Altered Steady-State Levels of the Messenger RNAs for c-myc and p53 in L1210 Cell Lines Resistant to Deoxyadenosine


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Abstract

L1210 cell lines, selected for resistance to deoxyadenosine due to the loss of allosteric inhibition of ribonucleotide reductase by dATP, had altered steady-state levels of the mRNAs for c-myc, fos, and p53. Wild-type L1210 cells had constitutive steady-state levels of c-myc and p53 with little or no fos mRNA. Two different deoxyadenosine-resistant cell lines (Y8 and ED2) had elevated steady-state levels of c-myc and fos but essentially no p53 mRNA. Hydroxyurea-resistant L1210 cells had the same levels of c-myc, fos, and p53 as the wild-type cells. There was no amplification of the gene for c-myc in the Y8 or ED2 cell lines. The half-life for c-myc mRNA was essentially the same in the wild-type and the Y8 and ED2 cells. Nuclear runoff experiments showed that the rates of transcription for c-myc in the Y8 and ED2 cells were elevated and could account for the increased steady-state levels of c-myc in these two cell lines. The transcription rate for p53 mRNA was not decreased in the Y8 and ED2 cells and therefore did not account for the loss of the steady-state levels of p53 in the cells. Cycloheximide treatment of the Y8 and ED2 cells resulted in a marked increase in the steady-state p53 mRNA level, indicating that a protein which was rapidly turned over was responsible for the extremely short half-life of p53 mRNA in these two cell lines.

Introduction

Mouse leukemia L1210 cell lines were selected for resistance to inhibitors directed at the subunits of ribonucleotide reductase (1–3). The drug resistance was specific for the particular drug and the subunit to which the drug was directed. Hydroxyurea-resistant cells (HU-7) were cross-resistant to IMPY but still sensitive to deoxyadenosine and 4-methyl-5-aminoisoquinoline thiosemicarbazone; deoxyadenosine-resistant cells (Y8) were sensitive to hydroxyurea, IMPY, and 4-methyl-5-aminoisoquinoline thiosemicarbazone; the ED2 cell line was resistant to deoxyadenosine, IMPY, and hydroxyurea. In these L1210 cell lines the biochemical bases for resistance to hydroxyurea and deoxyadenosine were different; hydroxyurea resistance was associated with increases in ribonucleotide reductase activity, while deoxyadenosine resistance was associated with the loss of sensitivity of ribonucleotide reductase to the negative effector dATP. In these studies, we determined the steady-state levels of the mRNAs for the EB and NHI subunits of ribonucleotide reductase (4) and how these mRNA levels changed during the cell cycle (5). In carrying out these studies we used the time course of the changes in the mRNA levels for c-myc (6) and p53 (7) to determine the time frame of the changes in the mRNA levels for NHI and EB mRNAs during the cell cycle traverse.

When the steady-state levels of c-myc and p53 were determined in the drug-resistant cell lines, unexpected observations were made. The mRNA for c-myc was markedly elevated in the deoxyadenosine-resistant cell lines (Y8 and ED2), while the mRNA for p53 was virtually absent; the levels of c-myc and p53 in the hydroxyurea-resistant cell line were essentially the same as in the wild-type cells. In this report, we present the results of studies which follow up on these observations.

Materials and Methods

L1210 Cell Lines. The wild-type mouse leukemia L1210 cell line was originally purchased from the American Type Culture Collection (Rockville, MD); the resistant cell lines were generated from the wild-type L1210 cells and characterized as previously described (1–3). The L1210 cells were grown in RPMI 1640, which was supplemented with 10% horse serum, sodium bicarbonate (2 g/liter) and gentamycin sulfate (50 mg/liter). The drug-resistant cells were maintained in the same culture medium supplemented with the appropriate drugs (HU-7, hydroxyurea; Y8, deoxyadenosine/EHNA; ED2, deoxyadenosine/EHNA plus IMPy/Desferal).

