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Transcriptional Regulation of TNF Family Receptors and Bcl-2 Family by Chemotherapeutic Agents in Murine CT26 Cells

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Abstract Various chemotherapeutic agents have been shown to sensitize cancer cells to members of the tumor necrosis factor (TNF) family. However, it is unclear whether sensitization by chemotherapeutic agents involves the transcriptional regulation of apoptosis-related genes. In this study, we investigated mRNA regulation of TNF family receptors and Bcl-2 family members after treating the murine colon cancer cell line, CT26, with various apoptosis inducers. We found that treatment with cycloheximide, a protein synthesis inhibitor, remarkably increased CD40 mRNA levels by semi-quantitative RT-PCR. Other protein synthesis inhibitors, such as anisomycin and emetine, also enhanced CD40 mRNA expression, which was significantly blocked by a NF-κB antagonist and a p38 MAP kinase antagonist. After treatment with cycloheximide, and further cultivation in fresh medium, CD40 protein levels were found to increase by flow cytometry. Additionally, we found that cycloheximide treatment appeared to downregulate the Bcl-xL mRNA level but not the Bax mRNA level by RNase protection assay. Because the upregulation of CD40 mRNA and the downregulation of Bcl-xL correlated with CT26 cell death, our results suggest that chemotherapeutic agents, including cycloheximide, may exert their synergistic effects on the TNF family treatment of cancer cells by regulating the mRNA levels of apoptosis-related genes. J. Cell. Biochem. 91: 410–422, 2004.

Key words: chemotherapy; apoptosis; TNF; cycloheximide; CD40; Bcl-xL; Bax; CT26

Apoptosis can be induced by various stimuli, such as, chemotherapeutic agents and death ligands that belong to the tumor necrosis factor (TNF) family [Herr and Debatin, 2001; Sartorius et al., 2001].

Recently, apoptosis induction through the death receptor was suggested as a new approach to cancer therapy [Fisher, 1994; Fung and Fisher, 1995]. TNF-α and Fas ligand (FasL), which are efficient at killing a variety of cancer cells, were the first TNF family members to be tested for their anti-cancer activity, although they do cause some damage to normal tissues [Galle et al., 1995; Havell et al., 1998]. Two other molecules, CD40 ligand (CD40L) and TNF-related apoptosis-inducing ligand (TRAIL) also induce apoptosis in cancer cells and have minimal toxicity to normal tissues [Hirano et al., 1999; Walczak et al., 1999; Jo et al., 2000; Ghamande et al., 2001; Vonderheide et al., 2001]. However, there is still remaining problem that many cancer cells are resistant to CD40L- or TRAIL-mediated apoptosis.

To enhance host safety and to sensitize cancer cells to death ligands, the efficacy of the TNF family ligands has been examined in combination with various chemotherapeutic drugs [Eliopoulos et al., 2000; Ghamande et al., 2001; Jazirehi et al., 2001; Mizutani et al., 2002; Munshi et al., 2002]. For example, the anti-cancer effect of CD40 ligand was synergistically increased by subtoxic treatments with cisplatin, an anti-cancer drug or with cycloheximide (CHX), a cytotoxic agent to ovarian carcinoma cells [Eliopoulos et al., 2000; Ghamande et al., 2001]. The effect of TRAIL

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was also found to be enhanced by various chemotherapeutic agents such as cisplatin, etoposide, and adriamycin in prostate cancer cells [Munshi et al., 2002] and by adriamycin in human multiple myeloma cells [Jazirehi et al., 2001]. These synergistic effects suggest that combination therapies of TNF family ligands and chemotherapeutic agents may have potential applications in cancer therapy.

Although numerous studies have investigated the chemotherapy-induced apoptosis of cancer cells, the molecular events concerned and the synergistic effects of chemotherapeutic agents on the anti-cancer activity induced by the TNF family ligands are poorly understood. Some studies have demonstrated that several chemotherapeutic agents can alter the expressions of apoptosis-related molecules at the both transcription and translation levels [Muller et al., 1997; Higami et al., 2000; LeBlanc et al., 2002]. In human hepatoma HepG2 cells, anticancer drugs, such as bleomycin, cisplatin, and methotrexate, were found to induce apoptosis by upregulating Fas receptor [Muller et al., 1997]. Another cytotoxic agent, CHX, induced apoptosis and upregulated the expression of p53 and Fas receptor in the rat liver in vivo [Higami et al., 2000]. In addition, etoposide and camptothecin also sensitized Bax-deficient human colon carcinoma cells to TRAIL by upregulating the expression of DR5, a TRAIL receptor, and Bak, a pro-apoptotic Bcl-2 family member, in vitro and in vivo [LeBlanc et al., 2002]. Therefore, it became evident that an investigation into altered apoptosis-related gene expressions by chemotherapeutic agents was warranted.

In this study, we examined whether chemotherapeutic agents can modulate the mRNA expression of apoptosis-related genes, including the TNF family receptors and the Bcl-2 family members, in mouse colon carcinoma CT26 and breast carcinoma TA3HA cells. We found that CHX treatment significantly upregulated CD40 mRNA expression in both cell-lines, but appeared to downregulate Bcl-xL, without altering the Bax mRNA level, in a dose- and time-dependent manner. Because the upregulation of CD40 mRNA and the downregulation of Bcl-xL mRNA have been previously correlated with cancer cell death, our data suggest that the synergistic effect of CHX treatments in combination therapy with TNF family ligands is associated with the altered mRNA levels of apoptosis-related genes, such as CD40 and Bcl-xL.

