Changes in c-myc and c-fos Expression in a Human Tumor Cell Line following Exposure to Bifunctional Alkylating Agents

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ABSTRACT

This study was initiated to determine if DNA-damaging chemotherapeutic agents can suppress the expression of oncogenes. The effects of three structurally related bifunctional alkylating agents on the steady state mRNA levels of c-myc, c-fos, N-ras, and β-actin in the human colon carcinoma cell line Colo320HSR were examined. Colo320HSR has an amplified c-myc oncogene, which is highly overexpressed, and is assumed to be one of the transforming genes of this cell line. Two concentrations of mechlorethamine, L-phenylalanine mustard, and 4-hydroperoxycyclophosphamide, which produced 1 or 3 log cell kills were used to examine the effects of drug exposure on the expression of specific genes. Steady state mRNA levels were measured by Northern blot analysis. Following a h drug exposure, RNA was isolated from cells at 0, 6, 12, and 24 h following drug removal. The agents used produced changes in the expression of specific genes, and all three did so in a similar fashion. Immediately following drug removal, the steady state expression of c-myc in treated cells was increased 2- to 3-fold compared to control. At 6 and 12 h following drug removal, c-myc levels were depressed 2.5- to 5-fold. By 24 h, c-myc expression approached, but remained below, control levels. Immediately following drug removal, c-fos levels were increased 3- to 4-fold, and from 6 to 24 h following drug removal, c-fos levels gradually returned to, or fell below, low basal levels. During the 24-h time course, drug treatment had little or no effect on the steady state levels of N-ras or β-actin. These data support the hypothesis that alkylating agents may suppress the expression of specific transforming genes.

INTRODUCTION

In recent years, a number of investigations have demonstrated that perturbations in normal cellular protooncogenes can transform them into activated oncogenes. These changes may occur by a variety of mechanisms, (e.g., amplification, translocation, point mutation) and typically result in aberrant gene expression or an altered protein product (1-3). Once activated, it appears that oncogenes play a critical role in the tumorigenic phenotype (4-6).

Although much is known of the role oncogenes play in carcinogenesis, a paucity of data exists regarding the effects of clinically efficacious antitumor agents on oncogene expression. It is conceivable, however, that different antitumor agents may selectively inhibit the expression of specific oncogenes. For example, a preliminary report indicated that exposure to HN2 suppressed c-myc expression, but did not affect ras expression, in the Burkitt lymphoma cell line Daudi (7). Another report examined the effect of a variety of clinical antileukemic chemotherapeutic regimens on c-myc expression in patients with leukemia (8). Although no alkylating agents were included in the chemotherapeutic regimens used, this study demonstrated that depression of c-myc expression 24 h after treatment correlated with tumor response in patients with acute myelogenous leukemia or acute lymphocytic leukemia (8). These data suggest that drug effects on oncogene expression may serve as indicators of prognosis in specific types of cancer (8).

In this report, we have examined the effects of three structurally related bifunctional alkylating agents on oncogene expression in the colon carcinoma cell line Colo320HSR. Colo320HSR is a human colon tumor cell line of neuroectodermal origin isolated from a patient prior to the onset of chemotherapy (9). Contained within the homogeneously staining region (HSR) are approximately 30 to 40 copies of the c-myc oncogene, which in turn is highly overexpressed, and is assumed to be one of the transforming genes of this cell line (10). Low levels of expression of c-fos and N-ras can also be detected (this study).

The compounds used in this study are the clinically useful agents, HN2, L-PAM, and a form of cyclophosphamide active in vitro, 4-HC (11). These electrophilic agents react with a number of cellular macromolecules, including protein and RNA, but it appears that they exert their cytotoxic effects by producing lesions in the DNA, particularly DNA interstrand and intranstrand cross-links (12-16). We have examined the effects of the aforementioned alkylating agents on the expression of selected genes in Colo320HSR. These genes are the amplified c-myc oncogene, the N-ras and c-fos oncogenes, as well as the gene encoding β-actin. The three alkylating agents produced changes in the expression of specific genes, and each did so in a similar fashion. Immediately following drug treatment, steady state levels of c-myc and c-fos transcripts are increased 2- to 3-fold and 3- to 4-fold, respectively. From 6 to 24 h following drug treatment, c-myc expression decreased to below control levels, and c-fos gradually declined to its low basal levels. Drug treatment did not have any discernible effects on the steady state levels of either N-ras or β-actin.