RNA Isolation and Northern Blotting. Total RNA was isolated from log-phase L1210 cells by the method of Chomczynski and Sacchi (8). A minimum of 4 × 10^7 cells were used for each RNA isolation. RNA (20 μg) was subjected to electrophoresis on 1% agarose gels containing 0.66 M formaldehyde and 3-(7N-morpholino)propanesulfonic acid using the method of Fourney et al. (9). After electrophoresis for 4 h at 4 V/cm, the RNA was transferred overnight to a nylon membrane (Nytran; Schleicher and Schuell, Keene, NH) by capillary blotting techniques in 10× SSC. The RNA was cross-linked to the membrane by UV radiation, and the membrane was heated at 80°C for 1 h.

Probing Northern Blots. The blots were prehybridized for 1–3 h in 1 ml/10 cm^2 of Hybrisol I (Oncor, Inc., Gaithersburg, MD) containing 50% formamide at 45°C. The 32P-labeled probe (10^6 cpm/ml of hybridization solution) was added, and the hybridization was carried out at 45°C overnight. The membranes were then washed at room temperature with 0.1× SSC and 0.1% sodium dodecyl sulfate for 15 min (three times) and finally washed with 0.1× SSC and 0.1% sodium dodecyl sulfate at 60°C for 1 h. The membranes were exposed to XAR5 film (Eastman Kodak Co., Rochester, NY) with an intensifying screen at −85°C for various periods of time.

The c-myc probe (ATCC 41029) and the fos probe (ATCC 41040) were purchased from the American Type Culture Collection (Rockville, MD). The p53 probe (2325-base pair BamHI fragment) was a gift from Dr. David Meek (Salk Institute, La Jolla, CA). The glyceraldehyde 3-phosphate dehydrogenase probe was obtained from Drs. Ray Wu, J. Tao, and Xiao-Hong Sun of Cornell University (Ithaca, NY). The cDNAs for the NHI and EB subunits of ribonucleotide reductase were obtained from Dr. Lars Thelander (University of Umeå, Umeå, Sweden).

The cDNA probes were labeled using Pharmacia's oligolabelling kit (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) and [α-32P] dCTP.
p53 in Wild-type and Variant L1210 Cells. Fig. 1 shows the blot for dCTP (3000 Ci/mmol). After a 2-h reaction period the labeled cDNA probes were separated using Select-D G-50 columns (5 Prime-3 Prime, Inc., West Chester, PA).

For quantitation, the X-ray film was scanned on a video densitometer (Biomed Instruments, Inc., Fullerton, CA).

Half-life of c-myc. Wild-type, Y8, and ED2 cells in log phase were collected by centrifugation and resuspended in fresh culture medium (50 x 10^6 cells/20 ml). Actinomycin D (5 µg/ml) was added to the flasks, and the cells were incubated at 37°C in a CO2 incubator in the presence of actinomycin D for 0, 15, 30, and 45 min. The cells were collected by centrifugation and washed once with phosphate-buffered saline. RNA was isolated as described (8).

Treatment of Cells with Cycloheximide. Wild-type, Y8, and ED2 cells in log phase were incubated in culture (40 x 10^6 to 50 x 10^6 cells/40 ml) in the presence and absence of cycloheximide (20 µg/ml) for 3 h. The cells were harvested by centrifugation and washed once with phosphate-buffered saline, and the RNA was isolated (8).

Southern Analysis. Genomic DNA was prepared from the wild-type and resistant cell lines using the DNA extraction kit from Oncor Sure Blot (Gaithersburg, MD). A portion of the DNA was completely digested with the restriction endonucleases BamHI, EcoRI, or HindIII. The DNA concentrations were determined spectrophotometrically using Hoechst dye (no. 33258) and a set of DNA standards. The digested genomic DNA (10 µg) was run on a 0.7% gel in Tris-acetate/EDTA buffer under vacuum to the nylon membrane (Oncor Sure Blot). After the DNA transfer, the nylon membrane was heated at 80°C for 1 h in a vacuum oven. The blots were prehybridized in Hybrid sol I and hybridized overnight with the ^32P-labeled probe as described for the Northern blots.