MATERIALS AND METHODS

Cell Culture

CT26 cells were maintained in DMEM medium (Life Technologies, Inc., Gaithersburg, MD), supplemented with 10% FBS, L-glutamine, glucose, pyridoxin hydrochloride, 110 mg/ml sodium pyruvate, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B. TA3HA were maintained in RPMI 1640 medium (Life Technologies, Inc.), supplemented with 10% FBS, L-glutamine, 25 mM HEPES buffer, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B. Cells were cultured in a 5% CO_2 incubator at 37°C.

Chemotherapeutic Agents and Chemicals

Camptothecin, dexamethasone, cycloheximide, and etoposide were purchased from BioVision, CA; cisplatin, staurosporine, and actinomycin D from Sigma (St. Louis, MO); and the protein synthesis inhibitors, anisomycin, and emetine from Calbiochem (San Diego, CA). To examine the effects of chemotherapeutic agents on the cancer cells, CT26 and TA3HA cell-lines were exposed to culture medium containing either camptothecin (2 μM), cisplatin (100 μM), dexamethasone (10 μM), cycloheximide (10 μg/ml), staurosporine (1 μM), actinomycin D (10 μg/ml), or etoposide (10 μM) for 24 h. In terms of protein synthesis inhibitors, cells were treated with anisomycin (10 μg/ml) or emetine (20 μg/ml) for 24 h. The involvements of the NF-κB signaling pathway and of p38 MAP (mitogen-activated protein) kinase were examined by pretreating with 1 μM SN50 (Calbiochem), a NF-κB inhibitor, or 1 μM CFPD (2-(4-chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1, 2-dihydropyrazol-3-one, Calbiochem), a p38 MAP kinase inhibitor, for 1 h followed by adding with chemotherapeutic agents.

Total RNA Preparation and RT-PCR Assay

Total RNA was isolated from the lysates of murine cancer cell lines (CT26, TA3HA) using Tri-reagent (Sigma). About 1 μg of total RNA was used in a 20 μl cDNA synthesizing reaction using oligo$(dT)_{18}$ primer and Maloney-murine leukemia virus (MMLV) reverse transcriptase

(Promega, WI). One microgram of the cDNA mixture was used for PCR amplification. For the amplification of CD40, RT-PCR was conducted with CD40 specific primers 5′- AAG CTT ATG GTG TCT TTG CCT CGG CTG T-3′ (mCD40-F) and 5′-GAT ATC GAC CAG GGG CCT CAA GGC TAT (mCD40-R). To amplify Fas, we used the Fas specific primers 5′-GAA TTC ATG CTG TGG ATC TGG GCT GTC C-3′ (mFas-F) and 5′-AAG CTT TCA CTC CAG ACA TTG TCC TTC ATT-3′ (mFas-R). TNF receptor (TNFR)1 was amplified by TNFR1 specific primers 5′-GAA TTC ATG GGT CTC CCC ACC GTG CC-3′ (mTNFR1-F) and 5′-AAG CTT TCA GGC CAC TTT GAC TGC AAT CT-3′ (mTNFR1-R); and TNFR2 with the specific primers 5′-GAA TTC ATG GCG CCC GCC GCC CTC T-3′ (mTNFR2-F) and 5′-AAG CTT TCA GGC CAC TTT GAC TGC AAT CT-3′ (mTNFR2-R). These primers produced 882, 996, 1,377, and 1,437 bp PCR products for CD40, Fas, TNFR1, and TNFR2, respectively. As a quantitative control, mouse β-actin cDNA was amplified to determine the integrity of the RNA and the efficiency of the cDNA synthesis, using the primers 5′-GGC TAC AGC TTC ACC ACC ACA G-3′ (mβ-actin F) and 5′-GGT GCT AGG AGC CAG AGC AGT A-3′ (mβ-actin R). A RT-PCR negative control without cDNA was included in all experiments. Ethidium bromide-stained agarose gels were scanned using a gel documentation system 1000 (BioRad, Hercules, CA) and band intensity was analyzed using Sigma Gel 1.0 (Sigma).

Preparation of Probes and RNase Protection Assay (RPA)

The expression of Bcl-2 family mRNA was determined by RNase protection assay using mouse Bcl-2 family multi-probe template sets (mAPO-2; Pharmingen, San Diego, CA) according to the manufacturer's instruction. Briefly, each sample was washed twice with PBS to remove medium protein. Total RNA from cells was extracted using Tri-reagent (Sigma). Ten microgram RNA samples were hybridized with ^{32}P-labeled antisense mRNA probes against Bcl-w, Bcl-xL, Bak, Bax, Bad, L32, and GAPDH, and digested with RNase and T1 nuclease. The final protected hybridized probe fragments were resolved on 5% TBE urea polyacrylamide gels and the radioactivity in fixed, dried gels were detected by phosphorimager analysis (FUJI-BAS 1000, Tokyo, Japan). The relative mRNA levels were determined by normalizing band intensities of Bcl-w, Bcl-xL, Bak, Bax, and Bad with those of the L32 and GAPDH probes.

Flow Cytometry Analysis

After treating with 10 μg/ml of CHX for indicated times, CT26 cells were harvested, washed twice with PBS, and then incubated for 30 min at 4°C with either a control anti-mouse IgG (H+L)-FITC (Pharmingen) or rat anti-mouse CD40-PE (Pharmingen). After washing in PBS containing 0.1% BSA, cells were analyzed on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA). A minimum of 5,000 cells was collected for each sample.

Assay of Cell Viability

Cells viability was determined by Trypan Blue dye exclusion assay. All the assays were performed in triplicate and results are indicated as mean ± the standard deviation (SD).

