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DNA Repair Initiation Induces Expression of Ribonucleotide Reductase in Human Chronic Lymphocytic Leukemia Cells

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Abstract

Mammalian ribonucleotide reductase (RR) is a heterodimer enzyme responsible for maintaining levels of deoxynucleotides needed for DNA replication. The M2 subunit of RR is known to increase in tandem with progression of cells into S phase, whereas the M1 subunit is expressed at steady-state. Since the expression level of the M2 subunit increases because the cells need deoxyribonucleoside triphosphates (dNTPs) for replication, it is logical to hypothesize that the same increase will be seen during DNA repair. To test this, we used CLL cells, which are replicationally quiescent and have low endogenous levels of RR and dNTPs. Cyclophosphamide was selected as the DNA damaging agent because of its clinical use in the treatment of CLL. DNA repair, measured by [³H]thymidine incorporation after 4-h treatment with 4-hydroperoxycyclophosphamide increased in a dose-dependent manner at 3, 10, and 50 μ M. The induction of DNA repair concomitantly increased the mRNA and protein levels of M2 subunit (median 1.6 fold; range 0.9-5.3). Maximum induction occurred at 10 μ M after 4 h and correlated with [³H]thymidine incorporation ($p = 0.02$). In contrary, no change was observed in mRNA or protein levels of M1 subunit. We conclude that RR is regulated not only during replication but also during DNA repair and in both cases M2 subunit expression was increased.

Keywords

chronic lymphocytic leukemia; cyclophosphamide; deoxynucleoside triphosphate; ribonucleotide reductase

INTRODUCTION

Ribonucleotide reductase (RR) is the enzyme responsible for producing and maintaining four deoxyribonucleoside triphosphates (dNTPs) necessary for DNA synthesis [1]. This reaction is the rate-limiting step for the *de novo* synthesis of dNTPs. Mammalian RR is a heterodimer consisting of two non-identical subunits, M1 and M2 [2]. The active catalytic site of the enzyme is formed by elements of both M1 and M2 subunits.

The fidelity of DNA replication is maintained by controlled synthesis of dNTP and this in turn is sustained by interactive regulatory mechanisms of RR. One of the most vital regulations on RR is the allosteric regulation exerted by its substrates and products. Another important regulation imposed on RR is by the cell cycle. Levels of RR activity and DNA synthesis have been shown to be closely correlated [3,4]. Much of the enzyme's capacity to synthesize the dNTPs needed for DNA is restricted to S phase; however, in certain situations, DNA synthesis is needed outside of S phase. The prime example for this scenario is the induction of DNA synthesis after DNA damage. Nucleotide excision repair, mismatch repair, recombinational repair, and post-replicative repair all require a step in DNA synthesis involving the synthesis or utilization of stored cellular dNTPs [5-8].

Previous studies have shown specific channeling of certain dNTPs in DNA repair. For example, deoxycytidine triphosphate (dCTP) synthesized from the salvage pathway was specifically used for DNA repair, whereas the dCTP pool from the *de novo* pathway, in which RR is used, was shown to be preferred for DNA replication [9-11]. It is not clear, however, whether such channeling occurs for all four dNTP pools.

In contrast to the channeling of dNTPs for specific processes in DNA synthesis, several lines of evidence suggest that, in a variety of cell types, dNTPs produced through the *de novo* pathway are used for DNA repair in yeast [12] in mouse [13], and in human [14]. Consistent with these observations, other studies have shown that RR inhibition results in a decrease in the DNA repair capability [15-17]. Although these studies strongly suggested involvement of RR in DNA repair, it is difficult to rule out the influence of DNA replication on RR perturbations. An element of RR perturbation by DNA replication exists in these systems because all the investigations cited above were conducted with exponentially growing cell populations. The optimal system for studying the relationship between RR and DNA repair may be, therefore a cell population of non-replicating cells.

