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Effects of Transcription and Translation Inhibitors on a Human Gastric Carcinoma Cell Line

POTENTIAL ROLE OF Bcl-X_S IN APOPTOSIS TRIGGERED BY THESE INHIBITORS

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ABSTRACT. The effects of the macromolecular synthesis inhibitors 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB), actinomycin D, and cycloheximide on the human gastric cancer TMK-1 cell line were studied. These agents inhibited DNA, RNA, or protein synthesis efficiently and induced cell death rapidly in a wide range of concentrations. After 8 hr of exposure to these agents, the cells exhibited morphological features of apoptosis, including cell shrinkage, nuclear condensation, DNA fragmentation, and formation of apoptotic bodies. Western blot analysis revealed that these inhibitors altered the protein levels of apoptosis-related gene products such as c-Myc, Bcl-X_s, and the mutant p53 (mp53) in TMK-1 cells markedly. The c-myc mRNA and protein levels were decreased initially and were then induced markedly to a new level after 4 hr of exposure to DRB, a RNA polymerase II inhibitor. The Bcl-X_S levels were increased rapidly after treatment with all of these agents, whereas the levels of Bcl-X_L and Bax remained largely unchanged. Northern blot analysis indicated that the *c-myc* overexpression is concomitant to DRB-induced DNA fragmentation and that the increased mp53 protein level was mainly a posttranscriptional event. Our observations suggest that the up-regulation of Bcl-X_S may serve as an important mechanism for the apoptosis triggered by these inhibitors. This study also provides evidence for the notion that interference with the cellular survival pathway may lead to apoptosis. BIOCHEM PHARMACOL 53;7:969–977, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. TMK-1 cells; 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole; DNA fragmentation; cell death; *c-myc; bcl-X; bax*

Apoptosis, or programmed cell death, is an important mechanism for the differentiation, development, defense, and survival of eukaryotic organisms. The biochemical features of this type of cell death include chromatin condensation, cell membrane blebbing and activation of endonucleases, which degrade chromatin into oligonucleosomes [1, 2]. In addition to normal physiological processes, cell death through apoptosis has been demonstrated by the use of an extensive array of nonphysiological agents such as cytotoxic drugs, X-irradiation, heat shock, hyperthermia, calcium influx, therapeutic agents, and viral invasion [3–5]. Apoptosis can also be triggered by the deprivation of some factors that function to prevent this process [6]. As the molecular mechanism of apoptosis has been characterized more thoroughly, evidence indicates that distinct forms of apoptosis exist [3–5, 7]. Regulatory genes such as *p53*, *c-myc*, *bcl-2*, and *bcl-2*-related genes, were shown to play important roles in the regulation of apoptosis, although the detailed mechanism is still not understood [7, 8]. The *bcl-2*-related gene *bcl-X* can code for two proteins, Bcl-X_L and Bcl-X_S, by alternatively spliced transcripts [9]. Bcl-X_L inhibits apoptosis triggered by a wide range of stimuli as effectively as Bcl-2, whereas Bcl-X_S facilitates apoptosis by inhibiting the death suppressor activity of Bcl-2 and Bcl-X_L [9, 10]. It has been suggested that certain cells may be more susceptible to entering apoptosis when the expression of these genes is deregulated [5].

It has been demonstrated that protein synthesis is required for certain forms of apoptosis because they can be blocked by metabolic inhibitors including Act D§ and CHX [11]. However, many studies also indicated that apoptosis could be triggered or potentiated by these inhibitors [12–15]. In this study, we investigated the effects of transcription and translation inhibitors including the RNA polymerase II specific inhibitor (DRB) [16, 17], Act D, and CHX on the human gastric cancer cell TMK-1. The changes in the expression of *c-myc*, *p53*, *bcl-2*, *bcl-x_L*, *bcl-*

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§ *Abbreviations:* Act D, actinomycin D; CHX, cycloheximide; DRB, 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole; BrdU, bromodeoxyuridine; and GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

X_S, and *bax* were also examined because few of the previous studies on apoptosis triggered by macromolecular synthesis inhibitors have analyzed the alterations of expression of apoptosis-related genes in parallel. Here we show that alterations in the expression of these genes correlated well with cell death induced by these inhibitors. These data indicate that the effect of DRB may be c-*myc* dependent and that the up-regulation of *bcl-x*$_S$ is essential for apoptosis triggered by all these inhibitors in this cell line.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, U.S.A.). Culture plasticware was purchased from Costar (Cambridge, MA, U.S.A.). [α-^{32}P]dCTP (6000 Ci/mmol) was purchased from Dupont NEN (Boston, MA, U.S.A.). The ^{32}P-labeled cDNA probes were prepared by random primer extension with [α-^{32}P]dCTP to a specific activity of about 10^8 cpm/μg of DNA. The cDNA templates for c-myc, p53, and GAPDH in random priming and antibodies against human c-Myc, Bcl-2, Bax, Bcl-$X_{L/S}$ and p53 were purchased from Oncogene Sciences (Uniondale, NY, U.S.A.) and Santa Cruz Biotech., Inc. (Santa Cruz, CA, U.S.A.). DRB, Act D, and CHX were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals used were purchased from either Sigma or E. Merck (Darmstadt, FRG).

