness to treatment with alkylating anticancer drugs.

CLL represents a model particularly suited to study the development of drug resistance. At first presentation, CLL patients are usually either untreated or on oral CLB chemotherapy and become resistant to this regimen later on. In extracts of lymphocytes from nonresponsive CLL patients, increased expression of the DNA excision repair protein ERCC1 and enhanced repair of DNA cross-links were observed (21, 22). Other studies, however, did not find a relation between repair of...
cross-links or the expression of DNA repair proteins and treatment outcome in leukemia patients (23–25). The clinical significance of DNA repair as a major determinant of drug resistance thus remains controversial.

Methods for the measurement of DNA repair in individual cells have been developed recently (19, 20, 26–30). One of these techniques, the SCGE “comet” assay, has been applied for the measurement of DNA damage and repair at the single-cell level (26–30). Following neutral or alkaline lysis of cells, this assay uses the electrophoretic mobility of DNA fragments in agarose gels that, after staining with a fluorescent DNA dye, appear as “comets” emerging from the compact nuclear DNA. The formation and gradual disappearance of “comets” correspond, respectively, to the number of DNA double-strand breaks (neutral lysis) or alkali-labile lesions and SSBs (26–30). Several different SCGE protocols exist, using either irradiation or various DNA-reactive agents for the induction of DNA damage and, furthermore, various lysis conditions, incubation periods, and end points (27, 30).

The SCGE assay has been used for the single time assessment of (unprocessed) DNA damage [corresponding to the time of incision and rejoining of DNA strands during excision repair. In the present protocol, CLL lymphocytes were pulse-exposed to EtNU and analyzed at different time points for comet formation. One advantage of using EtNU as a DNA-damaging agent, although it is not used clinically as a chemotherapeutic drug, is that all of its major DNA reaction products, the alkylating product, 06-EtGua, in the nuclear DNA of individual cells: ICA. Lymphocytes (5 × 10^7/10 μl) were suspended in low-melting-point agarose (0.6%, Biozym). The cell suspension was pulse-exposed for 20 min to EtNU at 37°C. Cells were then lysed twice in PBS and resuspended in RPMI 1640 kept at 37°C in a humidified atmosphere. Samples were taken immediately or at several time points up to 9 h after EtNU treatment. After sampling, cells were washed with PBS and suspended in low-melting-point agarose (0.6%, Biozym). The cell suspension was spread evenly onto a fully frosted microscope slide covered with a thin layer of 0.6% LE agarose. A top layer of 0.5% low melting point agarose was used to protect the cells. Cells were then lysed by 2.5 mM NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, and 1% sodium sarcosinate (pH 10) at 4°C overnight. Afterward, slides were incubated in an alkaline electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH 12) for 20 min with two buffer changes to unwind the DNA. The slides were then subjected to electrophoresis in the same solution for 20 min at 4°C and 4 V/cm, neutralized (3 × 10 min in 0.4 mM Tris-HCl, pH 7.5), and stained with ethidium bromide (2 μg/ml).

Measurement of O6-EtGua in the Nuclear DNA of Individual Cells: ICA. Lymphocytes were exposed to EtNU essentially as described for the SCGE assay. After 20 min of incubation, cells were washed twice in PBS and resuspended in fresh, prewarmed RPMI 1640. Samples were taken immediately or 1.5, 3, 6, 9, and 24 h after EtNU treatment and transferred to microscopic slides. The immunofluorescence staining of O6-EtGua in nuclear DNA was performed as described previously (19, 20). Briefly, cells on slides were fixed with methanol, and DNA was partly denatured by alkali treatment. Cells were then incubated with the (anti-O6-alkylguanine) monoclonal antibody ER-17 at a concentration of 0.2 μg/ml in PBS/1% BSA for 16 h at 4°C. Cells were washed and stained with goat anti-rat IgG (Fab)2 fragment conjugated with rhodamine isothiocyanate (Dianova; 2 μg/ml PBS/1% BSA) for 45 min at 37°C. Nuclear DNA was counterstained with the fluorescent dye 4,6-diamidino-2-phenylindole (3 × 10⁻⁷ M in PBS for 10 min).

