Alterations in the Activity and Regulation of Mammalian Ribonucleotide Reductase by Chlorambucil, a DNA Damaging Agent*

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Ribonucleotide reductase provides the four deoxyribonucleotides required for the synthesis of DNA. In this study, we examined the hypothesis that alterations in the regulation of ribonucleotide reductase activity may be necessary to provide the deoxyribonucleotides required for DNA repair, following exposure of mammalian cells to DNA damaging agents such as the antitumor agent chlorambucil. We observed a marked transient increase in ribonucleotide reductase activity within 2 h of exposing BALB/c 3T3 mouse cells to DNA damaging concentrations of chlorambucil. Northern blot analysis showed that elevations in activity were accompanied by transient increases in the mRNA levels of both genes (R1 and R2) that code for ribonucleotide reductase. Western blot analysis indicated that only the protein for the limiting component for enzyme activity, R2, was significantly elevated in chlorambucil treated cultures. The chlorambucil effects upon activity and regulation of ribonucleotide reductase occurred without any detectable changes in the rate of DNA synthesis, as would be expected if the elevation in enzyme activity is required for DNA repair. The chlorambucil-induced elevations in R1 and R2 message levels were blocked by treatment of cells with actinomycin D or the tumor promoter 12-O-tetradecanoylphorbol-13-acetate, indicating the importance of the reductase transcriptional process in responding to the action of chlorambucil and providing evidence for the involvement of a protein kinase C pathway in the regulation of mammalian ribonucleotide reductase. In addition to the chlorambucil-induced elevations in enzyme activity, message, and protein levels, the drug was also shown to be an inhibitor of ribonucleotide reductase activity in cell-free preparations. Separation of ribonucleotide components on an affinity column followed by selective exposure of the protein components to chlorambucil showed that both R1 and R2 proteins were targets for chlorambucil, in keeping with the known alkylation abilities of the drug. These observations provide the first direct demonstration of a link between the regulation of mammalian ribonucleotide reductase and the process of DNA repair and contribute to our understanding of the mode of action of a class of drugs represented by chlorambucil, in which chemotherapeutic activity has been attributed to DNA damaging effects.

Deoxyribonucleotides, precursors for DNA synthesis and DNA repair, originate from the direct reduction of the 2'-carbon on the ribose moiety of ribonucleotides. In mammalian cells, this reduction occurs at the ribonucleoside diphosphate level in the presence of R1 and R2, two structurally dissimilar protein components of the enzyme (1, 2). The R1 protein is a dimer with a molecular weight of 170,000 and contains subunit and complex effector binding sites (3). Protein R2 is also a dimer, has a molecular weight of 88,000, and contains non-heme iron and a unique tyrosyl free radical required for enzyme activity (4, 5). Alterations in ribonucleotide reductase are often associated with major changes in the biological properties of cells (1, 6). For example, it has been reported that changes in ribonucleotide reductase regulation can affect the balance of deoxyribonucleotide pools (7), modify spontaneous mutation rates in mammalian cells (8), may be involved in certain immunodeficiency diseases in man (9), participate in early events important in the process of tumor promotion (10), and is involved in a mechanism of altered growth factor response exhibited by malignant cells (11). In addition, the importance of ribonucleotide reductase in the synthesis of DNA and cell proliferation has led to investigations correlating enzyme activity with possible mechanisms of tumorigenesis (12) and has encouraged the development of antitumor agents that target enzyme activity (1, 2, 6, 9).