Leukemic lymphocytes from patients with chronic lymphocytic leukemia (CLL) are primarily in the G₀ and G₁ phase of the cell cycle [18]. Their indolent nature was instrumental in alleviating the problem of background DNA synthesis in our evaluation of DNA repair induction. We hypothesized that the DNA damage caused by agents such as cyclophosphamide in quiescent CLL cells will require RR activation to provide the deoxyribonucleotides needed for DNA repair synthesis. Additionally, the fludarabine (a ribonucleotide reductase inhibitor) and cyclophosphamide couplet is standard of care for patients with CLL which provides a treatment objective to test role of RR during DNA repair.

EXPERIMENTAL PROCEDURES

Patient Samples

The samples used in this study were obtained from peripheral blood of patients diagnosed with CLL. The samples were collected by registered nurses after patients signed an informed consent. The protocol to obtain peripheral blood samples from patients with CLL was approved by the Institutional Review Board of the MD Anderson Cancer Center. To obtain a sufficient number of CLL lymphocytes, only patients with $> 10,000$ WBC/ μ l in peripheral blood were selected.

Drugs and Other Materials

The alkylating agent cyclophosphamide was selected as a DNA-damaging agent for all experiments. Cyclophosphamide itself has minimal to no intrinsic activity; it exhibits no cytostatic effect in vitro and to be effective, must be activated in vivo by hepatic microsomal cytochrome P-450. Hence, in in-vitro cell culture experiments, 4-hydroperoxycyclophosphamide (4-HC) is used as a surrogate for cyclophosphamide. For our studies, 4-HC was provided by Dr. Susan Ludeman (Duke University Comprehensive Cancer Center, Durham, NC). Hydroxyurea was purchased from Sigma Chemical Co. (St. Louis, MO); [methyl- 3 H]thymidine purchased from ICN Radiochemicals (Irvine, CA) had a specific activity of 50 Ci/mmol.

Cell Culture

The leukemia cell line ML-1, used either as positive control or standard, was provided by Dr. M. B. Kastan (Duke University, Durham, NC). The cell line was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in an incubator with 5% CO₂. The doubling time of these cells is approximately 24 h. To ensure their integrity, the cells were subjected to mycoplasma testing every 3 months using the Mycoplasma Rapid Detection kit (Gen-Probe Inc., San Diego, CA).

Isolation of CLL Lymphocytes

Whole blood was collected in heparinized tubes from patients with B-CLL. Leukemic lymphocytes were isolated by Ficoll-Hypaque (specific gravity 1.086) density centrifugation [19]. Cells were counted and resuspended at a concentration of 5×10^6 cells/ml in RPMI 1640 supplemented with 10% fetal calf serum and 1% penicillin-streptomycin (GIBCO, Gaithersburg, MD). Cells were incubated overnight at 37°C to allow acclimatization to culture conditions and for the removal of monocytes by plastic adherence.

Drug Treatment

For every experiment, a fresh solution of 4-HC was prepared in H₂O. Generally, cells were either incubated with 10 μ M of 4-HC at the indicated times or with indicated concentrations of 4-HC for 4 hours. At chemotherapeutic doses of cyclophosphamide, such concentrations are achieved in plasma [20].

Cell Cycle Analysis

An evaluation of the cell population in each cell-cycle phase was performed by flow-cytometric analysis of 1×10^6 cells after propidium iodide staining. Analysis was done with a Becton Dickinson Flow Cytometer (Mountain View, CA) using Lysys II software to detect DNA content.