Cell Culture

The cultured human gastric carcinoma cell line TMK-1, derived from a poorly differentiated adenocarcinoma [18], was a gift from Dr. Eiichi Tahara (Hiroshima University, Japan). The cells carry a mutated form, G to A point mutation at codon 173, of the *p53* gene (*mp53*) as described [19]. In this study, TMK-1 cells were cultured at 37° in DMEM supplemented with 15 mM HEPES, 26 mM sodium bicarbonate, 2 mM L-glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin, and 10% charcoal-dextran-treated FBS with 5% CO_2. The growth curve of the cells was determined by counting the viable cells by trypan blue exclusion using a Coulter counter. Cells in the logarithmic growth phases were used in this study. The cells were seeded into culture dishes and allowed 24 hr prior to treatment. RNA or protein synthesis inhibitors were added to the medium as a concentrated stock in ethanol. Control cells were fed with medium containing the same amount of ethanol.

Treatment of TMK-1 Cells with RNA and Protein Synthesis Inhibitor

For experiments in which the effects of DRB, Act D, and aphidicolin on RNA synthesis were determined, TMK-1 cells were plated into 24-multiwell plates at a density of 2 $\times$ 10^4 cells/well for 24 hr and then incubated with various RNA synthesis inhibitors for the time period and at the concentrations indicated. [5,6-^{3}H]Uridine (2 μCi/mL) was then added into the culture medium and the mixture was further incubated for 1 hr. Treatment of the cells with the inhibitors was continued during the period of pulse labeling. The cells were washed twice with PBS and three times with cold 5% trichloroacetic acid (TCA) and then twice with absolute ethanol. The dried cells were dissolved in 200 μL of 0.2 N NaOH at 37° for 10 min with gentle shaking. Radioactivity incorporated in a 100-μL sample was determined by liquid scintillation counting. The effect of CHX on protein synthesis was determined similarly, but [^{3}H]leucine was used to label the newly synthesized proteins. The cells were washed twice with PBS and then solubilized in 200 μL of cell lysis buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.5% BSA, 0.5% Nonidet P-40, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride) at 4° for 1 hr. Insoluble material was removed by centrifugation at 12,000 *g* for 5 min at 4°, and 100-μL aliquots of supernatant were precipitated with 10% TCA. The acid-insoluble material was pelleted at 12,000 *g* at 4° for 10 min and then dissolved in 0.1 mL of 10% SDS. Radioactivity incorporated into acid-insoluble material was determined.

Analysis of DNA Fragmentation

DNA was isolated from cells treated with or without various agents using the procedures described [20]. Briefly, cells were washed and lysed (10 mM Tris–Cl, pH 8.0, 10 mM EDTA, 10 mM NaCl, 0.5% SDS, and 1 mg/mL proteinase K) and incubated overnight at 37°. Then the lysed cells were extracted twice in phenol:chloroform:isoamyl alcohol (25:24:1, by vol.) and ethanol-precipitated. The precipitate was resuspended and digested with RNase at 37° for 2 hr. The RNase-treated lysates were extracted with phenol:chloroform:isoamyl alcohol and ethanol-precipitated. The DNA was resuspended in 10 mM Tris–HCl, pH 8.0, 5 mM EDTA, and quantitated. The same amount of DNA of each sample was loaded and electrophoresed in a 2% agarose with TBE (Tris-borate, EDTA) buffer. Gels were stained with ethidium bromide and photographed. Cellular DNA fragmentation was assayed using the cell death detection ELISA reagents from Boehringer Mannheim (Mannheim, Germany) following the manufacturer's instructions. Briefly, TMK-1 cells grown exponentially were labeled with 10 μM BrdU for 24 hr, and the labeled cells were stripped and seeded into 96-well plates at a density of 2 $\times$ 10^4 cells/well. Different concentrations of agents were added into triplicate wells and incubated for the time indicated in the figures. To determine the amount of BrdU-labeled mono- and oligonucleosomal DNA released from cell nuclei into the cytoplasm, cells were lysed by adding 100 μL/well of lysis buffer and incubated for 30 min at room temperature. The microtiter plates were centrifuged at 250 *g* for 10 min, and 100 μL of supernatant was removed for ELISA.

Western Blot Analysis

TMK-1 cells were plated at 6-cm dishes at a density of 1 × 10^6 cells/dish for 24 hr prior to drug treatment, and then were incubated with DRB (25 μg/mL), Act D (50 ng/mL), or CHX (1 μg/mL) for the indicated time periods. Next, cells were washed and lysed in 0.2 mL of lysis buffer (1% Nonidet P-40, 50 mM Tris–HCl, pH 7.4, 180 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 10 mM Na_3VO_4) for 30 min at 4°. After centrifuging at 17,500 *g* for 15 min, equal amounts of proteins in the lysate supernatants were separated by 12% SDS–PAGE gel and transferred. Immunoblotting was performed using rabbit anti-human antibodies for c-Myc, p53, Bcl-2, Bcl-$X_{L/S}$ and Bax. Horseradish peroxidase-conjugated goat anti-rabbit antibody was used as secondary antibody. Signals were visualized by an enhanced chemiluminescence kit (ECL, Amersham, U.K.) followed by exposure to X-ray films.