MATERIALS AND METHODS

Cell Culture, Drug Treatment and Survival Assays. The cell line Colo320HSR was purchased from American Type Culture Collection, and cultured in RPMI 1630 (Hazelton-Dutchland) supplemented with 15% heat inactivated bovine calf serum (HyClone), glutamine, penicillin, and streptomycin at 37°C, 95% air/5% CO2. The cells have a doubling time of approximately 24 h and were passaged twice a week.

For survival assays and gene expression experiments, exponentially growing cells at 5 × 105 cells/ml were exposed to the respective alkylating agents for 1 h at 37°C, and then washed free of drug. For survival assays, cells were seeded into soft agar culture tubes as described by Chu and Fisher (17). Colony forming efficiency of untreated controls was typically 75%. For measurements of drug effects on gene expression, following drug exposure cells were washed, resuspended in fresh media, and grown at 37°C before cell lysis at designated intervals.

Alkylating Agents. HN2 and L-PAM were obtained from the Drug Development Branch, National Cancer Institute. Both compounds were dissolved in sterile filtered 0.1 N HCl and were maintained as a frozen stock solution. 4-HC was the generous gift of Dr. Michael Colvin of The Johns Hopkins School of Medicine. 4-HC was dissolved in sterile RPMI 1630 immediately prior to use.
RNA Isolation. Total cellular RNA was isolated essentially by the procedure of Chirgwin et al. (18). Briefly, cells were washed with ice-cold phosphate buffered saline, and then lysed with a solution composed of 4 M guanidium isothiocyanate (Bethesda Research Laboratories), 0.02 M sodium acetate, 0.01 M dithiothreitol, and 0.5% Sarkosyl (Sigma). Chromosomal DNA was sheared by repeated passage through a 20-gauge needle. RNA was recovered by sedimentation through a 5.7 M CsCl, 0.1 mM EDTA cushion in an SW 40Ti rotor centrifuged at 35,000 rpm for 16 h. RNA was resuspended in diethyl pyrocarbonate treated deionized water and quantitated by absorbance measurements at 260 nm.

Northern Blot Analysis. RNA from control and drug treated cells was denatured by treatment with 50% dimethyl sulfoxide, 1 M glyoxal (Sigma), 0.01 M NaPO4, pH 6.8, at 50°C for 1 h. Identical amounts of denatured RNA were size fractionated through 1% agarose gels in a recirculated running buffer containing 0.01 m NaPO4, pH 6.8. RNA was transferred to GeneScreenPlus membranes (New England Nuclear) by capillary blotting with 10x standard saline citrate (1.5 M NaCl, 0.15 M trisodium citrate) for 18-24 h. The glyoxal reaction was reversed and the membrane was neutralized by sequentially treating the membrane with 50 mM NaOH (15 s) and 0.2 M Tris (pH 7.5), 2x standard saline citrate (35 s).

Prehybridization of the membrane was carried out in 50% formamide, 10% dextran sulfate, 5x SSPE 1% SDS, 1x Denhardt’s solution (0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin (Pentax Fraction V), and 250 µg/ml denatured salmon sperm DNA at 42°C for 6-24 h in a shaking water bath. Hybridization was carried out in the same prehybridization solution, at 42°C for 18-24 h, to which 10⁶ cpm/ml of denatured ³²P-labeled DNA probe had been added.

Following hybridization, the membrane was first washed in a large volume of 2x SSPE, 0.5% SDS at room temperature. The membrane was then washed in a large volume of 0.1x SSPE, 0.5% SDS at 60°C (except for c-fos blots, in which case the final wash was 0.2x SSPE, 0.5% SDS at 60°C).