Quantitation of Fluorescence Signals and Data Analysis. A fluorescence photomicroscope (Zeiss Axiosplan) equipped with a HBO 100-W mercury arc lamp and Zeiss
standard filter combinations 02 (for 4,6-diamidino-2-phenylindole), 14 (for tetramethylrhodamine isothiocyanate), and 15 (for ethidium bromide) were used. Fluorescence signals were amplified and recorded by a dual-mode charge-coupled device camera (Phototonics, Hamamatsu City, Japan) and fed into a multiparameter image analysis program (ACAS Cytometry Analysis System; Ahrens Electronics, Bargteheide, Germany). In case of the SCGE, the amount of DNA damage in single cells was determined by measuring the total area of stained nuclear DNA and the fluorescence intensity by image analysis. Comet sizes were defined as the relative increase of fluorescence area of EtNU-treated lymphocytes compared to the area of nuclei of untreated control cells.

MTT Assay. For the tetrazolium dye (MTT) assay, cells were seeded into 96-well microtiter plates using 200 μl/well of a cell suspension containing 10^6 lymphocytes/ml of RPMI 1640 supplemented with 10% FCS. Dissolved drugs were added in 20 μl of PBS. On day 4, 20 μl of a solution of 5 mg MTT/ml of PBS were added to each well, and the plates were further incubated for 5 h. Thereafter, the plates were centrifuged for 10 min at 100 × g. The medium was removed from each well, 200
RESULTS

Formation and Repair of SSB and O\textsuperscript{6}-EtGua in the Nuclear DNA of CLL Lymphocytes. CLL lymphocytes were exposed to EtNU for 20 min and analyzed for the initial increase, and subsequent decrease, in electrophoretic mobility of nuclear DNA (\(t_0\ \text{SCGE}\)). The initial comet size, representing the number of DNA SSBs and alkali-labile sites after 20 min of EtNU exposure, was determined by relating the increased stained DNA area of treated cells to the area of untreated control cells from the same donor (Fig. 1). The time periods required to reduce the "initial" comet area by 50% (\(t_{50\%}\ \text{SCGE}\)), were determined as a measure for the cellular capacity to process secondary DNA lesions. Typical examples of the DNA repair kinetics for induced DNA strand breaks are shown in Fig. 2. Initial comet areas varied interindividually between 26 and 435% (Fig. 3). Similarly, the kinetics of comet formation and disappearance showed considerable interindividual differences, with the \(t_{50\%}\ \text{SCGE}\) values varying by factors up to 8.2 between CLL specimens (Fig. 3). No correlation was found between "initial" DNA damage (\(t_0\ \text{SCGE}\)) and the \(t_{50\%}\ \text{SCGE}\) repair values (\(P > 0.05\)). The pattern of comet formation was generally uniform among CLL lymphocytes within a given sample (coefficients of variation, 7.2–19.6). Subpopulations displaying degrees of DNA damage deviating significantly from the mean value of a specimen were not observed.

In parallel, the repair kinetics of the alkylation product \(O^6\)-EtGua from DNA were measured after 20 min of exposure to EtNU in selected specimens using the monoclonal antibody-based ICA (Fig. 2). For each time point, antibody-mediated fluorescence signals were corrected for nuclear DNA content. The time periods required to eliminate 50% (\(t_{50\%}\ \text{ICA}\)) of \(O^6\)-EtGua residues from DNA were determined as a measure of the efficiency of early DNA repair steps contributing to adduct removal. In specimens from four CLL patients, cellular DNA repair kinetics were determined by