Isolation of RNA and Northern Blot Analysis

Total RNA was isolated with the guanidine isothiocyanate procedure [21]. The cells were lysed with 1.2 mL of 4 M guanidine isothiocyanate/0.5% sodium dodecylsarcosine/2.5 mM sodium citrate (pH 7.0)/0.1 M 2-mercaptoethanol. After shearing the DNA, total RNA was pelleted by centrifugation through a 5.7 M CsCl cushion. For northern analysis, 30 μg of total RNA was treated with glyoxal/dimethyl sulfoxide at 50° for 1 hr and size-fractionated on 1.2% agarose gel. The RNA was transferred to nylon filters and prehybridized in 50% formamide/50 mM NaH_2PO_4/Na_2HPO_4 (pH 6.8)/0.5% SDS/0.1× Denhardt's solution/1% Ficoll/1% polyvinylpyrrolidone/1% BSA/0.1 mg/mL salmon sperm DNA at 42° for 4 hr. Labeled probe was added to a concentration of 2 × 10^6 cpm/mL, and the incubation was continued for 20 hr at 42°. The filters were washed and autoradiographed.

RESULTS

Cell Growth and Morphology

TMK-1 cells exhibited significant morphological changes when exposed to transcription and translation inhibitors including Act D, DRB (see Fig. 1, A–C), aphidicolin, CHX, and puromycin. After incubation with these agents for 6–8 hr, the cells began to disintegrate and most cells died within 24 hr of treatment. After first rounding up, the cells then developed plasma membrane projections in about 6–8 hr, and eventually formed cellular fragments after about 12 hr (Fig. 1). Among these agents, Act D was the most effective in eliciting cell death. The concentration ranges of these agents used in this study were 0.5 to 2 μg/mL (1.75 to 7 μM) for CHX, 10 to 50 ng/mL (8 to 40 nM) for Act D, and 6.25 to 25 μg/mL (1.96 to 7.83 × 10^{-5} M) for DRB. These agents induced similar cell death effects in this cell line, although the onset and extent of cell death were concentration dependent. The results also indicated that the

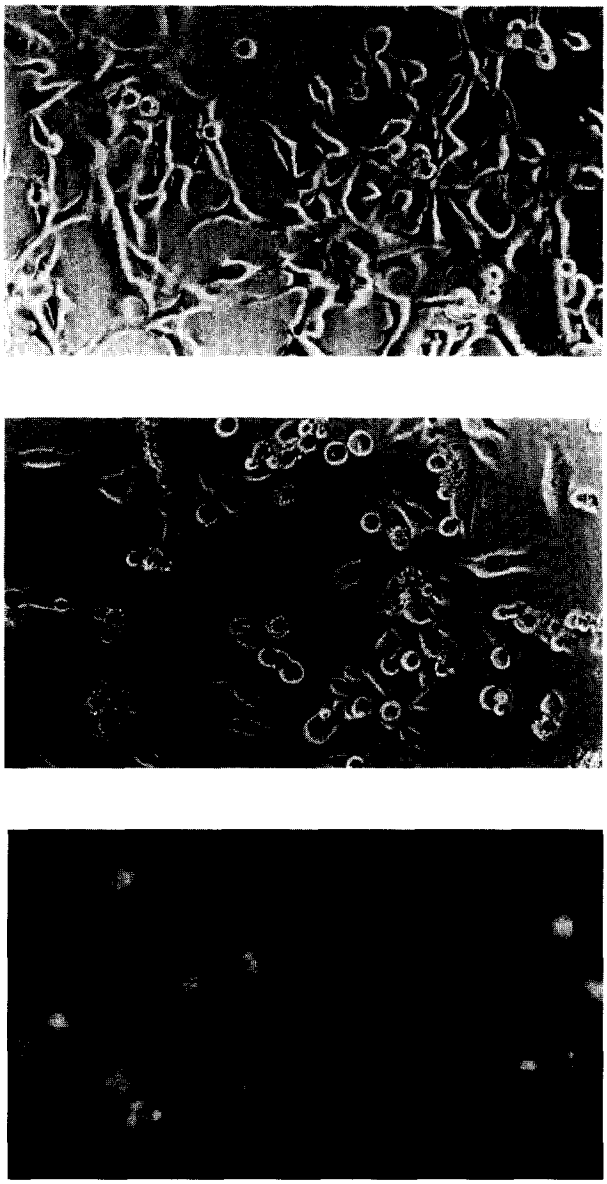

FIG. 1. Morphological effects of DRB on TMK-1 cells. Cells (2 × 10^6) were seeded into 10-cm culture dishes for about 24 hr and then were incubated in the absence (A) or presence (B) of 78 μM DRB for 8 hr. The cells were analyzed by phase-contrast microscopy (400×). (C) DRB-induced condensed and fragmented cell nuclei. The cells were treated with 78 μM DRB for 8 hr, fixed with cold 30% methanol/70% acetone, and stained for DNA with propidium iodide (1 μg/mL) for 30 min. Then slides were mounted and analyzed by fluorescence microscopy (510×).