Transcription Rate Analysis. The cells were cultured in the same manner as for total RNA isolation. Nuclei were isolated over sucrose cushion by the method of Marzluff (10) using approximately 10^6 cells. Pellets containing the nuclei were drained and frozen in storage buffer (25% glycerol; 5 mM magnesium acetate; 50 mM Tris-HCl, pH 8; 5 mM dithiothreitol) in liquid nitrogen until used, to a maximum of 1 month.

The labeling of nuclei was carried out by the methods of Greenberg and Ziff (11) and Groudine et al. (12). Briefly, 200 µl of nuclei were quickly thawed and subsequently incubated in 200 µl of reaction buffer (10 mM Tris-HCl, pH 8; 5 mM MgCl2; 0.3 M KCl; 10 mM dithiothreitol) with 1 µM unlabeled nucleotides (ATP, CTP, and GTP) and 10 µl of [α- ^32P]UTP (800 Ci/mmol; 10 mCi/ml) for 30 min at 30°C with agitation. Following the incubation period, the transcripts were isolated as described in detail (13). The transcripts used in these studies yielded more than 5 x 10^6 cpm/sample. The cpm/ml were equalized in all samples prior to hybridization using N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid/NaCl solution [10 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.4; 10 mM EDTA; 0.2% sodium dodecyl sulfate; 0.6 mM NaCl]. Blots were routinely hybridized using 1-2 x 10^6 cpm/ml hybridization fluid.

Nitrocellulose slot blots containing linearized plasmid cDNAs were baked at 80°C in a vacuum oven. The cDNAs were obtained using plasmids from which inserts were derived for use as probes as described earlier; pUC18 plasmid DNA was used as a negative control. The blots were hybridized with the ^32P-labeled transcripts for 36 h at 65°C. Following hybridization, the blots were washed twice in 2x SSC at 65°C and once in 2x SSC containing 10 µg/ml RNase A at 37°C. Blots were allowed to air dry and were then exposed to XAR5 film for 3-10 days. The autoradiographs were quantitated by densitometry using a scanning video densitometer.

Results

Northern Analysis for Steady-state Levels of c-myc, fos, and p53 in Wild-type and Variant L1210 Cells. Fig. 1 shows the blot analysis of the RNAs isolated from log-phase wild-type, ED2, HU-7, and Y8 cells. The wild-type L1210 cell lines had constitutive levels of c-myc and p53 mRNAs; the ED2 and Y8 cell lines had elevated levels of c-myc mRNA and observable levels of fos mRNA; p53 mRNA was essentially absent in the ED2 and Y8 cell lines. The hydroxurea-resistant cells showed levels of c-myc, fos, and p53 similar to those of wild-type L1210 cells. Quantitation of the levels of c-myc in the four cell lines showed that c-myc mRNA was elevated 4- to 6-fold in the ED2 and Y-8 cells compared to the wild-type cells. Included in Fig. 1 is the mRNA for GAP to indicate equal loading of the RNA samples on the gels.

Southern Analysis of Wild-type and Variant L1210 Cell Lines for c-myc. Since the increased levels of c-myc mRNA are often associated with gene amplification, Southern analysis of the DNA from these cell lines was carried out. Genomic DNA treated with either HindIII, EcoRI, or BamHI and then probed with [32P]c-myc cDNA (Fig. 2A) did not show any amplification of the DNA for c-myc in the ED2 or Y8 cell lines. For comparison, the membrane was probed with the cDNA for the NHI subunit of ribonucleotide reductase (Fig. 2B). As reported before (4), there was amplification of the NHI subunit gene in the HU-7 cell line. Neither the effector-binding subunit gene of ribonucleotide reductase nor the p53 gene was amplified in these cell lines (data not shown). The increased steady-state
ALTERED STEADY-STATE LEVELS OF THE mRNAs FOR c-myc AND p53

levels of mRNA for c-myc in the Y8 and ED2 cell lines were not the result of gene amplification.