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\frac{\mu l}{340} \text{ of DMSO were added to dissolve the crystals, and the plates were shaken for 10 min. Absorbances at 540 and 690 nm were read using a dual wavelength Dynatech MR 7000 plate-reader. In all experiments, four replicate wells were used for each drug concentration. The drug concentration required to reduce the absorbance at 540 nm to 50% of the control was taken to be the ID\textsubscript{50} of the sample.}
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Statistics. The Spearman rank correlation coefficient was applied to \(ID_{50}\) and \(t_{50\%}\) values. The Student's \(t\) test was used to calculate statistical significance of any differences between various groups of patients.
Fig. 5 Correlation between chemosensitivity in vitro and DNA repair time for SSBs in CLL lymphocytes. The Y-axes display ID_{50} values for EtNU, BCNU, and CLB, respectively, and the X-axes the time interval required for the reduction of initial comet formation by 50% (t_{50\%} SCGE values). The Spearman rank correlation coefficients (r_{s}) for DNA repair time and cytotoxicity were r_{s} = -0.71 for EtNU, r_{s} = -0.54 for BCNU, and r_{s} = -0.09 for CLB, respectively (P < 0.001).

Table 1 Chemosensitivity of isolated lymphocytes derived from untreated (U), treated sensitive (TS), and treated resistant (TR) CLL patients

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<th>U</th>
<th>TS</th>
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<tr>
<td>EtNU (μg/ml)</td>
<td>78.4 ± 32.0</td>
<td>89.4 ± 21.7</td>
<td>144.0 ± 39.0</td>
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<tr>
<td>BCNU (μg/ml)</td>
<td>5.6 ± 2.7</td>
<td>6.6 ± 1.4</td>
<td>9.2 ± 2.6</td>
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<tr>
<td>CLB (μg/ml)</td>
<td>2.1 ± 0.7</td>
<td>2.5 ± 0.9</td>
<td>3.7 ± 1.1</td>
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*P < 0.001.

Relationship of DNA Repair Capacity to Chemosensitivity to Alkylation Agents in Vitro. DNA repair capacities, as determined by SCGE, were related to the chemosensitivity of CLL lymphocytes to the alkylation agents EtNU, BCNU, and CLB. A significant ranking correlation was found between the t_{50\%} SCGE and ID_{50} values for these DNA-reactive drugs (Fig. 5). No relationship was found between t_{50\%} SCGE values and in vitro drug resistance (P > 0.05). The ID_{50}s of EtNU, BCNU, and CLB were positively correlated (EtNU/BCNU, r_{s} = 0.51; EtNU/CLB, r_{s} = 0.70; BCNU/CLB, r_{s} = 0.52; P < 0.001; n = 26) and suggested cross-resistance of CLL lymphocytes to alkylation agents forming different DNA adducts.

Relationship of DNA Repair Capacity to Clinical Status. Fig. 6 displays the DNA repair rates of CLL lymphocytes in relation to the patients’ clinical status. The mean t_{50\%} SCGE values for secondary DNA lesions were not significantly different between specimens derived from untreated (n = 12) or treated sensitive (n = 10) CLL patients. In contrast, high rates of DNA strand break processing were observed in specimens from 10 CLL patients resistant to treatment with alkylation agents. The respective mean t_{50\%} SCGE values were significantly lower in comparison to untreated or treated sensitive CLL patients. The degree of initial DNA damage (t_{0} SCGE) was

SCGE and ICA simultaneously (Fig. 4). A positive correlation was observed between the t_{50\%} values for both the elimination of O^6-EtGua from nuclear DNA and the repair of SSBs (r = 0.98, P < 0.015).
unrelated to clinical status \( (P > 0.05) \). Table 1 shows the ID\(_{50}\) for alkylating agents determined in CLL lymphocytes in relation to clinical status. The mean ID\(_{50}\) for EtNU, BCNU, and CLB were significantly elevated in lymphocytes from CLL patients resistant to chemotherapy with CLB in comparison to untreated or treated sensitive patients.