Western Blot Analysis

Monolayer cells were washed twice with PBS and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) in the presence of protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany). After 30-min incubation on ice, the lysates were cleared by centrifugation at 15,000 rpm for 10 min. The proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked with TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat milk and followed by standard immunoblot procedure. The membranes were incubated either with anti-Bcl-xL antibody (Santa Cruz, Inc.), anti-Bax antibody (Santa Cruz, Inc.), anti-β-actin antibody (Sigma), or anti-active p38 antibodies (Promega), and then washed with TBST, followed by incubation with HRP-conjugated goat anti-rabbit IgG or sheep anti-mouse IgG secondary antibodies (Amersharm Bioscience).

RESULTS

Effect of Various Apoptosis Inducers on the mRNA Expression of TNF Family Receptors in Mouse CT26 or TA3HA Cancer Cells

To examine the effect of apoptosis inducers on the mRNA expression of the TNF family

receptors, mouse colon CT26 cells and mouse breast TA3HA cells were treated with various apoptosis inducers, including camptothecin, dexamethasone, cisplatin, cycloheximide (CHX), staurosporine, actinomycin D, and etoposide. Total RNA was collected at 24 h after treatment and RT-PCR was performed using the primers specific for CD40, Fas, TNFR1, and TNFR2. The effects of apoptosis inducers on the mRNA expressions of the TNF family receptors were variable, and depended upon cell types and the apoptosis inducers used (Fig. 1). In CT26 cells, some of the inducers downregulated the mRNA expressions of TNFR1 and TNFR2, whereas CHX upregulated the mRNA expressions of CD40 and Fas. In particular, CHX treatment markedly increased CD40 mRNA expression (Fig. 1). In the case of the TA3HA cell-line, Fas mRNA was significantly upregulated by treatment with dexamethasone and CD40 mRNA was modestly induced by treatment with CHX. However, some inducers downregulated the mRNA expressions of Fas and TNFR1 (Fig. 1).

To investigate this CHX-induced CD40 expression, we examined various human cancer cells established from the stomach, colon, lung, liver, and blood. By semi-quantitative RT-PCR, we observed the induction of CD40 mRNA in 27 human cancer cell lines after treatment with CHX (Table I). CD40 mRNA was significantly increased (more than threefold increases) in 25 out of 27 cell lines. Thus, these results indicate that the CHX can upregulate CD40 mRNA