TMK-1 cells were very sensitive to these transcription and translation inhibitors since many other cultured cells, including MCF-7, HeLa, and BC-M1 [22], showed much lower toxicity to these agents at the same concentrations used in our studies. In addition, the concentrations of these agents needed to induce a similar extent of cell death were comparable or lower than those used in previous studies in other cell lines [12–15]. The growth curve shown in Fig. 2 demonstrated that DRB and CHX induced cell death effi-

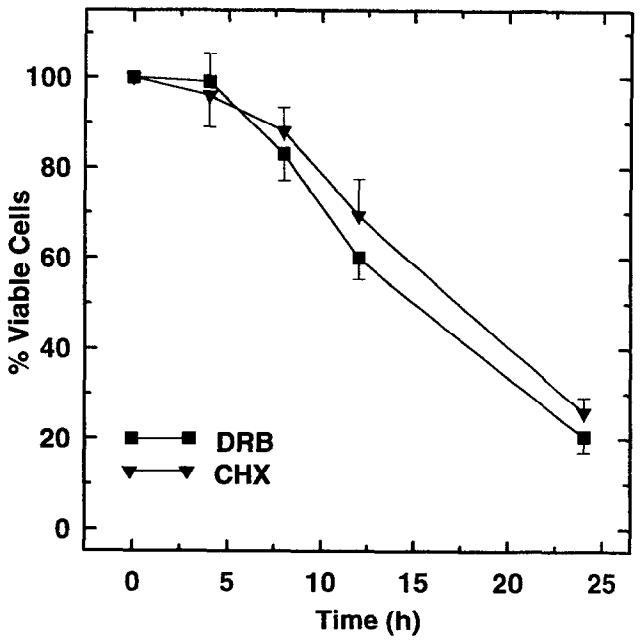

FIG. 2. Effects of DRB and CHX on the growth and viability of TMK-1 cells. TMK-1 cells were subcultured into 6-well plates at a seeding density of 10^5 cells/well for 48 hr. The cells were treated with 78 μM DRB or 7.1 μM CHX for the indicated time periods before being harvested to count the cell numbers. Viability was determined by trypan blue exclusion. Results are means ± SEM (N = 4).

ciently. The cell death effects were most prominent after exposing the cells to these agents for over 12 hr. The pattern of DRB-induced cell death was analyzed by morphological changes in cell nuclei and DNA fragmentation. The cells were treated with 78 μM DRB for 8 hr, stained with propidium iodide, and viewed by fluorescence microscopy. The DRB-treated apoptotic cells were identified with the features of formation of membrane-bound vesicles (apoptotic bodies), condensed chromatin, and fragmentation of cell nuclei into discrete masses (Fig. 1). Act D- or CHX-treated cells also showed similar features of apoptosis (data not shown). These results indicated that these metabolic inhibitors triggered cell death by apoptosis.

DNA Fragmentation

Initiation of cell death is associated with the activation of endogenous endonucleases that extensively cleave chromatin DNA into oligonucleosomal DNA fragments with lengths of about 160–220 bp. The apoptotic nature of cells treated with metabolic inhibitors was further confirmed by agarose gel electrophoresis of the extracted DNA. A characteristic pattern of internucleosomal ladder profiles, which characterizes the programmed cell death, was detected in DRB-, Act D-treated (Fig. 3A), and CHX-treated (Fig. 3B) TMK-1 cells. Distinct nucleosome ladders were detectable after 6 hr, and were evident after 8 hr of exposure to these agents. No DNA fragmentation occurred in an Ara-C-treated sample although Ara-C shares a similar nucleoside structure with DRB and has been shown to induce apoptosis in many cell types [23]. CHX, from 0.5 μg/mL (1.8 μM) to 5 μg/mL (17.5 μM), induced internucleosomal DNA fragmentation in this cell line after 8 hr of incubation, and the extent of fragmentation was concentration dependent (Fig. 3B).

To express DNA degradation quantitatively, we employed the ELISA method in which the fragmented and BrdU-incorporated DNA were detected by anti-BrdU-DNA monoclonal antibody. Results shown in Fig. 3C demonstrated that the fragmentation of DNA occurred after 4 hr in all three drug treatments. The time courses of fragmentation were similar in these agents, suggesting that a similar apoptotic mechanism may exist. As shown in Fig. 3D, the concentrations of these inhibitors required to produce maximal DNA fragmentation at 10 hr of incubation were 25 ng/mL for Act D, 1 μg/mL for CHX, and 25 μg/mL for DRB. Incubation of TMK-1 cells with these agents for 10 hr produced over 1 15-fold increase in the mono- and oligonucleosomal DNA fragments in cytoplasm as compared with untreated cells. In this study, the extent of DNA fragmentation, as shown in the time–course results in Fig. 3C, correlated well with the percentage of apoptotic cells, judged by the morphological changes under a fluorescence microscope as well as the flow cytometric analysis (data not shown). Taken together, these results demonstrate that exposure of TMK-1 cells with transcription and translation inhibitors led to DNA fragmentation and apoptosis.