DNA Synthesis Assay

Unscheduled DNA synthesis (DNA repair) was measured by the incorporation of [^3H]thymidine into DNA exposed to 4-HC. Because CLL cells are quiescent, no replicative DNA synthesis is expected to occur, and for this reason, patients with a high percentage of circulating CLL lymphocytes in peripheral blood were selected. To reduce the number of contaminating blood cells, cells were kept overnight in culture and monocytes were removed. Further, to prevent replicative DNA synthesis that might occur in contaminating nonquiescent blood cells, 2 mM hydroxyurea was added to each sample [19], before treatment with various concentrations of 4-HC. To quantitate DNA synthesis, 5 μCi of [^3H]thymidine was added to each sample for 4 h. Because thymidine is phosphorylated to mono-, di-, and tri-phosphate using nucleoside and nucleotide kinases by salvage pathway, it does not require ribonucleotide reductase activity. We expect that cellular reserved dNTPs will provide deoxynucleotides needed for this 4 h time period of unscheduled DNA synthesis while ribonucleotide reductase is inhibited by hydroxyurea. Thymidine incorporation in DNA was measured using perchloric acid extraction followed by radioactivity counting, as described elsewhere [19]. Radioactivity counts in control sample (hydroxyurea treated but without 4-HC) is expressed as 100%. Remaining data, obtained after 4-HC treatment, are expressed as percent of control and represent unscheduled (DNA repair) DNA synthesis. This assay is adapted from previously published method of unscheduled DNA synthesis measurement after radiation treatment [21].

Ribonucleotide Reductase Protein

CLL lymphocytes or ML-1 cells were harvested at different time points after incubation with varying concentrations of 4-HC and centrifuged at 1500 rpm for 5 min at 4°C. The cells (1×10^7) were lysed in 250 μl of lysis buffer (1% v/v Triton, 0.5% w/v deoxycholate, 0.1% w/v sodium dodecyl sulfate, 5 mM EDTA, 100 mM NaCl, 10 mM PO_4 -buffer, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 5 mM aprotinin) by sonication. Supernatant was collected and protein determination performed with the Bio-Rad protein assay (Hercules, CA). Cell extracts containing 30 μg of protein were resolved on 10% SDS-PAGE and transferred to an Immobilon-P membrane at 250 mAmps for 2.5 h. The membrane was cut so that the region containing the M1 protein (88 kDa) could be blotted separately from the region containing the M2 and actin proteins (40-45 kDa). The membrane was blocked with 4% milk/TBS-T solution for 4 h, followed by overnight probe of primary antibody and washing. Secondary mouse antibodies were added to the respective blots for 1 h followed by washing. Monoclonal mouse anti-ribonucleotide reductase (Accurate Chemical & Scientific Corporation, Sussex, England) for the M1 subunit at a dilution of 1:100, a monoclonal rat anti-tubulin as a surrogate for the M2 subunit at a dilution of 1:70, as well as polyclonal rabbit anti-ribonucleotide reductase for the M2 subunit (Dr. T.J.

Kinsella, Department of Radiation Oncology, Rhode Island Hospital, Providence, RI) at a dilution of 1:100 were used as primary antibodies. Actin antibody (Sigma Chemical Co.) was used at a dilution of 1:4000. Several secondary antibodies were used: a mouse antibody (Bio-Rad) at a dilution of 1:5000, secondary rat antibody (Santa Cruz) at 1:500 dilution, and secondary rabbit antibody (KPL, Gaithersburg, MD) at a dilution of 1:500. The immunoblot was developed using the ECL chemiluminescent kit (Amersham Life Sciences, Arlington Heights, IL). Immunoblots of the M2 subunits were stripped and reprobed with the actin antibody. The relative expression of the protein of each subunit was determined using a Molecular Dynamics personal densitometer (Sunnyvale, CA). To evaluate the quantitation of the M1 and M2 subunits, the values obtained from the same immunoblots for actin protein were used to normalize the protein loaded.