Due to the close association between ribonucleotide reductase, DNA synthesis, and cell proliferation, compounds that can modulate the levels of ribonucleotide reductase are of interest (1, 2). For example, previous studies have shown that the tumor promoter 12-O-tetradecanoylphorbol-13-acetate and the antitumor drug hydroxyurea can alter the levels of ribonucleotide reductase components in mammalian cells, and the mechanisms underlying these changes are of considerable interest (2, 4, 10, 13, 14). Furthermore, changes in ribonucleotide reductase gene expression have been observed in yeast following exposure to DNA damaging agents (15). However, to the best of our knowledge, there have not been any reports describing alterations in ribonucleotide reductase gene expression, in mammalian cells, following treatment with drugs that target DNA directly, even though the cytotoxicity of many chemotherapeutic compounds involve DNA modifications. As an example, chlorambucil is an alkylating agent frequently used in the treatment of chronic lymphocytic leukemia, and much of the antineoplastic activity of this compound appears to be due to DNA damage, resulting from the formation of cross-links (16-18). Indeed, a variety of mechanisms have been described, which provide resistance to chlorambucil, and
most of them appear to involve protection from the DNA damaging effects of the drug (16-18). In this report, we show for the first time that chlorambucil, at concentrations known to damage mammalian DNA, is capable of markedly altering ribonucleotide reductase gene expression. These results are in keeping with the view that ribonucleotide reductase plays an important role in the DNA repair process by supplying the required deoxyribonucleotides and suggests that this enzyme is important in cellular mechanisms designed to overcome the cytotoxic DNA damaging effects of chemotherapeutic compounds like chlorambucil.

**EXPERIMENTAL PROCEDURES**

**Cell Culture Conditions**—Mouse cell lines were routinely cultured at 37 °C on plastic tissue culture plates (Lx Scientific) in α-minimal essential medium (Flow Laboratories) supplemented with antibiotics and 10% (v/v) fetal bovine serum (Gibco Laboratories). Logarithmically growing BALB/c 3T3 cells were treated with medium containing varying concentrations of chlorambucil (Sigma) and for different time periods. Chlorambucil was prepared as a fresh stock solution prior to each experiment by dissolving the drug in a 1% acidified ethanol solution. Control experiments were carried out with cultures receiving only the solvent.

**Western Blot Analysis**—A rapid RNA extraction method was used to prepare total cellular RNA (19), which was subjected to electrophoresis through a 1% formaldehyde-agarose gel followed by transfer to nylon membranes (Nytran, Schleicher and Schuell). Blots were prehybridized and hybridized as described previously (4, 5, 11). Hybridization occurred in the presence of either a 32P-labeled NcoI-generated fragment containing the cDNA of clone 65 (R1) or the PstI fragment of clone 10 (R2) (4, 11). Probes were labeled using an oligolabeling kit (Pharmacia LKB Biotechnology Inc.) and α-32P-labeled dCTP (Moravek Biochemicals) was used as the substrate, and snake venom (Sigma) was used to hydrolyze the nucleotides (10, 14). The reaction mixture contained in a final volume of 150 μl: [32P]dCTP, 0.05 μCi, 7.5 μmol; dithiothreitol, 900 μmol; magnesium acetate, 600 nmol; ATP, 300 nmol; and enzyme preparation. Enzyme reactions were initiated by addition of enzyme and carried out for 20 min at 37 °C for ribonucleotide reductase and for 1 h at 37 °C for snake venom phosphodiesterase. Reactions were terminated by boiling for 5 min and then diluted with 500 μl of H2O, centrifuged to remove debris, and passed over a Dowex 1 (Bio-Rad) column equilibrated with saturated sodium borate solution; fractions containing radioactivity were collected and analyzed (10, 14, 24). In some experiments, partially purified preparations containing either the R1 and R2 protein components of ribonucleotide reductase were prepared, mixed, and assayed for ribonucleotide reductase activity as described above. The R1 and R2 proteins were separated from enzyme preparations obtained from logarithmically growing cultures of a mouse L cell line, SC2, which has previously been shown to contain elevated levels of ribonucleotide reductase proteins (4, 13). The protein components were separated by chromatography on blue dextran-Sepharose as described (25). Protein concentrations were estimated using the Bio-Rad determination kit (Technical Bulletin 1051) with bovine serum albumin as a standard.