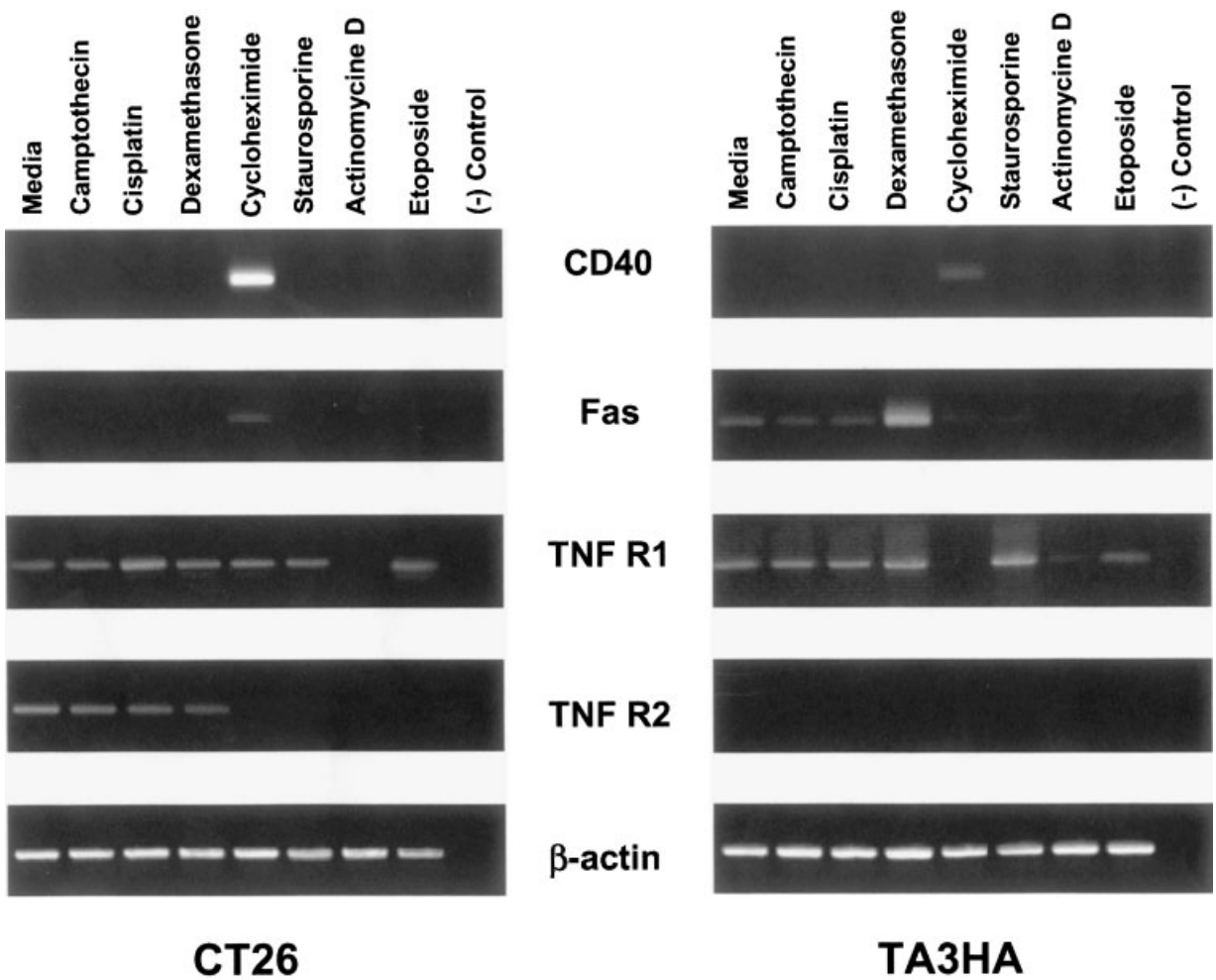

Fig. 1. Effect of chemotherapeutic agents on the mRNA expressions of TNF family receptors in CT26 and TA3HA mouse cancer cells, assayed by RT-PCR. Cells were treated with camptothecin (2 μM), cisplatin (100 μM), dexamethasone (10 μM), cycloheximide (10 μg/ml), staurosporine (1 μM), actinomycin D (10 μg/ml), or etoposide (10 μM) for 24 h and total RNA was isolated. The products of the PCR reaction, after 30 cycles, are shown. Similar results were obtained in three independent experiments.

TABLE I. CD40 Exression Levels in Various Human Cancer Cell Lines Treated With CHX

Cell line	Origin	CD40 mRNA expression[a] −CHX	CD40 mRNA expression[a] +CHX
SNU638	Stomach, adenocarcinoma	−	+ (3.2)
KATO III	Stomach, carcinoma	−	−
SNU5	Stomach, adenocarcinoma	+	+ (7.1)
SNU719	Stomach, adenocarcinoma	−	+ (13.2)
SNU484	Stomach, adenocarcinoma	+	+ (11.3)
Jurkat	Acute T-cell leukemia	−	+ (3.2)
CCRF-CEM	T-cell leukemia	−	+ (3.5)
Molt4	T-cell leukemia	+	+ (14.4)
CEM-CM3	T-cell leukemia	−	−
CCRF-HSB II	T-cell leukemia	−	−
Ramos	B-cell, Burkitt's lymphoma	++	+ (3.4)
Jijoye	B-cell, Burkitt's lymphoma	++	+ (3.7)
SW480	Colon, adenocarcinoma	+	+ (3.6)
DLD-1	Colon, adenocarcinoma	−	−
SNU C4	Colon, adenocarcinoma	+	− (3.2)
SNU8	Ovary, adenocarcinoma	+	+ (3.3)
SK-OV-3	Ovary, adenocarcinoma	+	+ (27.2)
SNU387	Liver, carcinoma	−	+ (4.2)
SNU449	Liver, hepatocellular carcinoma	+	+ (6.3)
SNU423	Liver, hepatocellular carcinoma	+	+ (14.8)
MCF-7	Breast, adenocarcinoma	−	+ (3.1)
MDA-MB-231	Breast, adenocarcinoma	++	− (4.7)
NCI-H358	Nonsmall lung cancer cell	+	+ (3.8)
H29	Lung, carcinoma	−	+ (2.2)
293	Embryonic kidney	+	+ (3.4)
ME180	Cervix, carcinoma	+	+ (3.3)
Capan	Pancreas, adenocarcinoma	+	+ (3.7)

[a]CHX-induced CD40 mRNA level changes were assessed in various human cancer cell lines. The cells were treated with CHX (10 μg/ml) for 24 h and total RNA was isolated and analyzed by semi-quantitative RT-PCR. The PCR products, after 28 cycles of amplification, were separated on 2% agarose gel. Relative increases in the levels of CD40 mRNA were analyzed by measuring the intensities of PCR bands visualized by ethidium bromide. Numbers in parentheses indicate folds of increase.

levels although mRNA levels of the TNF family receptors could be regulated differently in response to apoptosis inducers.

CHX and Anisomycin Remarkably Induced CD40 mRNA Expression in CT26 Cells via NF-κB Activation and p38 MAP Kinase Pathway

Because CHX, a potent protein synthesis inhibitor, highly induced CD40 mRNA expression in CT26 cells, we further investigated the phenomenon of CHX-induced CD40 mRNA expression.

The effect of CHX in CT26 cells was examined by semi-quantitative RT-PCR. Although the specific PCR product of CD40 cDNA increased according to the number of amplification cycles, the amount of the PCR product was linearly proportional to the number of PCR cycles only over a certain range (from 22 to 30 cycles). Thus, 28 cycles of amplification were used for the quantification of mouse CD40 mRNA. At this level, we were able to compare at least 30∼80-fold differences by densitometric quantification of the PCR products as visualized by ethidium bromide.

As shown in Figure 2, CHX effects on CD40 mRNA induction were both time- and dose-dependent in CT26 cells. Treatment with CHX (10 μg/ml) resulted in a clear accumulation of CD40 mRNA, which started at 6 h, peaked at 24 h, and then decreased up to 72 h (Fig. 2A). At 24 h after CHX treatment, CD40 mRNA expression was observed at CHX concentrations as low as 1.0 μg/ml, and was strongly induced 35- to 80-fold by high concentrations of CHX (10∼30 μg/ml) (Fig. 2B).

Several protein synthesis inhibitors, such as anisomycin and emetine as well as CHX, have been shown to enhance mRNA expression in response to various stimuli [Lau and Nathans, 1987; Edwards and Mahadevan, 1992; Ma et al., 2000]. We further investigated whether anisomycin and emetine could induce CD40 mRNA expression. As shown in Figure 3A, anisomycin and emetine did induce CD40 mRNA expression, although their effects were weaker than that of CHX. Therefore, these results suggest that a common signaling pathway triggered by the protein synthesis inhibitors might be involved in the CD40 mRNA induction.