Ribonucleotide Reductase mRNA Transcripts

Before and after drug treatment, ML-1 cells or CLL lymphocytes (5×10^6 per sample) were harvested and RNA was extracted using Trizol reagent as described by the manufacturer (GIBCO BRL). RNA was quantified using a Pharmacia Biotech Ultraspec 3000 spectrophotometer (Piscataway, NJ) at 260-nm wavelength. Uniquely designed M1 and M2 subunit oligonucleotide probes and primers were purchased from Perkin Elmer (Branchburg, NJ). Glyceraldehyde phosphate dehydrogenase (GAPDH)-specific oligonucleotide probe was also obtained from Perkin Elmer. In designing these primers and probes, MacVector software was used. Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using an ABI Prism 7700 Sequence Detection System in the presence of 10 μ M of a GAPDH-specific oligonucleotide probe (Perkin Elmer). 10 μ M of M1 and M2 subunit oligonucleotide probes were used in conjunction with the GAPDH-specific probe. The assay was prepared using the TaqMan PCR Core Reagent Kit along with MuLV reverse transcriptase and RNase inhibitor (Perkin Elmer). RNA samples from leukemic lymphocytes of patients with CLL were loaded at 100 ng, then RNA from each patient sample was mixed, in duplicate, with probes and primers of GAPDH, M1 subunit, or M2 subunit. The GAPDH probe was used as an endogenous control to normalize the fluorescence data obtained for each subunit. Gene expression was quantitated by the relative standard-curve method, as suggested by the manufacturer. In this method, quantity is expressed relative to a basis sample. ML-1 standards were used as a basis sample. The standard curve produced by the ML-1 cell extracts was used to obtain the amount of target in each sample. The standard curve produces C_T values (which denote the first cycle in which fluorescence can be detected above a threshold value set on the detector) that can then be associated with known RNA values. The amplification plots are reported in the change of normalized reporter dye versus the cycles of amplification, in which an increase in the reporter fluorescence is proportional to the amount of PCR product. The C_T values produced by each patient sample are then used with the standard curve to interpolate the amount of RNA.

Statistical Analyses

P values were calculated by using the one-tailed Student's t-test for paired samples. Linear regression analyses for r values and p values for correlation of protein level and thymidine incorporation level were done using Prism Software Version 3.0 (GraphPad Prism, Inc., San Diego, CA)

RESULTS

Cell Cycle Analysis of CLL Cells and Effect of 4-HC

To determine whether the majority of cells was in the quiescent phase of cell cycle, cells from 5 patients were stained with propidium iodide and analyzed with a flow cytometer. Similarly, to determine effect of 4-HC on the cell cycle, cells were treated with 10 μ M 4-HC for 4 h before propidium iodide staining. In the 5 samples examined (Table I), the percent of cells in G₀/G₁ phase is high in the untreated cell population (median 99.2%, range 98.7 - 99.8%). Furthermore, in each patient sample, the percentage of cells in each cell-cycle phase remained fairly constant from before to after treatment. Although, in 4 of 5 samples, a higher percentage of cells were in S phase in the treated than in the untreated samples, the difference was not significant ($p = 0.14$). Thus, 4-HC treatment did not affect the cell-cycle profile of these quiescent cells. Although patient sample number 4 showed a higher percentage of cells with DNA content less than G₁ cells, indicating dead cells, this percentage did not increase with drug treatment.

Induction of DNA Repair Activity by 4-HC Treatment

As described above, the majority of the cell population was in G₀/G₁ phase, indicating a quiescent nature of the cell. However, there is a small fraction (less than 3%) of cells that were in S phase. Because these cells are in S phase, they will incorporate [³H]thymidine more efficiently than quiescent cells in which DNA repair was induced with 4-HC treatment. To block the DNA replication signal, all cells were treated with 2 mM hydroxyurea for 30 min. The capacity of thymidine incorporation was then considered as the extent of DNA repair that could be induced in CLL. Unscheduled DNA synthesis was taken as an assessment of DNA repair[22]. Cells were incubated with varying concentrations of 4-HC for 4 h, and unscheduled DNA synthesis was measured by incorporation of [³H]thymidine into DNA (Figure. 1). The thymidine incorporation value obtained in untreated cells was taken as 100%. Compared to this control value, a $19.9 \pm 6.7\%$ ($n=5$) increase was seen in thymidine incorporation after treatment with 3 μ M 4-HC ($p = 0.02$), this was further increased at the 10- μ M concentration. In a cohort of 23 patients, there were 7 patient samples whose cells did not show an increase in thymidine incorporation after 4-HC treatment. The mean increase in thymidine incorporation signal after 4-h 10- μ M 4-HC treatment was 125% ($n=23$), significantly different from the value in untreated cells ($p = 0.0001$). Samples from 4 patients analyzed after incubation with 50 μ M showed a mean increase of 136% ($p = 0.07$). These data suggested that 4-h treatment with these concentrations of 4-HC initiated DNA repair synthesis.