**RESULTS**

**Effect of Chlorambucil on the Level of Ribonucleotide Reductase Activity**—We have hypothesized that the induction of ribonucleotide reductase activity may be necessary to provide the deoxyribonucleotides required for DNA repair when cells are exposed to a DNA damaging agent like chlorambucil. To test this idea, we cultured mouse BALB/c 3T3 fibroblasts in the presence of 200 μM chlorambucil, a drug concentration known to cause DNA lesions in mammalian cells (16-18). Table I shows that a significant elevation in ribonucleotide reductase activity occurred in chlorambucil-treated 3T3 cells within 2 h, with a maximum increase of 4.5-fold observed after 8 h of drug treatment. We also observed that the chlorambucil effect on enzyme activity appeared to be transient since the level of ribonucleotide reductase activity dropped to less than 2 times the untreated controls within 24 h.

**DNA Synthesis in the Presence and Absence of Chlorambucil**—Since ribonucleotide reductase is markedly elevated in mammalian cells during DNA synthesis (1, 2, 6), we examined the possibility that treatment of 3T3 cells with chlorambucil determined by liquid scintillation spectroscopy using a model LS7800 scintillation counter (Beckman).

**Preparation and Assay of Ribonucleotide Reductase**—Enzyme preparations containing 2-4 mg of protein/ml were used to assay ribonucleotide reductase activity. BALB/c 3T3 cells were cultured in the absence or presence of 200 μM chlorambucil for 2, 4, 8, and 24 h and then removed from culture plates with a phosphate-buffered solution containing trypsin (Difco) and EDTA (Mallinckrodt). Cells obtained by centrifugation were washed three times with ice-cold phosphate-buffered saline and disrupted by sonication. The extract was cleared of cellular debris by centrifugation (14,000 g) x g for 30 min). The remaining solution was assayed for ribonucleotide reductase activity by a modified method of Steeper and Stuart (23), as we have described previously (10, 14). [32P]CDP (Moravek Biochemicals) was used as the substrate, and snake venom (Sigma) was used to hydrolyze the nucleotides (10, 14). The reaction mixture contained in a final volume of 150 μl: [32P]CDP, 0.06 μCi, 7.5 μmol; dithiothreitol, 900 μmol; magnesium acetate, 600 nmol; ATP, 300 nmol; and enzyme preparation. Enzyme reactions were initiated by addition of enzyme and carried out for 20 min at 37 °C for ribonucleotide reductase and for 1 h at 37 °C for snake venom phosphodiesterase. Reactions were terminated by boiling for 5 min and then diluted with 500 μl of H2O, centrifuged to remove debris, and passed over a Dowex 1 (Bio-Rad) column equilibrated with saturated sodium borate solution; fractions containing radioactivity were collected and analyzed (10, 14, 24).

**TABLE I**

| Hours of chlorambucil treatment | Ribonucleotide reductase activity Increase nM CDP reduced/h/mg protein *fold | nm CDP reduced/h/mg protein
|-------------------------------|---------------------------------|-------------------------|
| 0                             | 0.72 ± 0.14                     | 1.0                     |
| 2                             | 1.54 ± 0.09                     | 2.8                     |
| 4                             | 2.12 ± 0.28                     | 3.0                     |
| 8                             | 3.22 ± 0.11                     | 4.5                     |
| 24                            | 1.25 ± 0.35                     | 1.8                     |

*The average ± S.E. of four independent determinations of ribonucleotide reductase activity.

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1 The abbreviations used are: TBS, Tris-buffered saline; TPA, 12-O-tetradecanoylphorbol-13-acetate.
may shift a significant proportion of the cell population into S phase, which could be responsible for the increased enzyme activity. This point was investigated directly by measuring the incorporation of \( ^{3}H \)thymidine into DNA at several time periods over an 8-h exposure of 3T3 cells to the drug, during which a maximum level of ribonucleotide reductase activity is attained (Table I). Fig. 1 shows that there were no significant differences in DNA synthesis rates, between cells treated with chlorambucil for up to 8 h and cells grown in the absence of the drug. Therefore, the elevation in ribonucleotide reductase activity occurs in the absence of any detectable changes in the rates of DNA synthesis, and this elevation is not due to an unusual shift of the cell population into S phase.