Inhibition of Macromolecular Synthesis

Inhibition of RNA synthesis was determined by the incorporation of [^{3}H]uridine into the cells. TMK-1 cells were incubated in the absence or presence of the indicated concentrations of macromolecular synthesis inhibitors for 6 hr and pulse-labeled for another hour. Results shown in Table 1 indicated that treatment with 25 and 50 ng/mL of Act D caused over 97% reduction of [^{3}H]uridine uptake by these cells. The inhibitory effect of DRB was less marked than that of Act D, and the overall inhibition of RNA synthesis was about 60–70% at concentrations of 12.5 and 25 μg/mL. A similar extent of RNA synthesis inhibition by DRB also has been demonstrated in human HeLa and mouse L cells [16, 17]. The moderate effect of DRB is due to the highly selective inhibition on the synthesis of heterogeneous nuclear RNA without much interference with the synthesis of ribosomal and transfer RNA [16, 17]. The effect of various concentrations of CHX on protein synthesis in TMK-1 cells was determined by [^{3}H]leucine incorporation, as shown in Table 2. At 0.5 μg/mL, CHX caused a 60% inhibition in the uptake of [^{3}H]leucine into the cells. At concentrations greater than 1 μg/mL, over 80% of protein synthesis was inhibited after 6 hr of CHX treatment. Taken together, these results indicated that macromolecular synthesis was inhibited effectively by these agents at the concentrations studied.

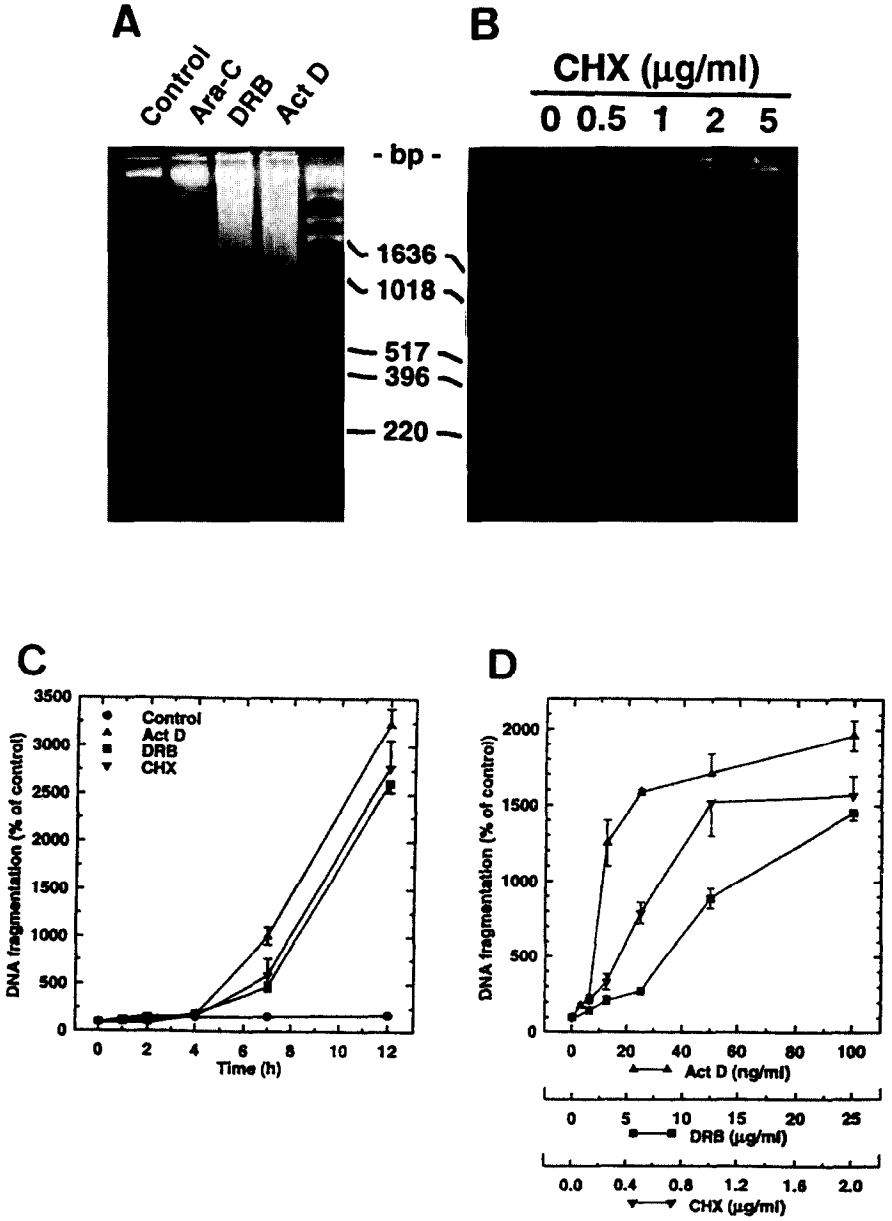

FIG. 3. Internucleosomal DNA fragmentation. Cells (2×10^6) were subcultured into a 100-mm culture dish for 24 hr prior to the experiments. (A) Cells were incubated in the absence (control) or the presence of 1 mM Ara-C, 78 μM (25 μg/mL) DRB, or 40 nM (50 ng/mL) Act D for 8 hr. (B) Cells were treated for 8 hr with the indicated concentrations of CHX. Internucleosomal DNA fragmentation was analyzed by agarose gel electrophoresis. For (C) and (D), BrdU-prelabeled cells were subcultured into 96-well plates and treated with 78 μM DRB, 40 nM Act D, or 7.1 μM CHX for the indicated time periods (C); or treated for 10 hr with the indicated concentrations of DRB, Act D, or CHX (D). Results represent means ± SEM of two independent experiments, each consisting of triplicate wells.