Effect of 4-HC on M2 Protein Expression—Dose-dependent Response

Thymidine incorporation data suggested that 4 h of 3, 10, and 50 μ M 4-HC treatment resulted in unscheduled DNA synthesis in a majority of patient samples. To determine whether this DNA repair synthesis resulted in an induction of the RR proteins to synthesize dNTPs, protein expression levels were measured by western blot analysis. Figure. 2A is a representative immunoblot of the dose response. As evident from the immunoblot, the expression of the M2 protein level gradually increased at 1, 3, and 10 μ M 4-HC. At 30 and

100 μ M, the level of M2 protein decreased. In contrast, there was no increase in M1 protein level (data not shown).

To test whether such dose-dependent perturbations occurred in these proteins in all patient samples, similar experiments were done with CLL cells from 5 patients (Figure. 2B). At all concentrations tested, the M1 subunit protein levels were similar to those of untreated samples (data not shown). This is in contrast to the M2 subunit, which showed a gradual dose-dependent increase (Figure. 2B). Densitometric measurements indicated a 1.65-, 1.76-, and 1.45-fold change for the 3, 10, and 30 μ M concentrations, respectively, compared with the untreated control sample. Cells treated with 10 μ M 4-HC displayed the highest elevation in expression of M2 protein levels, while concentrations of more than 10 μ M produced descending levels of protein, albeit still higher levels than those of the control. The increases seen at the 3 and 10 μ M concentrations were statistically significant, with *p* values of 0.01 (*n* = 7) and 0.05 (*n* = 10), respectively.

Effect of 4-HC on M2 Protein Expression–Time-course Response

Since CLL cells treated with 10 μ M 4-HC demonstrated the most notable increase in protein levels, this concentration was used in time-course experiments. Figure. 3A shows a representative immunoblot of a time course with 10 μ M 4-HC. The expression level of the M1 subunit did not increase throughout the time course (data not shown), and the M2 protein level, starting with minimal expression in the control lane, demonstrated an increase beginning at 1 h, continuing up to 8 h and decreasing at 24 h. Again, evaluations of the actin protein levels were done for normalization purposes.

Similar evaluations of CLL cells from 18 patients were done to investigate whether the pattern was uniform in most patients. Although a slight perturbation occurred in the protein level throughout the time course, the M1 subunit did not show an increase in expression (data not shown); protein remained at basal levels throughout the time course, with insignificant fluctuations (*p* = >0.05). With 10 μ M of 4-HC, the M2 subunit protein level (Figure. 3B) started to increase at 2 h, most distinct increases being seen at 4 and 8 h. Quantitative analysis by densitometer at these time points indicated significant 1.55-fold (*p* = 0.02, *n* = 18) and 1.58-fold (*p* = 0.04; *n* = 6) increases, respectively.

To evaluate the validity of the increase in M2 protein level and the absence of increase in M1 seen in the immunoblots, 20 paired samples were used to compare the fold increase of the M2 protein level with the increase in the M1 level. A paired Student *t*-test reflected a significant increase in the M2 (*p* = 0.03) and no significant increase in the M1 level (*p* = 0.07). Concomitantly, a Wilcoxon signed-rank test was done to examine the validity of the protein increase in the M2 subunit. Data in each subgroup (M1 and M2) were compared to a theoretical median of 1.0. The actual median of the M1 subunit was 1.0 and that of the M2 subunit 1.4. The *p* value for deviation from the theoretical median was 0.16 for the M1 subunit and <0.0001 for the M2 subunit. These data validated the conclusion that the M2 subunit protein levels increased following DNA damage by an alkylating agent.