**Effect of Chlorambucil on R1 and R2 mRNA Levels**—To determine if the drug-induced increase in ribonucleotide reductase activity was accompanied by elevations in message levels for the two components of ribonucleotide reductase, BALB/c 3T3 cells were cultured in the presence of various concentrations of chlorambucil, which have been shown to cause DNA damage (17, 18). Densitometric measurements indicated 3-, 4.5-, and 5-fold elevations of R2 message in cells treated with 25, 50, and 100 \( \mu \)M chlorambucil for 6 h.

Results of experiments in which R1 mRNA levels were determined in chlorambucil-treated BALB/c 3T3 cells are shown in Fig. 2. It is clear that R1 gene expression is elevated following exposure to 200 \( \mu \)M drug. A 3-, 5-, 6-, 9-, and 1.2-fold elevation in R1 mRNA levels was determined by densitometry during drug exposure of 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h, respectively. This rapid but transient increase in R1 gene expression paralleled the changes observed with R2 gene expression, although the magnitude of the increase observed for R1 was lower. As observed with R2 gene expression, the increase in R1 message levels also occurred after exposure to 25, 50, or 100 \( \mu \)M chlorambucil (Fig. 3B). A 2-, 3-, and 4-fold increase in R1 mRNA was detected following exposure for 6 h to 25, 50, and 100 \( \mu \)M drug, respectively.

**Effect of Chlorambucil on R1 and R2 Protein Levels**—To determine if the increases in ribonucleotide reductase message levels resulted in elevations in protein levels, BALB/c 3T3 cells were cultured in the presence of 200 \( \mu \)M chlorambucil for 2, 4, 8, and 24 h. Protein levels were determined by Western blot analysis. Fig. 4 clearly shows an increase in protein R2 in response to chlorambucil treatment. Measurements by densitometry indicated an increase of 2-, 4-, and 6-fold over untreated cells following exposure to the drug for 2, 4, and 8 h, respectively. The level of R2 protein after a 24-h exposure to chlorambucil declined to approximately the untreated con-

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**Fig. 1.** \( ^{3}H \)Thymidine incorporation into DNA as a measure of DNA synthesis during early induction of R1 and R2 gene expression. BALB/c 3T3 cells (10\(^{4}\)/well) were cultured in the absence (−) or presence (+) of chlorambucil (200 \( \mu \)M) for the times indicated. Cells were then pulsed with \( ^{3}H \)thymidine for 2 h, and the incorporation of label into trichloroacetic acid-precipitable material was determined. The data shown are from two independent experiments done in duplicate.

**Fig. 2.** Elevation in R2 and R1 message levels at various times in the presence of 200 \( \mu \)M chlorambucil. Northern blot analysis of R2, R1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in BALB/c 3T3 cells grown in the absence or presence of chlorambucil for 30 min (\( \frac{1}{2} \)), 1, 2, 4, 8, and 24 h, respectively. The R2, R1, and glyceraldehyde-3-phosphate dehydrogenase autoradiograms were exposed for 24 h, 72 h, and 24 h, respectively, at \(-70^\circ C\) with intensifying screens.

**Fig. 3.** Elevations in R2 and R1 message levels at various concentrations of chlorambucil. A. Northern blots of R2 mRNA levels in BALB/c 3T3 cells cultured in the absence (a) or presence of 25 \( \mu \)M (b), 50 \( \mu \)M (c), or 100 \( \mu \)M (d) chlorambucil, respectively. B. Northern blots of R1 mRNA levels in cells cultured in the absence (a) or presence of 25 \( \mu \)M (b), 50 \( \mu \)M (c), or 100 \( \mu \)M (d) chlorambucil, respectively. Loading controls for R2 and R1 Northern blots were performed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and autoradiograms were exposed for 24 h, 72 h, and 24 h, respectively, at \(-70^\circ C\) with intensifying screens.