Effects of Act D, DRB and CHX on c-myc, p53, bcl-X *and* bax *expression*

The products of c-*myc* and *p53* genes have been shown to be involved in many types of cell apoptosis [24–27]. Recently, it also has been found that cell death is controlled, at least in part, by the interplay of the expanding family of Bcl-2-related, apoptosis-regulating, proteins [8, 28, 29]. To further understand the effects of Act D, DRB, and CHX on this cell line, we examined the alterations in the expression of apoptotic-related genes including c-*myc*, *mp53*, *bcl-2*, *bcl-X*, and *bax* associated with TMK-1 cell death. The changes in the protein levels of these genes in response to the three inhibitors are summarized in panels A and B of Fig. 4. While the Bcl-X_L and Bax levels remained largely unchanged, both the mp53 and Bcl-X_S protein levels were induced rapidly by all three compounds. These results indicated that the regulation of the expression of *mp53* and *bcl-X* genes by these inhibitors might share similar mechanisms. The *bcl-2* gene might be absent or not expressed in this cell line since both bcl-2 protein and mRNA were not detected in this study (data not shown). Since all of the inhibitors tested in this study affected the Bcl-X_S expression in a similar fashion (Fig. 4), it is likely that Bcl-X_S plays an essential role in apoptosis triggered by these inhibitors in TMK-1 cells. In addition, it should be noted that TMK-1 cells expressed an unusually high basal level of Bcl-X_S since most tumor cell lines do not appear to express such high levels of this protein [28, 29]. In contrast to mp53 and Bcl-X_S, the alterations of c-Myc levels induced by these inhibitors were different. As shown in Fig. 4, Act D and CHX produced a moderate induction in c-Myc protein lev-

TABLE 1. Inhibitory effects of Act D and DRB on [^{3}H]uridine incorporation in TMK-1 cells

Inhibitor	[^{3}H]Uridine incorporation (cpm)	% Inhibition
Control	2400 ± 260	
Act D		
25 ng/mL	53 ± 7	98
50 ng/mL	25 ± 5	99
DRB		
12.5 μg/mL	930 ± 20	61
25 μg/mL	720 ± 160	70

Cells (2 × 10^4/well) were seeded into a 24-well plate. After 24 hr, the cells were treated with Act D or DRB for 6 hr and then pulsed-labeled with [^{3}H]uridine (2 μCi/mL) for 1 hr. [^{3}H]Uridine incorporated into acid-precipitable material was determined. The results are means ± SEM (N = 3).

els. The effect of DRB was unique; after an initial down-regulation to a minimal level, the c-Myc protein level was induced rapidly and significantly to more than 3-fold of the original level after 5 hr of DRB exposure (Fig. 4). These results indicated that c-Myc may also play an important role at least in DRB-induced apoptosis in this cell line [30].

To know whether the increased c-Myc and mp53 protein levels were due to increased mRNA levels, we performed northern blot analysis. The stability of c-myc and mp53 mRNA was determined by using Act D, which inhibited over 98% of total RNA synthesis in this cell line (Table 1). As shown in Fig. 5 (A and B), the half-lives of c-myc and mp53 mRNA transcripts in these cells were about 30 min and 8 hr, respectively. The mp53 mRNA level was down-regulated, which suggests that the increased mp53 protein induced by these three inhibitors is mainly independent of transcription. It is possible that the increased mp53 protein levels during apoptosis is a posttranscriptional event caused by increasing mp53 protein half-life as described [31]. Consistent with the remarkable alterations in c-Myc protein product, the c-myc mRNA was similarly regulated by DRB (Figs. 4 and 5). These results indicated that alterations in c-*myc* gene expression may be crucial for the biochemical effects of DRB in this cell line. Taken together, these results suggest that the rapid up-regulation of Bcl-X_S protein induced by the inhibitors may be an important mechanism

TABLE 2. Effect of CHX on [^{3}H]leucine incorporation in TMK-1 cells

Inhibitor	[^{3}H]leucine incorporation (cpm)	% Inhibition
Control	470 ± 57	
CHX		
0.5 μg/mL	190 ± 26	60
1 μg/mL	85 ± 15	82
2 μg/mL	67 ± 7	86

Cells (2 × 10^4 c/well) were seeded into a 24-well plate. After 24 hr, the cells were treated with CHX for 6 hr and then pulsed-labeled with [^{3}H]leucine (2 μCi/mL) for 1 hr. [^{3}H]leucine incorporated into acid-precipitable material was determined. The results are means ± SEM (N = 5).

for the apoptosis in this cell line. In addition, in the presence of DRB, a potent and specific RNA polymerase II inhibitor, the cell proliferation-related c-*myc* gene was still actively transcribed.