Half-life of mRNA for c-myc in Wild-type and Y8 Cell Lines. Wild-type, Y8, and ED2 cell lines were incubated in culture in the presence of actinomycin D (5 μg/ml) for various periods of time ranging from 0 to 45 min. The half-life for c-myc mRNA in the wild-type cells was approximately 15–18 min; the Y8 and ED2 cells had essentially the same half-life for c-myc mRNA (18–20 min).

Transcription Rates. The rates of transcription of the genes for c-myc, fos, and p53 were studied in nuclei from wild-type, ED2, HU-7, and Y8 cells. As seen in Fig. 3, the nuclei from the L1210 cell lines synthesized the transcripts for c-myc, fos, p53, and GAP. The levels of production of the c-myc transcript were elevated (3-fold) in the nuclei from the ED2 and Y8 cells relative to the nuclei from the wild-type or HU-7 cells. The level of fos transcript production was very high in the four cell lines, even though steady-state levels of fos mRNA were not seen except in the ED2 and Y8 cells. The transcript for p53 was synthesized to a slightly greater extent by the nuclei from the ED2 and Y8 cells than that seen in the nuclei from wild-type and HU-7 cells.

Effect of Cycloheximide on mRNA Levels for c-myc and p53. As seen in Fig. 4, treatment of Y8 and ED2 cells with cycloheximide for 3 h caused a marked increase in the steady-state level of p53 mRNA relative to the untreated Y8 and ED2 cells, which showed essentially no measurable levels of mRNA for p53. Treatment of wild-type cells with cycloheximide did not cause a significant increase in the p53 mRNA level. Cycloheximide treatment of the wild type, Y8, and ED2 cells did not result in the superinduction of c-myc mRNA levels (data not shown).

Discussion

A series of L1210 cell lines was selected for resistance to inhibitors directed at the ribonucleotide reductase site. Since ribonucleotide reductase consists of two nonidentical protein subunits (14) encoded by different genes (15, 16), it was possible to specifically develop drug-resistant cell lines with alterations at either the NHI or EB subunit (1–3) of the enzyme. We chose for detailed study the three variant cell lines: a hydroxyurea-resistant cell line (HU-7); a deoxyadenosine-resistant cell line (Y8); and a deoxyadenosine/IMPY-resistant cell line (ED2). The common biochemical features of these resistant cell lines with respect to ribonucleotide reductase are: the HU-7 and ED2 cell lines have elevated levels of ribonucleotide reductase along with an increase in the mRNA for the NHI subunit (1, 2); and ribonucleotide reductase activity in cell-free extracts from the Y8 and ED2 cell lines is not sensitive to dATP as a negative effector of reductase activity (2, 3). In the course of our studies, we compared the time frame for the increases in the mRNA levels for the NHI and EB subunits during the cell cycle. c-myc and p53 mRNAs were used as the “internal time markers” for these changes (17). When these studies were extended to the variant L1210 cell lines, unexpected observations were made regarding the steady-state levels of c-myc, fos, and p53 in the variant cell lines.

As seen from the data in Fig. 1, the levels of the mRNAs for c-myc and fos were markedly elevated in the Y8 and ED2 cell lines relative to the wild-type cells. Conversely, the steady-state level of the mRNA for p53 was essentially eliminated in the Y8 and ED2 cell lines. The HU-7 cell line had essentially the same levels of c-myc and p53 as the wild-type cells. Southern analysis (Fig. 2) showed that the increased level of c-myc in the Y8 and ED2 cell lines was not due to gene amplification of the c-myc gene. Nuclear runoff experiments showed that the nuclei from the Y8 and ED2 cell lines synthesized the c-myc and fos transcripts at a higher rate than did the nuclei from the wild-type cells. These increased rates of synthesis of the c-myc and fos transcripts could account for the increased steady-state levels of the c-myc and fos mRNAs in the Y8 and ED2 cell lines. The half-life for the c-myc mRNA was determined in the wild-type, Y8, and ED2 cell lines and was shown to be the same (15–20 min) in the three cell lines. Since the rate of c-myc mRNA synthesis was increased with no change in the degradation rate of c-myc mRNA, the increased steady-state levels of c-myc mRNA in the Y8 and ED2 cells could be accounted for at the transcriptional level. Presumably, the same would be true for fos in the Y8 and ED2 cell lines, although mRNA half-life experiments were not carried out because of the lack of steady-state levels of fos mRNA in the wild-type cells upon which the comparison would be made.