**Fig. 4.** Effect of chlorambucil on cellular R2 and R1 protein levels. A. Western blots for R2 protein; 100 \( \mu \)g of cellular protein extract was loaded in each lane. B. Western blots for R1 protein; 200 \( \mu \)g of cellular protein extract was loaded in each lane. Each lane represents protein obtained from BALB/c 3T3 cells cultured in the absence (a) and the presence of 200 \( \mu \)M chlorambucil for 2 h (b), 4 h (c), 8 h (d), and 24 h (e).
Chlorambucil-induced Alterations in Ribonucleotide Reductase

Effect of Chlorambucil Treatment on Transcription of R1 and R2 Genes—The possibility that the increases in R1 and R2 message levels observed following exposure to chlorambucil were due to changes in gene transcription rates was tested by pretreating BALB/c 3T3 cells with the transcription blocker actinomycin D (26), prior to exposure of the cells to chlorambucil. As shown in Fig. 5, actinomycin D prevented the increase in R1 and R2 gene expressions previously observed following exposure to chlorambucil, suggesting that at least in part, chlorambucil increases ribonucleotide reductase gene expression by altering the transcriptional process.

Possible Role of a Protein Kinase in the Induction of R1 and R2 Gene Expression—The rapid and transient increase in R2 gene expression in chlorambucil-treated cells resembles a previous observation from our laboratory (10) of increased R2 gene expression following treatment of BALB/c 3T3 cells with the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA). The mechanism of action of TPA involves modulation of protein kinase C activity (27). Therefore, we were interested in evaluating the possibility that chlorambucil may be inducing alterations in ribonucleotide reductase gene expression through a mechanism that involves protein kinase C. To test this idea, 3T3 cells were treated with 0.1 μM TPA for 24 h to down-regulate protein kinase C activity (28). Previous studies have shown that R1 and R2 gene expressions are at approximately untreated control levels after 24 h of exposure to 0.1 μM TPA (10). Fig. 6 clearly demonstrates that pretreatment with TPA prevents the elevation in R1 and R2 message levels that are observed in the presence of chlorambucil (Figs. 2 and 3). This result suggests a possible role for protein kinase C in the chlorambucil-induced modulation of ribonucleotide reductase gene expression in BALB/c 3T3 cells.

Effect of Chlorambucil on the Activity of Ribonucleotide Reductase—Chlorambucil is an alkylating agent, and although its chemotherapeutic mode of action is through the interaction of the drug with DNA (16–18), it is also known that chlorambucil can bind to proteins, presumably through interactions with sulfhydryl groups (18, 29). Therefore, we tested the idea that chlorambucil may inhibit the activity of ribonucleotide reductase. Table II shows a drug dose-dependent decline in enzyme activity, with approximately 50% activity remaining in the presence of 500 μM chlorambucil. To determine the component(s) involved in the inhibition of ribonucleotide reductase activity, the R1 and R2 proteins from a mouse L cell line that overproduces these components (4, 13) were separated by affinity chromatography, each component was exposed separately to several chlorambucil concentrations or left untreated, and combinations of the two components were mixed to determine enzyme activity. These studies confirmed the sensitivity of mouse ribonucleotide reductase to chlorambucil inhibition and indicated that both protein components are targets for chlorambucil (Fig. 7).

**DISCUSSION**

DNA synthesis and DNA repair require the four deoxyribonucleoside triphosphates that originate from the direct reduction of the 2'-carbon atom on the ribose moiety of ribonucleotides, an activity catalyzed by the enzyme ribonucleotide reductase (1, 2, 6). The present study demonstrates for the first time that treatment of mammalian cells with the antitumor agent chlorambucil, at concentrations known to cause DNA damage (16–18), leads to transient elevations in ribonucleotide reductase activity and to alterations in the regulation of the two components of ribonucleotide reductase. The increased levels of R1 and R2 message are due, at least in part, to modifications in transcriptional efficiency of the...
R1 and R2 genes. Furthermore, we show that down-regulation of protein kinase C activity prevents the rise in R1 and R2 mRNA levels observed following chlorambucil exposure, providing evidence for an involvement of a protein kinase C pathway in the regulation of ribonucleotide reductase. In addition, we observed that chlorambucil is an inhibitor of ribonucleotide reductase activity, a finding that may not be too surprising in view of the alkylating abilities of this drug. The effects of chlorambucil on enzyme activity appear to be nonspecific since the drug interacted with both ribonucleotide reductase proteins, an observation that is consistent with previous studies showing that protection to the DNA damaging effects of the drug in resistant cells can involve increased cellular levels of sulfhydryl groups, through elevations in glutathione or metallothionein, both of which bind and inactivate chlorambucil. The concentrations of chlorambucil found to cause significant inhibition of ribonucleotide reductase activity were relatively high, suggesting that inhibition of the reductase is unlikely to be a major component in the mode of action of the drug. For example, 50% inhibition of ribonucleotide reductase was observed at 500 μM chlorambucil, a higher concentration than needed to produce DNA damaging or cytotoxic effects. However, these observations do not rule out the possibility of a minor role for chlorambucil inhibition of ribonucleotide reductase activity in the chemotherapeutic effects of the drug or in resistance mechanisms involving this class of compounds.