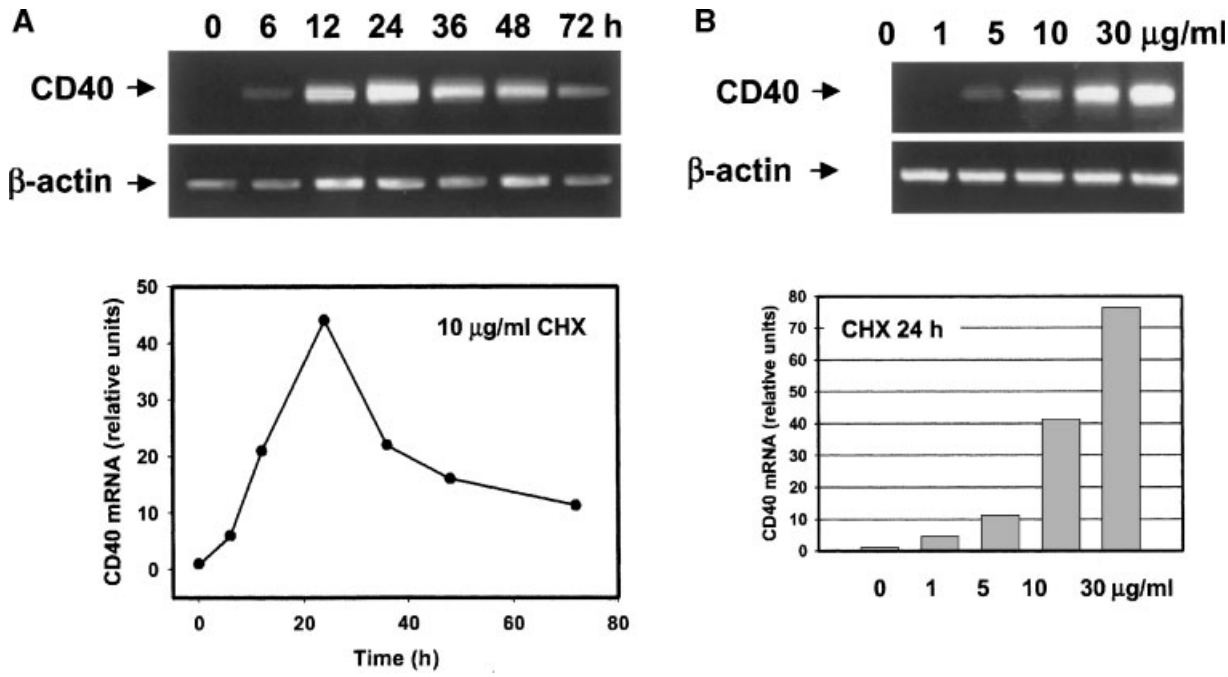

Fig. 2. Effects of cycloheximide on the expressions of CD40 mRNA in CT26 cells. The cells were treated with 10 μg/ml cycloheximide for the indicated times (**A**), and treated with the indicated doses of cycloheximide for 24 h (**B**). Total RNA was isolated from the cells and analyzed by semi-quantitative RT-PCR. The PCR products, after 28 cycles of amplification, were separated on a 2% agarose gel. Relative increases in the levels of CD40 mRNA were analyzed by measuring the intensities of PCR bands visualized by ethidium bromide.

Previously, it was demonstrated that protein synthesis inhibitors can superinduce the mRNA expressions of several genes by activating NF-κB [Newton et al., 1996; Krzesz et al., 1999] and that anisomycin is a potent activator of the p38 MAP kinase pathway [Zinck et al., 1995; Hazzalin et al., 1998]. So, we used a NF-κB inhibitor, SN-50, and a p38 MAP kinase inhibitor, CFPD, to examine whether NF-κB and the p38 MAP kinase pathway are involved in CHX-induced CD40 mRNA expression. CT26 cells were pretreated with either SN-50 (1.0 μM) or CFPD (1.0 μM) for 1 h, and CHX or anisomycin was added. As shown in Figure 3B, the effects of CHX or anisomycin on CD40 mRNA induction were significantly blocked by pretreating with SN50 or CFPD. We confirmed the effect of SN50 on NF-κB inhibition in CT26 cells (data not shown). To ascertain that CHX activates p38 in CT26 cells, we examined p38 activation by immunoblotting using anti-active p38 antibodies that recognize phosphorylated form of p38. As shown in Figure 3C, CHX treatment induced p38 activation and this activation was inhibited by CFPD. Therefore, these results showed that CD40 mRNA expression by protein synthesis inhibitors might be related to both NF-κB activation and the p38 MAP kinase pathway.

Since it is known that CHX is a protein synthesis inhibitor, we examined whether the CHX-induced CD40 mRNA could be translated to protein. CD40 expression in CHX-treated CT26 cells was determined by flow cytometry analysis using mouse CD40 monoclonal antibody. As shown in Figure 4A, the enhancement of the expression of CD40 mRNA did not result in an increase in its protein product in the presence of CHX (10 μg/ml) up to 24 h after treatment. However, CD40 protein as well as CD40 mRNA was significantly detected from CT26 cells that were further incubated in fresh medium without CHX for 24 h following 24 h pretreatment with CHX (Fig. 4B,C).