The average increase in M2 protein expression with 10 μ M 4-HC for 4 h was 1.6 (range was 0.9 to 5.3). Similarly, patient samples varied in thymidine incorporation, a measure of DNA

repair synthesis. To determine whether the increase in M2 protein level was proportional to the increase in thymidine incorporation, we correlated M2 subunit expression levels and unscheduled DNA synthesis (Figure. 4). Data from 20 patients showed a linear correlation between M2 protein expression and [³H]thymidine incorporation ($r = 0.45$; $p = 0.04$), suggesting a relationship between the two parameters. In contrast to M2 protein levels, M1 subunit expression did not show a relationship between protein expression and thymidine incorporation, (data not shown, $r = 0.14$ and $p = 0.66$).

Effect of 4-HC on M1 and M2 mRNA Expression

To determine whether the induction observed occurred only at the translational or at the transcriptional level as well, mRNA message levels for both RR subunits were quantitated. Because the largest increase in protein level occurred after 4 h of treatment with 10 μ M 4-HC, mRNA levels were quantitated at that time point and concentration. mRNA levels were evaluated by 5' $\rightarrow$ 3' exonuclease-based real-time quantitative RT-PCR assay. The amount of RNA used to produce the standard curves were 0.5, 2, 5, 20, 50, and 100 ng of RNA extracted from the ML-1 cells. The r^2 values of standard curves for the M1 subunit, M2 subunit, and GAPDH were 0.991, 0.942, and 0.997, respectively. The levels of M1 and M2 message, as well as GAPDH in each patient sample were determined from this standard curve.

Data from 11 patients were obtained. Table II shows the changes in the M1 and M2 mRNA expression levels which indicate the expression of M1 not showing an increase from control whereas the M2 expression displayed a significant increase ($p = 0.02$; paired t-test, Figure 5). To examine if the increase in mRNA correlated with the induction in protein, the data were plotted for corresponding patients. The relationship with all data points present did not show a linear relationship ($p = 0.353$) but when one of the mRNA data points that were much higher than the average, were removed the data showed a linear response for protein and mRNA (p value of 0.02).

DISCUSSION

In response to DNA damage, transcriptional and translational upregulation of genes or proteins involved in DNA repair is associated with increased survival of tumor cells [23,24]. Some of the genes encode cell-cycle regulators while others encode factors involved in replication or repair or both. For example, the expression of E2F, a transcription factor involved in migrating cells through S phase of the cell cycle, and the expression of DNA replication genes were found to increase after DNA damage [25,26]. Studies have also shown that DDB, a DNA repair protein, is associated with the activation domain of E2F [27]. Cisplatin-resistant human colon carcinoma cells found to overexpress enzymes involved in DNA replication that might also be implicated in repair synthesis [28].

Induction of DNA replication genes during DNA repair could best be studied in a model system in which cells are not cycling. The indolent nature of CLL cells allows investigators to investigate the effects of DNA repair induction on the expression of any gene, including RR, without the confounding effects of DNA replication that are caused by cell-cycle events in actively growing cells. The relatively low expression of the RR protein in untreated CLL

cells cultured *ex vivo* (Figure. 2) also supports use of these cells as a model system. Because circulating normal and CLL lymphocytes maintain low levels of endogenous dNTPs [29,30], we hypothesized that RR induction may be required to provide dNTP pools for DNA repair after a DNA damage.

The type of damage dictates the type of mechanism used to repair the damage. In mammalian cells, the exact mechanism for repair of DNA adducts after cyclophosphamide treatment is still not fully elucidated. However, the major cytotoxic lesions caused by 4-HC damage are known to be the formation of bulky DNA adducts intrastrand and interstrand crosslinks [31,32]. Of the repair systems that require a resynthesis step, both nucleotide excision repair that corrects bulky DNA adducts [7] and recombinational repair that removes the interstrand crosslinks [33-35] have been implicated. Because of the need to synthesize a repair patch of many nucleotides [36], one could expect that a supply of dNTPs is required to efficiently and correctly complete repair patch synthesis. Other alkylating agents such as chlorambucil and bendamustine initiate nucleotide excision repair need to be tested for induction of M2 subunit.