There is evidence that the activity of mammalian ribonucleotide reductase in logarithmically growing cells is controlled by the synthesis and breakdown of the R2 protein, the limiting component for enzyme activity, with relatively little change in protein R1. The importance of R2 in the scheme of ribonucleotide reductase regulation has been observed in drug resistance studies which have revealed that an elevation in the limiting levels of R2 message and protein are enough to increase enzyme activity. Similarly, a transient increase in ribonucleotides reductase activity following treatment of mammalian cells with the tumor promoter, TPA, is brought about primarily through a transient increase in message and protein levels of the R2 component. These studies show that the two components of ribonucleotide reductase can be controlled independently unlike, for example, the Escherichia coli situation, where the two genes for the corresponding proteins are located in a single operon, and their synthesis is coordinately regulated. However, there must be a mechanism in mammalian cells for controlling the relative levels of R1 and R2 as required in different biological situations. For example, in cells exhibiting very high resistance to hydroxyurea, both R1 and R2 message and protein levels are elevated to achieve enough active enzyme to allow proliferation in the presence of very high concentrations of the enzyme inhibitor. Another recent example of this type of regulation comes from investigations of R1 and R2 levels of the reductase in malignant cells exposed to transforming growth factor-α. In this case, elevation of the enzyme activity in transforming growth factor-α-treated cells was accompanied by increases in both R1 and R2 message and protein levels. Interestingly, treatment of mammalian cells with chlorambucil produces common alterations in R1 and R2 regulation at the message level, but different mechanisms at the protein level. Therefore, increased enzyme activity in cells exposed to chlorambucil was accompanied by elevations in both R1 and R2 mRNA levels, but only the increased R2 message led to a detectable increase in protein.

These results are in keeping with the complex regulation of mammalian ribonucleotide reductase at transcriptional and post-transcriptional levels, and the finding that the R2 protein is usually limiting for enzyme activity in actively growing cells. The cellular concentrations of deoxyribonucleotides required to repair DNA following chlorambucil exposure is likely to be quite small compared to the levels needed during DNA synthesis in the S-phase of the cell cycle, so that an increase in the limiting R2 protein alone should be sufficient to provide ribonucleotide reduction for this purpose. This view is strengthened by the novel observation that the chlorambucil effects upon ribonucleotide reductase gene expression occurred without any detectable changes in the rate of DNA synthesis, as is expected if the elevation in enzyme activity is required for DNA repair.

In conclusion, we have demonstrated for the first time that the activity and regulation of a critical enzyme in DNA synthesis, ribonucleotide reductase, is significantly modified when mammalian cells are exposed to the DNA damaging agent, chlorambucil. These novel observations are relevant to studies attempting to understand the mechanisms which regulate ribonucleotide reduction and DNA repair and contribute to our knowledge of the mode of action of a class of chemotherapeutic agents represented by chlorambucil.

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FIG. 7. Chlorambucil acts at both the R1 and R2 components of ribonucleotide reductase. Isolated enzyme proteins were individually preincubated with chlorambucil (250 μM and 750 μM) for 15 min at room temperature. Following preincubation, the drug-treated R1 and drug-treated R2 proteins were separated from chlorambucil using a Sephadex G-25 column. Prior to assay, the drug-treated R1 and R2 proteins were reconstructed with R2 and R1 proteins, respectively, which were unexposed to drug. Ribonucleotide reductase activity was determined as described under "Experimental Procedures."
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