Effect of Various Apoptosis Inducers on mRNA Expression of the Bcl-2 Family Members in Mouse CT26 Cells

Some available evidence suggests that apoptosis inducers sensitize cancer cells to TNF ligands by altering the expressions of the Bcl-2 family members [Gibson et al., 1999; Zhang et al., 1999; LeBlanc et al., 2002]. In order to investigate the effects of several apoptotic

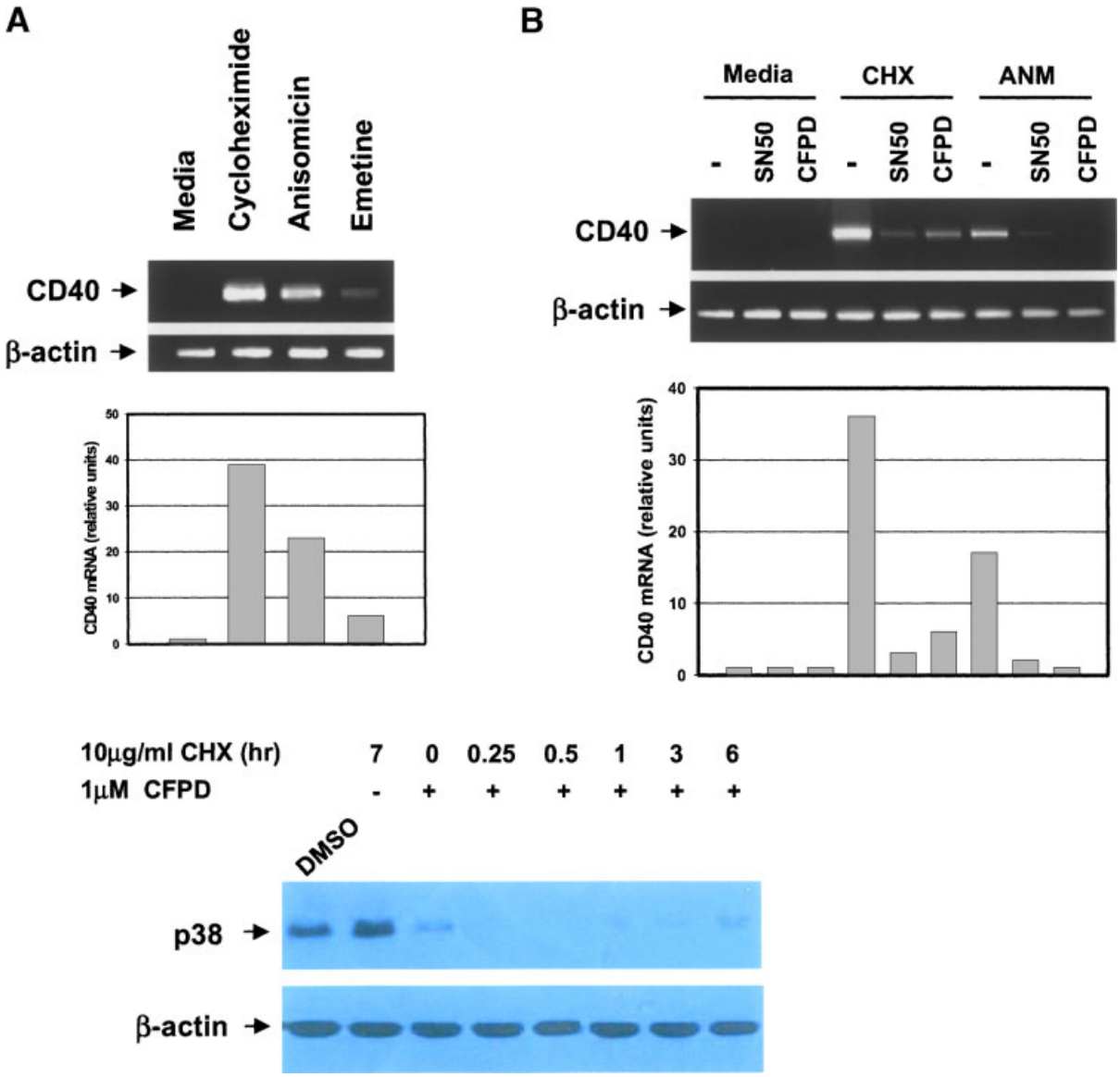

Fig. 3. Effects of protein synthesis inhibitors on the expression of CD40 mRNA in CT26 cells. **A**: The cells were treated for 24 h with cycloheximide (10 μg/ml; 35.5 μM), anisomycin (10 μg/ml; 37.7 μM), or emetine (20 μg/ml; 36.1 μM), or with media alone. **B**: Cells were pretreated for 1 h with an NF-κB inhibitor, SN-50 (1 μM), or a p38 MAP kinase inhibitor, CFPD (1 μM), and then incubated for 25 h with cycloheximide (10 μg/ml) or anisomycin (10 μg/ml). **C**: CT26 cells were pretreated with a p38 MAP kinase inhibitor, CFPD (1 (M), for 1 h, and then incubated with cycloheximide (10 (g/ml) for indicated times. Cells were lysed and total cellular proteins (40 μg) were resolved by SDS–PAGE and subjected to immunoblot analysis using anti-active-p38 or anti-β-actin antibodies. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

inducers on the mRNA levels of the Bcl-2 family members, we performed an RNase protection assay (RPA) in mouse CT26 cells. As shown in Figure 5A, CT26 cells constitutively expresses anti-apoptotic Bcl-xL, pro-apoptotic Bax, and Bak at high levels. Under normal conditions, the relative amount of Bax mRNA to Bcl-xL mRNA was about 1.2 by densitometric radioactivity determinations. However, at 24 h after treatment with CHX (10 μg/ml), the expression level of Bcl-xL mRNA was significantly reduced relative to that of Bax mRNA; the relative amount of Bax mRNA to Bcl-xL mRNA was about 3.3 (Fig. 5A). On the other hand, the treatment of CT26 cells with dexamethasone slightly upregulated Bcl-xL mRNA expression. Cisplatin or camptothectin did not have any effect on the mRNA expression of the Bcl-2 family, and staurosporin, etoposide, or actinomycin caused a nonspecific overall degradation