DNA Probes. DNA probes were made by uniformly labeling specific gene fragments with [α-³²P]dCTP (NEN; specific activity 3000 Ci/mmol), utilizing the random primer protocol of Feinberg and Vogelstein (19). Unincorporated nucleotide was removed by Sephadex G-50 filtration and specific activity was determined by trichloroacetic acid precipitation. The specific activity of labeled fragments was typically 0.7-1.0 x 10⁸ cpm/µg. Gene fragments used in hybridization studies were as follows: the 420-base pair PstI exon 2 fragment of human c-myc, the 1-kilobase PstI fragment of α-fos, the 770-base pair HindIII fragment containing exon 1 of human N-ras, and the 770-base pair fragment of a chicken cDNA encoding β-actin (20-23).

RESULTS

In this study we have examined the effects of three clinically useful antitumor alkylating agents on steady state levels of oncogene mRNA. 4-HC was used instead of cyclophosphamide because of the need of cyclophosphamide for microsomal activation. 4-HC spontaneously decomposes in vitro to 4-hydroxycyclophosphamide, the same intermediate resulting from the microsomal activation of cyclophosphamide (11). To determine drug exposures to be used for the measurement of effects on gene expression, colony formation assays were used (Fig. 1). The exposures chosen for gene expression experiments were equitoxic and produced approximately 1 or 3 log cell kills. The concentrations used were 2.1 and 6.3 µM HN2, 12.5 and 25 µM L-PAM, and 40 and 80 µM 4-HC.

Fig. 2 shows the effects of L-PAM, 4-HC, and HN2 on c-myc steady state transcripts, as measured by Northern blot analysis. Each agent produced an initial increase in c-myc transcripts immediately following drug removal. Densitometric measurements of each autoradiograph indicate this to be an approximate 2- to 3-fold increase in steady state transcripts of this highly overexpressed gene. At 6 and 12 h after drug removal, c-myc transcripts were consistently reduced, following drug exposures which produced 1 and 3 log cell kills. These exposures produced approximately 2.5- and 5-fold reductions in transcript levels, respectively. By 24 h, c-myc transcripts approached control levels.

The effects of drug treatment on steady state transcripts of
c-fos are shown in Fig. 3. Like c-myc, c-fos expression was elevated immediately following drug removal. In this case, levels of c-fos transcripts were seen to increase 3- and 4-fold, for 1 and 3 log cell kill exposures, respectively. At 6 to 24 h after drug exposure, c-fos levels returned to, or fell below, the very low basal levels observed in control cultures.

Fig. 4 shows Colo320HSR c-myc mRNA levels over a 24-h time course, after a mock drug treatment. Essentially, no alterations in steady state c-myc mRNA levels are detected, and the small differences (<20%) between the time points is probably due to experimental variability. Similarly, no differences in c-fos levels could be detected (data not shown).

In Figs. 5 and 6, the effects of drug treatment on N-ras and β-actin expression are shown. N-ras, like c-fos, is expressed at very low levels in untreated Colo320HSR. The three alkylating agents used in this study appeared to have no discernible effect on N-ras expression over the 24-h time course. Similarly, these
compounds had little or no effect on the expression of the constitutively expressed “housekeeping gene” β-actin.

**DISCUSSION**

A number of studies have examined the effects of various biological and chemical agents on gene expression, but very few have focused on the effects of cytotoxic, DNA damaging antitumor agents. In this report, we demonstrate that three structurally related alkylating agents produce strikingly similar responses in each of the genes examined.

The expression of the c-fos gene can be induced rapidly and transiently in a variety of normal cell types by a diverse group of compounds. These compounds include mitogens, such as serum, interleukins, and growth factors (e.g., platelet-derived growth factor, epidermal growth factor), tumor promoters, such as phorbol esters, and neuroactive compounds (24–28). In the present study, we demonstrate that c-fos steady state message is increased immediately following cytotoxic exposures to bifunctional alkylating agents.