Our initial experiments demonstrated that CLL cells are capable of undergoing DNA repair when exposed to 4-HC, as measured by [³H]thymidine incorporation. These data are consistent with previous reports in which normal quiescent lymphocytes were induced to undergo DNA repair by either UV [19] or cyclophosphamide [37]. The alterations of both subunits of RR have been studied extensively in yeast and mammalian cells. DNA damage has been induced by a variety of agents including IR, UV, chlorambucil, methyl methanesulfonate (MMS), and 4-nitroquinoline-1-oxide (4-NQO), induced an increase in the protein level of the M2 subunit with minimal or no increase in the M1 subunit [12-14]. The fluctuation in the protein levels of the M2 subunit of RR is also known to be cell-cycle dependent [38,39] with an increase in the S-phase. One reason for the subunit specific change during replication may be due to the turnover speed of the M2 subunit, which is much faster ($t_{1/2} = 3$ h) than that of the M1 subunit ($t_{1/2} = 20$ h) [40,41]. Consistently, after 4-HC treatment, only the M2 protein level was increased (Figures 2 and 3). The increase we observed in M2 protein expression, which coincided with a trend toward increased M2 mRNA expression, suggested that induction was a translational event but also a transcriptional regulation [42,43].

Consistent with an increase in M2 subunit of ribonucleoside reductase, studies have shown that the inhibition of this enzyme results in a decrease in the DNA repair capability. Inhibition of RR by hydroxyurea, a classic RR inhibitor, decreased dNTP pools and inhibited UV-induced DNA repair in human fibroblasts [15]. The hydroxyurea-mediated inhibition of DNA repair was restored with exogenous deoxyguanosine [16]. Similar investigations have suggested that specific depletion of purine deoxynucleotides by RR inhibitors decreases DNA repair capacity [17]. Taken together, these observations provide indirect evidence of the link between RR activity and DNA repair.

Single-cell gel electrophoresis (the comet assay) assay demonstrated that DNA damage was visible in quiescent human lymphocytes promptly after exposure to 4-HC [44]. The strand breaks, which indicate response to DNA damage if allowed, were repaired within 12 h.

However, if these damaged cells were preincubated with the nucleoside analog F-ara-A, which is known to be incorporated into DNA repair patches[19], DNA comets persisted for more than 12 h following drug exposure. These results suggested that repair mechanisms involving DNA resynthesis occur at times beyond the initial 15 minutes after exposure. During these times, expression level and functional activity of RR may play a vital role in DNA repair. As our time-course experiments demonstrated, RR protein expression began to increase at 2 h post 4-HC and reached maximum expression by 4-8 h (Figure. 3).

The critical role of RR in supplying dNTPs for cellular replication is well documented. Although, the role of RR in DNA repair scenarios has been suggested, the conclusion was diluted because of the contaminating effects of replicative DNA synthesis. The indolent nature of CLL cells provides a model system to investigate the role for RR in DNA repair. Our studies demonstrated a dose-dependent increase in unscheduled DNA synthesis with 4-HC treatment. Parallel to the unscheduled DNA synthesis, was an increase in protein levels of the M2 subunit while M1 subunit protein levels remained constant. The changes in protein levels resembled those of the enzyme subunits' levels of mRNA. We conclude that RR is regulated not only during replication but also during DNA repair and in both cases M2 subunit expression was increased. Strategies involving RR inhibitors in combination with DNA-damaging agents may prove effective in enhancing the therapeutic outcome of indolent malignant diseases. In fact both fludarabine and clofarabine, inhibitors of RR, show synergy with 4-HC in vitro in CLL cells [44] as well as during CLL therapy [45]. The fludarabine and cyclophosphamide couplet has become standard of care. Recent randomized studies in different cooperative groups have established that compared to single agent fludarabine, the fludarabine and cyclophosphamide combination resulted in superior clinical outcome [46-48].