DISCUSSION

Apoptosis in eukaryotic cells is an active process that is necessary for normal development and differentiation. Failure of apoptosis in tumor cells could play an essential role in the development of resistance to natural defense as well as cancer chemotherapy. As the molecular mechanism of apoptosis has been characterized more thoroughly, evidence indicates that distinct forms of apoptosis exist. In the present study, we demonstrated that inhibitors including DRB, Act D, and CHX induced apoptosis in cultured human gastric cancer TMK-1 cells. These results indicated that this cell line, along with many other tumor cells such as the human promyelocytic leukemia cell line HL-60 [13], is extremely sensitive to macromolecular synthesis inhibitors. In agreement with the extent of DNA fragmentation and RNA synthesis inhibition (Fig. 3 and Table 1), DRB was less effective than Act D in inducing apoptosis. This is because the mechanisms of action of DRB, Act D, and CHX are different in terms of their targets. DRB is known to selectively inhibit the synthesis of heterogeneous nuclear RNA with much less effect on the synthesis of ribosomal or transfer RNA [16, 17]. Mechanistic studies indicated that DRB is a CTD kinase inhibitor that specifically blocks the phosphorylation of the RNA polymerase II largest subunit [32, 33]. In contrast, Act D is not a protein kinase inhibitor; it arrests transcription by intercalating into template DNA. CHX is involved in a number of effects on cell metabolism and has been shown to block peptidyl transferase activity of the 60S ribosomal subunit. Despite the difference in mechanisms, all of these agents eventually led to the blocking of RNA or protein synthesis, which suggests that they may have similar mechanisms in triggering cell death. It has been suggested that in cells showing apoptosis in response to metabolic inhibitors, the apoptotic machinery is being actively suppressed by short-lived protective proteins that do not require RNA or protein synthesis to undergo apoptosis [13, 34]. It has been indicated that apoptosis is the default cellular option in higher organisms and that a continuous supply of survival signals is required to suppress cell death [35]. Thus, our results in the present study also support the notion that interference with the cellular survival synthesis pathway can induce apoptosis.

The mechanism of the biochemical activity of c-Myc and p53 proteins is possibly associated with their function as DNA binding proteins and transcription modulators in certain circumstances. p53 has been implicated in acting as a cell cycle checkpoint that induces growth arrest in response to DNA damage to allow repair or to trigger apoptosis [26, 27]. The present study indicated that, similar to the effect of DNA-damaging agents, the macromolecular synthesis inhibitors can up-regulate the p53 protein level. Since the

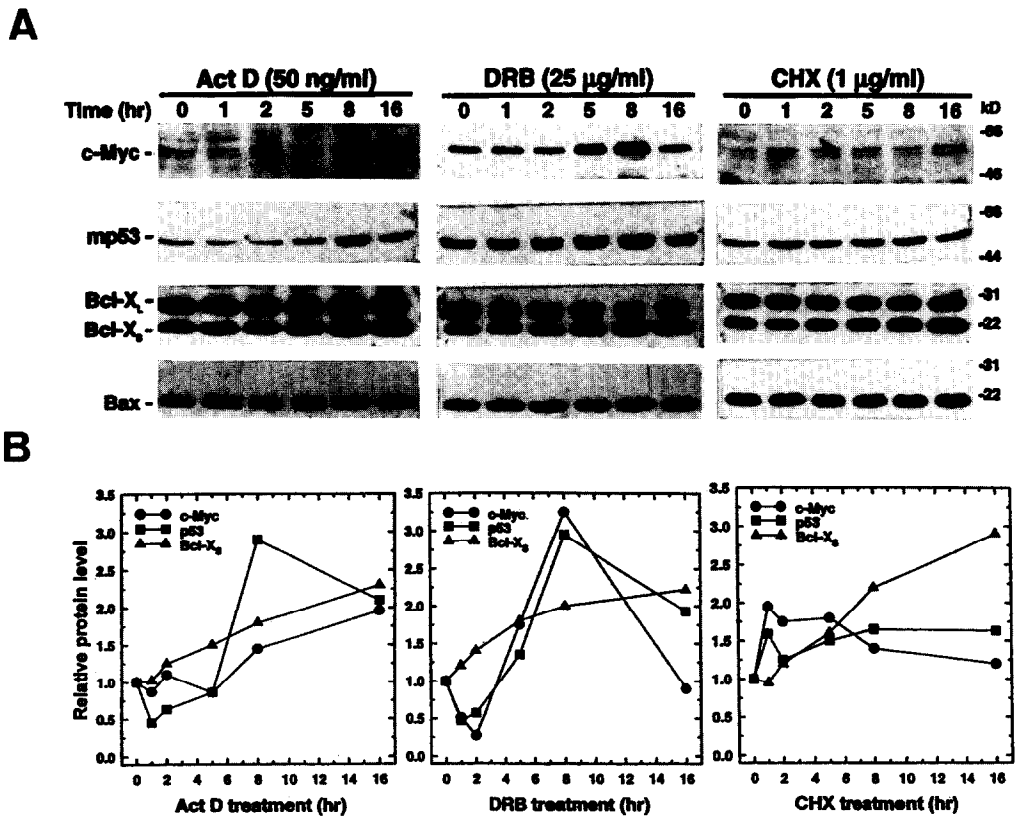

FIG. 4. Effects of Act D, DRB, and CHX on c-Myc, mp53, Bcl-X_L and Bcl-X_S expression in TMK-1 cells. (A) Whole cell extract (50 μg) of 78 μM DRB-treated TMK-1 cells from indicated time points were analyzed by immunoblotting for c-Myc, mp53, Bcl-X_L, Bcl-X_S, and Bax expressing as described in Materials and Methods. The immunoblots shown in (A) represent the typical result from 2–4 independent experiments. (B) The relative amounts of c-Myc, mp53, and Bcl-X_S proteins were determined from the immunoblots shown in (A). A Molecular Dynamics computing laser densitometer was used to quantify the immunoblot results. The results were the averages from two independent experiments.