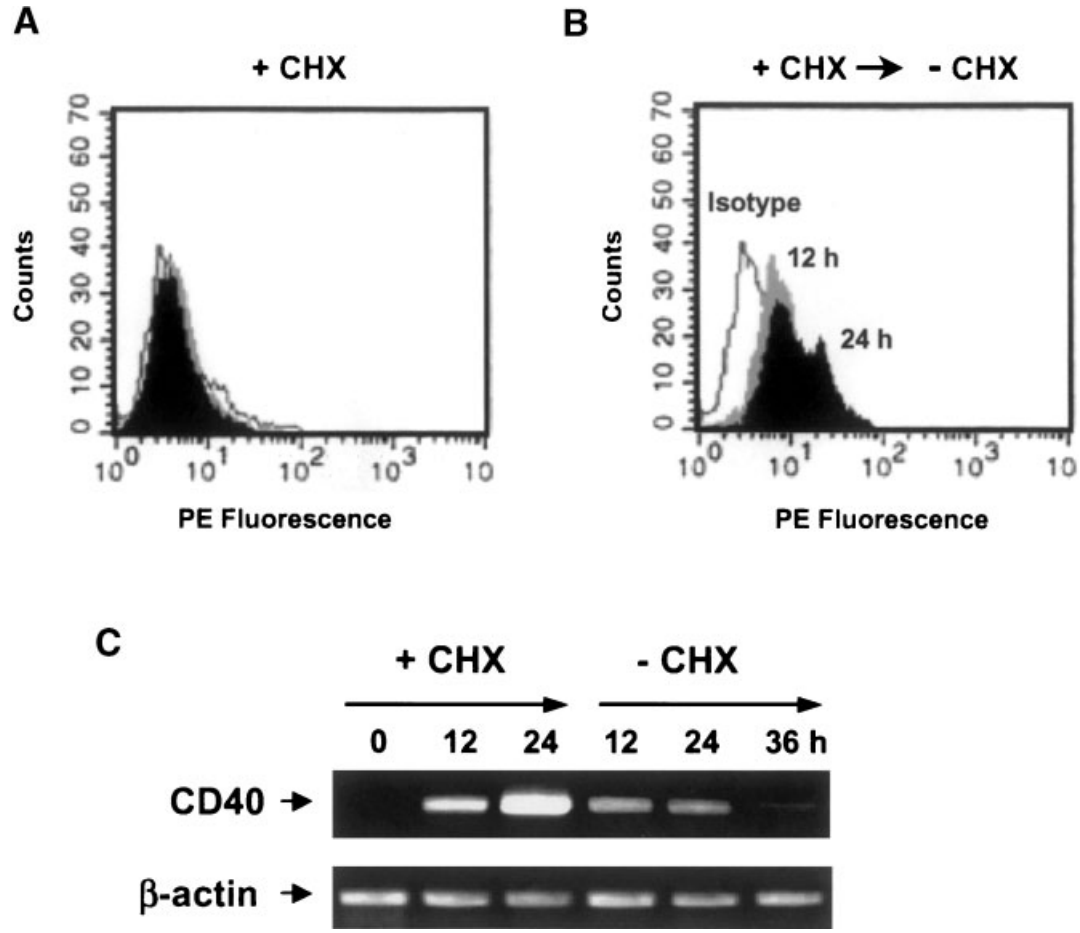

Fig. 4. Effects of cycloheximide on the expression of CD40 protein in mouse CT26 cells. **A**: CT26 cells were treated with 10 μg/ml CHX for 24 h. The expression levels of CD40 protein were determined using anti-CD40 mAb followed by anti-mouse IgG-FITC in conjunction with flow cytometry at 12 and 24 h after treatment. Isotype control (open area), cells treated with CHX for 12 h (gray area), and cells treated with CHX for 24 h (black area) are shown. **B**: CT26 cells were treated with 10 μg/ml CHX for 24 h and then further cultured in fresh medium without CHX. At 12 and 24 h after changing the medium, the expression of CD40 protein was determined by flow cytometry. Isotype control (open area), cells cultured for 12 h after medium change (gray area), and cells cultured for 24 h after medium change (black area) are shown. **C**: CD40 mRNA expression was analyzed by RT-PCR. Total RNA was isolated from cells treated with CHX (+CHX) and from cells cultured in fresh medium without CHX (−CHX) at the indicated times 24 h after pretreatment with CHX.

of mRNAs including L32 and GAPDH, which were used as control genes (data not shown). These results were reproduced in three independent experiments. We further investigated the effect of CHX treatment on Bcl-xL and Bax mRNA expressions, and on CT26 cell death. After CT26 cells had been treated with 0.1, 1, 5, and 10 μg/ml of CHX for 24 h, total RNA was harvested and analyzed by RNase protection assay. As shown in Figure 5B, Bcl-xL mRNA was specifically decreased by CHX treatment in a dose-dependent manner. However, Bax mRNA was not significantly affected by CHX treatment, and as a result, the relative ratio of Bax mRNA to Bcl-xL mRNA increased to 4.2 by densitometry radioactivity analysis (Fig. 5B).