Recently, a number of reports have been published which begin to define the functional role that the c-fos protein plays in the cell. It appears the c-fos protein interacts with the c-jun/AP-1 protein(s), which in turn acts to stimulate or repress transcription of genes under the influence of the AP-1 inducible enhancer (29–33). Furthermore, it has also been reported that genes inducible by the DNA damaging agents mitomycin C and UV light are also AP-1 inducible genes (34–36). From these observations, it is possible to speculate that the bifunctional alkylating agents may be initiating a cellular response to DNA damage mediated through the induction of c-fos.

Along with the immediate increase in c-fos expression, c-myc expression is also elevated. The rapid and transient increases in c-fos and c-myc expression are reminiscent of the mitogenic response seen in cells stimulated by a variety of mitogens (37, 38). In fact, it appears that both c-fos and c-myc levels are increased within 15 min following the addition of the bifunctional alkylating agent (data not shown). Although it is presently unknown if the increases in c-myc and c-fos steady state message are due to increased transcription; if they are, then this increase may be due to drug effects on the intracellular mediators of the proliferative response. Furthermore, if the immediate increases in c-myc and c-fos do reflect an “inappropriate” proliferative response, then it would be interesting to speculate that this response may act to “open up” critical areas of the genome necessary for growth, and in turn make these regions more accessible to drug-induced DNA damage. Finally, it is also possible that these increased levels are due to a drug-induced stabilization of both of these messages, but this type of effect may have the same biological consequences as increased transcription.

Each of the alkylating agents tested was able to profoundly suppress the level of c-myc transcripts at 6 and 12 h following drug removal. It is of interest to note that these are the time points for maximum DNA damage produced by these agents. Suppression of c-myc transcripts by a number of compounds has been previously reported. Specifically, differentiating agents such as dibutyryl cyclic 3'-5'-AMP, dimethyl sulfoxide, and 1,25-dihydroxyvitamin D, reduce c-myc expression in hematological tumor cell lines prior to terminal differentiation (39–41). Also, α-interferon can cause growth arrest in the Burkitt lymphoma cell line Daudi, and γ-interferon alone, and in cooperation with tumor necrosis factor, can cause growth arrest in HeLa cells. In both cases, the growth arrest has been closely linked to suppression of c-myc transcripts (42, 43). These data imply that reduction of c-myc expression may reflect the loss of cellular proliferation, or in the case of nitrogen mustards, this may be a precursor to cell killing. The cause of the alkylating agent induced suppression of c-myc transcripts is yet unanswered; it may be due to a specific cellular response to the drug challenge, as appears to be the case for the compounds mentioned above. Alternatively, suppression of transcript levels may be due to the accumulation of DNA damage within the c-myc oncogene. Interestingly, the reduced levels of c-myc transcripts observed at 6 and 12 h following drug removal are coincident with the time of peak DNA interstrand cross-links produced by these agents.

Over the 24-h time course, little or no effect of alkylating agent treatment could be seen on the expression of N-ras and β-actin. This may not be surprising since very few agents examined to date appear to alter expression of these genes (7, 44, 45). It is also possible that the nitrogen mustards may exert their effects, either directly or indirectly, on specific genes.

Data presented in this report suggest cytotoxic DNA-damaging agents differentially affect a subset of active cellular genes. Furthermore, these alterations may reflect efficient drug-induced cell kills. Future studies will be aimed at determining the direct cause of the initial elevation in c-myc transcripts, as well as the subsequent depression of c-myc. In addition, it may be worthwhile to determine if other early growth response genes (besides c-myc and c-fos), such as EGR2 (46, 47), or other AP-1 responsive genes, such as collagenase or metallothionein (34–36), are also induced by the bifunctional alkylating agents. Finally, it will be of interest to determine if different types of clinically useful, DNA cross-linking agents (e.g., nitrosoureas and platinum compounds) elicit responses similar to that of the nitrogen mustards.

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