**DISCUSSION**

The SCGE comet assay was applied to evaluate in vitro the efficiency of DNA excision repair in individual CLL lymphocytes in relation to DNA monoadduct elimination, chemosensitivity to alkylating agents, and to clinical status. EtNU-induced DNA monoadducts, such as 06-EtGua, are partly removed from DNA by the repair protein AT in a one-step process (3). Furthermore, 06-AlkGua is a substrate for mismatch repair processes, and excision repair also contributes to the elimination of 06-AlkGua from nuclear DNA (7–9). During these processes, SSB in DNA result from the incision by endonucleases as repair intermediates. Following pulse-exposure to EtNU, the initial numbers of SSBs varied considerably between CLL specimens. We have shown previously that this variation is not due to different levels of primary adduct formation but rather is related to different efficiencies of early steps in DNA repair (33). For example, EtNU failed to induce SSBs in vitro prior to the addition of DNA repair proteins. Widely varying time intervals were required for the disappearance of repair-induced SSBs, as observed among specimens from different CLL patients. This may reflect large interindividual differences in the concentrations/activities of DNA repair proteins in human cells, as reported previously (17–20). However, no obvious relationship was found between the initial comet size after EtNU \( t_0 \text{SCGE} \), used as an end point for DNA damage in most studies (27), and \( t_{50\%\text{SCGE}} \) or any other parameter tested. One explanation for this lack of correlation is that the number of SSBs measures at a given time point after drug exposure is dependent on several factors, such as the activity of early steps in DNA repair (glycosylases and endonucleases) and the efficiency of the downstream processing of DNA lesions by, e.g., DNA polymerases/ligases during the incubation period.

DNA repair phenotypes are heterogeneous not only between individuals but also among different cell types (34, 35). To monitor the DNA repair capacity of tumor cells from cancer patients, reliable, quantitative assays are required that allow the selective analysis of individual cells in heterogeneous biopsy material. The present approach does not permit the measurement of DNA repair efficiency in a given cell as a function of time; however, it enables us to evaluate cell-to-cell variation among a small number of cells by SCGE and ICA. Nevertheless, detectable subpopulations of CLL lymphocytes with DNA repair phenotypes significantly different from the average value for the total cell population were not observed among the lymphocyte specimens examined in this study.

ICA measures the efficiency of early steps of 06-EtGua repair including those effected by AT or excision repair proteins. Later stages of DNA damage processing, such as gap filling and rejoining of SSB during excision repair, are monitored by SCGE. It is, therefore, interesting to note that the relative repair rates determined by these functional assays covering different areas of the DNA repair network were correlated. This correlation suggests at least partial coordination of different rate-limiting repair components. It remains to be determined whether this reflects the predominant action of a certain repair pathway measured by both assays or coregulation of key components of distinct repair pathways. The latter possibility is supported by the observed cross-resistance of CLL lymphocytes to structurally unrelated alkylating agents inducing different patterns of DNA adducts.

In cell lines, an inverse relationship has been reported previously between the repair capacity of tumor cell lines for SSB or AT activity and the chemosensitivity to alkylating agents (36, 37). In the present study, increased in vitro chemoresistance to alkylating agents was observed in CLL lymphocytes exhibiting a fast DNA repair phenotype. This suggests the clinical importance of DNA repair in mediating cellular resistance to alkylating agents in human leukemic cells. It is not yet established whether the observed broad range of interindividual DNA repair capacities translates into clinical responsiveness to alkylating drugs. We have shown previously that CLL lymphocytes of chemotherapy-resistant patients displayed higher rates of adduct elimination in comparison to CLL lymphocytes of responsive patients (19). The present study has complemented this observation by the finding that CLL lymphocytes from nonresponsive patients exhibited faster processing of secondary, repair-induced DNA lesions, such as SSBs, compared to control cells. Both observations underline the clinical significance of DNA repair as an important mechanism of resistance to alkylating agents in leukemic cells. Thus far, the number of specimens studied is relatively small. Future studies should encompass a larger number of patients to exclude biased selection. In any event, use of functional assays such as SCGE and ICA will provide a means to monitor DNA repair in cancer patients to facilitate the rational design of chemotherapeutic regimens.

**ACKNOWLEDGMENTS**

We thank Bettina Baack for excellent technical assistance.

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