Interestingly, the Y8 and ED2 cell lines, which had elevated mRNA levels for c-myc and fos, had essentially no measurable levels of p53 mRNA (Figs. 1 and 4), although the wild-type and HU-7 cell lines had appreciable steady-state levels. Transcription rate analysis showed that the nuclei from the Y8 and ED2 cell lines synthesized the p53 transcript at a level essentially
and Methods." Linearized plasmid cDNAs for GAP, c-myc, fos, and p53 were loaded onto the nitrocellulose membrane; pUC18 plasmid DNA was used as the negative control. The 32P-labeled transcripts were prepared using nuclei from wild-type (WT), ED2 (ED), HU-7 (HU), and Y8 cells.

Fig. 3. Transcription rate analysis of nuclei from wild-type and variant L1210 cell lines. Nuclear runoff experiments were carried out as described in "Materials and Methods." Linearized plasmid cDNAs for GAP, c-myc, fos, and p53 were loaded onto the nitrocellulose membrane; pUC18 plasmid DNA was used as the negative control. The 32P-labeled transcripts were prepared using nuclei from wild-type (WT), ED2 (ED), HU-7 (HU), and Y8 cells.

Fig. 4. Effect of cycloheximide on p53 mRNA levels in Y8 and ED2 cell lines. The wild-type, Y8, and ED2 cells were incubated for 3 h in the presence (+) and absence (−) of cycloheximide (20 μg/ml). The RNA was isolated, electrophoresed, blotted, and probed as described in "Materials and Methods."

The lack of steady-state levels of p53 in the Y8 and ED2 cell lines therefore could not be accounted for by differences at the level of transcription. When the Y8 and ED2 cells were treated with cycloheximide, there was a marked increase in the steady-state level of p53 mRNA relative to the untreated Y8 and ED2 cells. This increase in p53 mRNA, induced by cycloheximide, was not seen in the wild-type cells, which already expressed a constitutive level of p53 mRNA. Furthermore, cycloheximide treatment of the wild-type, Y8, and ED2 cells did not result in the superinduction of c-myc RNA levels, as has been seen in other cell lines (6, 18, 19).

These data suggest that the lack of p53 mRNA in the Y8 and ED2 cell lines is due to a specific change in the posttranscriptional process and that a protein or series of proteins with a function.

The molecular bases for the alteration in the steady-state levels of the mRNAs for c-myc and p53 in the Y8 and ED2 cell lines are different. In the case of c-myc, the difference in the mRNA levels in Y8 and ED2 cells appears to be due to an alteration at the transcriptional level, while the decreased steady-state level of p53 mRNA in the Y8 and ED2 cells appears to be due to a modification at the posttranscriptional level. It is not clear at this point whether there is a direct relationship between the alterations seen in the steady-state mRNA levels for c-myc, fos, and p53. It is possible that the increased steady-state levels of the mRNAs for fos and/or c-myc in the Y8 and ED2 cell lines, and as a result the proteins encoded by the mRNAs for fos and/or c-myc are, in some way, involved in the turnover of p53 mRNA. Conversely, it is possible that the increased turnover of p53 mRNA, with the resultant rapid loss of p53 protein, could lead to the up-regulation of the c-myc and fos genes. It has been shown that the expression of p53 protein can cause the down-regulation of various promoters (20). The levels of fos, c-myc, and p53 proteins have not yet been determined in these cell lines. The role, if any, of deoxyadenosine resistance of the Y8 and ED2 cell lines to the alterations in mRNA levels for fos, c-myc, and p53 must still be established. These cell lines may provide useful approaches to answering questions related to the roles of c-myc, fos, and p53 in cellular function.

References

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