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Figure 1. Dose-dependent activation of unscheduled DNA synthesis by 4-HC

CLL lymphocytes from patients were treated with 3 (n=5), 10 (n=23), or 50 μM (n=4) 4-HC for 4 hours after treatment with 2 mM hydroxyurea for 30 minutes. Incorporation of [^3H]thymidine was measured as described in Experimental Procedures. Thymidine (thd) incorporation in control cells (0 μM 4-HC) was taken as 100%, and the percentage increase of thymidine incorporation was considered as unscheduled DNA synthesis (DNA repair).

B

Figure 2. Changes in ribonucleotide reductase protein levels show a dose response with 4-HC treatment

A. lymphocytes from a patient with CLL were incubated for 4 h with indicated doses of 4-HC. Cells were then lysed and processed for western blot assay as described in Experimental Procedures. Histograms B represent the mean values, ($\pm$ SD) after densitometric evaluations of protein level in CLL cells from 5 patients M2 subunit. The level of protein for the sample with 0 μM 4-HC (control) was taken as 1.

Figure 3. Time-dependent changes in ribonucleotide reductase protein levels with 10 μ M 4-HC

A. Lymphocytes from a patient with CLL were incubated with 4-HC for various times as indicated on the immunoblot. Cells were then lysed and processed for western blot assay as described in Experimental Procedures. Histograms **B** represent the mean values ($\pm$ SD) after densitometric evaluations of protein levels in CLL cells from 18 patients for M2 subunit protein levels. The level of protein for the sample with 0 μ M 4-HC (control) was taken as 1.

Figure 4. Relationship between DNA repair synthesis and increase in M2 subunit protein level
CLL cells from 18 patients were treated with 10 μ M 4-HC for 4 h. Data from protein immunoblots were obtained as described in Experimental Procedures and plotted against the thymidine incorporation data from the patient samples.

Figure 5. Quantitation of changes in RR message level after 4-HC treatment

RNA was extracted from lymphocytes of 11 CLL patient samples that were either treated with 10 μ M 4-HC for 4 h or left untreated. Values were interpolated from standard curves and normalized to GAPDH.

Table 1

Analysis of cell death and cell-cycle pattern in CLL lymphocytes before and after 4-HC treatment Five patient samples were treated with 10 μ M 4-HC for 4 h and processed for flow-cytometric analysis after staining with propidium iodide.

Patient no.	Treatment	% of cell population in each cell-cycle phase		
		Sub-G1	G0/G1	S
1	Control	0.82	98.85	0.10
	4-HC	0.57	97.97	0.87
2	Control	4.72	94.45	1.01
	4-HC	6.22	93.36	1.19
3	Control	0.70	99.05	0.35
	4-HC	0.62	98.52	2.12
4	Control	25.09	73.81	1.90
	4-HC	22.32	77.31	2.70
5	Control	1.90	96.78	2.70
	4-HC	1.85	96.74	1.89

Table II

Changes in M1 and M2 mRNA level after treatment with 4- HC Eleven patient samples were either untreated (Pre) or treated with 10 μ M 4-HC for 4 hrs (Post) and processed for quantitative real time RT-PCR analysis. GAPDH was used to normalize the reported values.

Patient no.	Subunit	Pre	Post	Change
1	M1	0.355	0.392	1.10
	M2	0.240	0.460	1.92
2	M1	1.220	1.130	0.93
	M2	0.018	0.033	1.83
3	M1	1.011	0.757	0.75
	M2	0.027	0.110	4.07
4	M1	0.509	0.951	1.87
	M2	0.003	0.017	5.67
5	M1	0.860	0.887	1.03
	M2	0.019	0.031	1.63
6	M1	0.749	0.313	0.42
	M2	0.025	0.009	0.36
7	M1	0.716	0.624	0.87
	M2	0.007	0.004	0.57
8	M1	1.258	1.126	0.90
	M2	0.008	0.016	2.00
9	M1	1.267	1.100	0.87
	M2	0.040	0.051	1.28
10	M1	0.935	1.076	1.15
	M2	0.004	0.004	1.0
11	M1	0.894	0.651	0.73
	M2	0.076	0.072	0.95