p53 gene in TMK-1 cells is a mutated form, it is unlikely that the apoptosis induced by these inhibitors is *p53* dependent [36, 37]. The relationship between alterations in c-Myc expression and apoptosis has been demonstrated in many studies. However, previous studies also indicated that c-*myc* and *p53* are not indispensable for the induction of apoptosis, and the cellular level of c-Myc and p53 proteins may vary significantly in different cells [38]. In this study, the increase in c-Myc levels is associated with DNA fragmentation and apoptosis induced by these inhibitors. The effect of DRB on the c-Myc level is most significant since an identical pattern of induction was shown for c-myc mRNA (Fig. 5). Thus, the mechanism of the induction of c-*myc* expression by the CTD kinase inhibitor, DRB, is distinct from that of Act D or CHX. Associated with the increase in c-Myc and mp53 was the rapid up-regulation of Bcl-X_S by inhibitors used in the present study (Fig. 4). The expression of both Bax and Bcl-X_L was largely unchanged in this study except for the down-regulation of Bcl-X_L in the DRB-treated cells (Fig. 4). Bcl-X_S has been shown recently to be a crucial component in the pathway of apoptosis, which promotes cell apoptosis by antagonizing the protective effect of Bcl-2 and Bcl-X_L [9, 10]. In addition to the up-regulation of Bcl-X_S, we found a fairly strong constitutive level of this apoptotic-promoting variant of Bcl-X in this cell line (Fig. 4). Since most of the tumor cells studied express little or no detectable levels of Bcl-X_S [28, 29], the high basal level of this protein in TMK-1 cells may correlate with the low threshold of the cells to apoptosis caused by these inhibitors. In this study, although the expression of Bax, Bcl-X_L, and Bcl-X_S could be detected in this cell line, we were unable to detect the expression of endogenous bcl-2 at either the protein or mRNA level (data not shown). Taken together, our study demonstrated that apoptosis of TMK-1 cells triggered by transcription and translation inhibitors is perhaps mainly mediated by up-regulation of Bcl-X_S.

In summary, we demonstrated that the transcription and translation inhibitors Act D, DRB, and CHX induce apoptosis in TMK-1 cells. Our study also serves to reinforce the emerging concept that interference with the cellular survival pathway can induce apoptosis. In addition, we showed that the process of apoptosis is accompanied by increased c-Myc, mp53 and Bcl-X_S protein levels. The c-*myc*, *mp53* and *bcl-X* genes may be involved as the molecular targets of apoptosis triggered by these inhibitors, although the direct

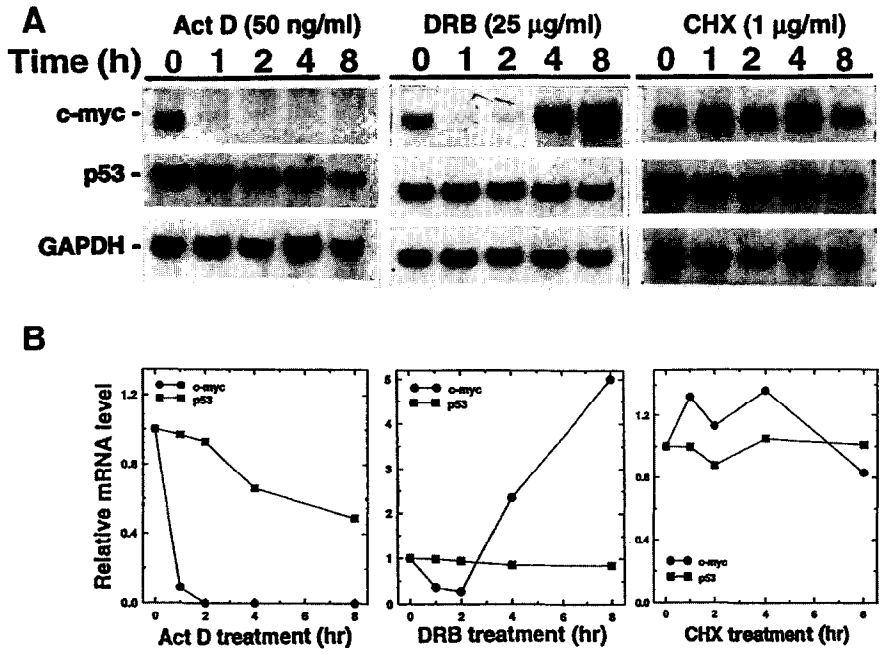

FIG. 5. Effects of Act D, DRB, and CHX on c-myc and mp53 mRNA levels. TMK-1 cells were treated with Act D, DRB, or CHX for the time indicated. (A) Total RNA (30 μg) from each sample was prepared, fractionated, transblotted, hybridized with the ^{32}P-labeled human DNA probes for *c-myc*, *mp53* and GAPDH, and autoradiographed as described in Methods and Materials. (B) Densitometric quantitation of the c-myc and mp53 mRNA levels in cells. Values for the mRNA levels were normalized with respect to GAPDH mRNA. Results are expressed as the amount relative to initial mRNA levels.

correlation awaits to be proven in this cell. Further investigations are required to clarify the possible roles of Bcl-X in controlling the cell apoptosis triggered by transcription and translation inhibitors in this and other cell lines.

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