Correlation of Specific Downregulation of Bcl-xL mRNA Expression With CT26 Cell Death by CHX

Twenty-four hours after treatment with CHX (10 μg/ml), no cytotoxic effect was observed on CT26 cells. However, further incubation of the cells in CHX-containing media caused a rapid apoptosis 36 h after treatment. Since the balance of the expressions of Bax and Bcl-xL is known to be a critical index for the occurrence of apoptosis, we examined whether the decrease in the mRNA expression of Bcl-xL correlates with CT26 cell death. To measure the cell death, we performed Trypan Blue dye exclusion assay and simultaneously performed RPA in CHX-treated

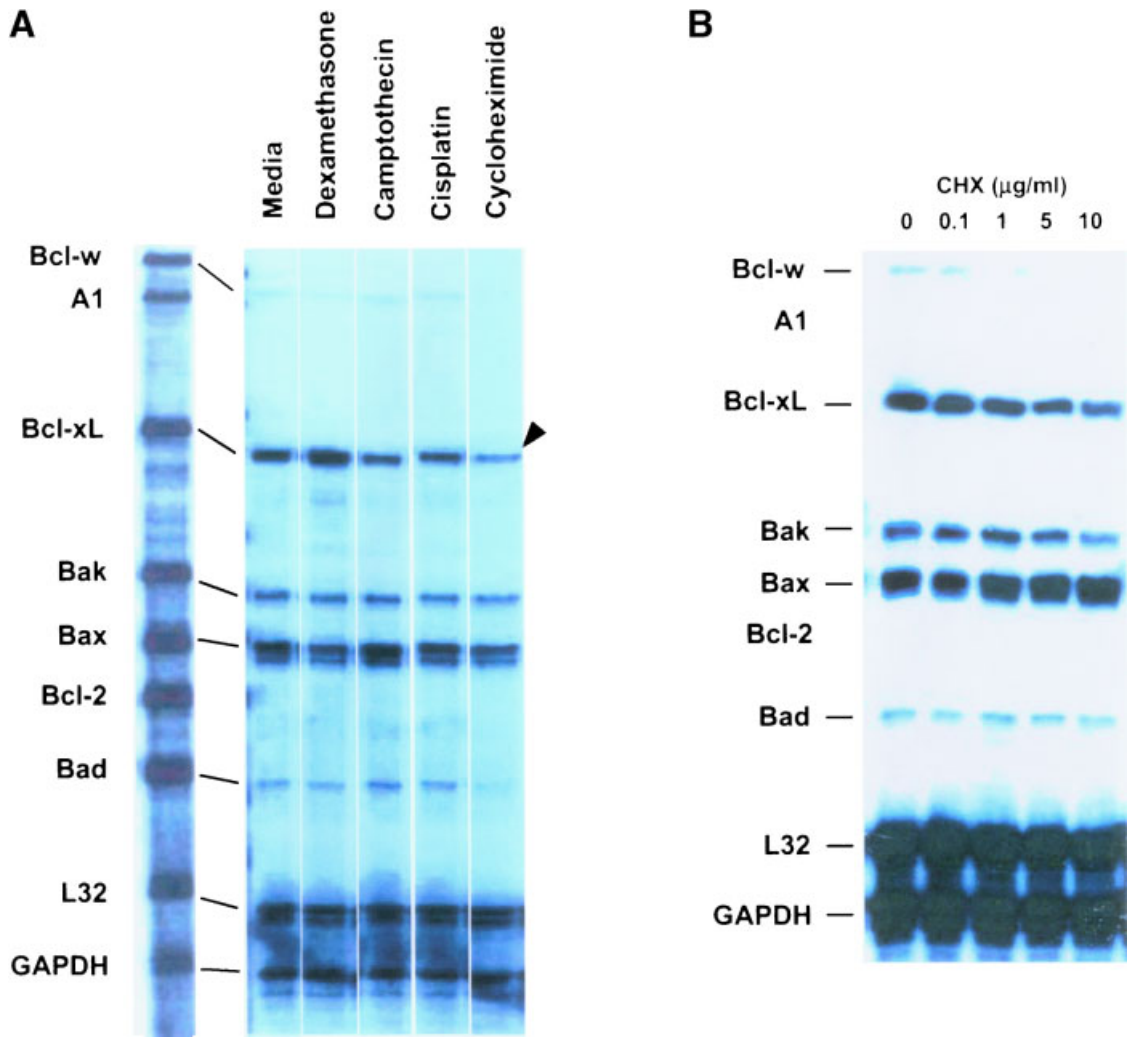

Fig. 5. Effects of chemotherapeutic agents on the mRNA expression of the Bcl-2 family in CT26 cells. **A**: Cells were treated for 24 h with camptothecin (2 μM), cisplatin (100 μM), dexamethasone (10 μM), or cycloheximide (10 μg/ml), or with media alone. **B**: Cells were treated with the indicated doses of cycloheximide for 24 h. Total RNA was extracted from the cells, and 10 μg of RNA was analyzed by using an RNase protection assay with multiple RNA probes against the Bcl-2 family genes. The obtained bands were quantitated with a phosphoimager system (FUJI-BAS 1000). Similar results were obtained in three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CT26 cells. Interestingly, we found that there was a correlation between an increased ratio of Bax mRNA to CT26 cell death versus time after CHX treatment (Fig. 6A,B). Although both Bcl-xL mRNA and Bax mRNA expressions were decreased by prolonged treatment with CHX, the relative ratio of Bax mRNA to Bcl-xL mRNA was significantly increased up to 72 h after CHX treatment. These changes of CHX-induced Bax and Bcl-xL mRNA were correlated with their protein levels as shown in Figure 6C. These results suggest that an apoptosis inducer, CHX, has a potential role for sensitizing cancer cells by modulating the mRNA expression of the Bcl-2 family members.

DISCUSSION

The stimulation of TNF family receptors leads to diverse cellular responses, ranging from proliferation and differentiation to growth suppression and apoptosis [Budd, 2002]. Some of these receptors including TNFR1, Fas, TRAIL-R1, TRAIL-R2, and DR3 share a death domain homology in their cytoplasmic tails, through which they transduce apoptotic signals [Sartorius et al., 2001; Budd, 2002]. Other members of the TNF family receptors, such as TNFR2, CD30, and CD40, despite lacking the death domain in their cytoplasmic regions, have also been reported to suppress growth and

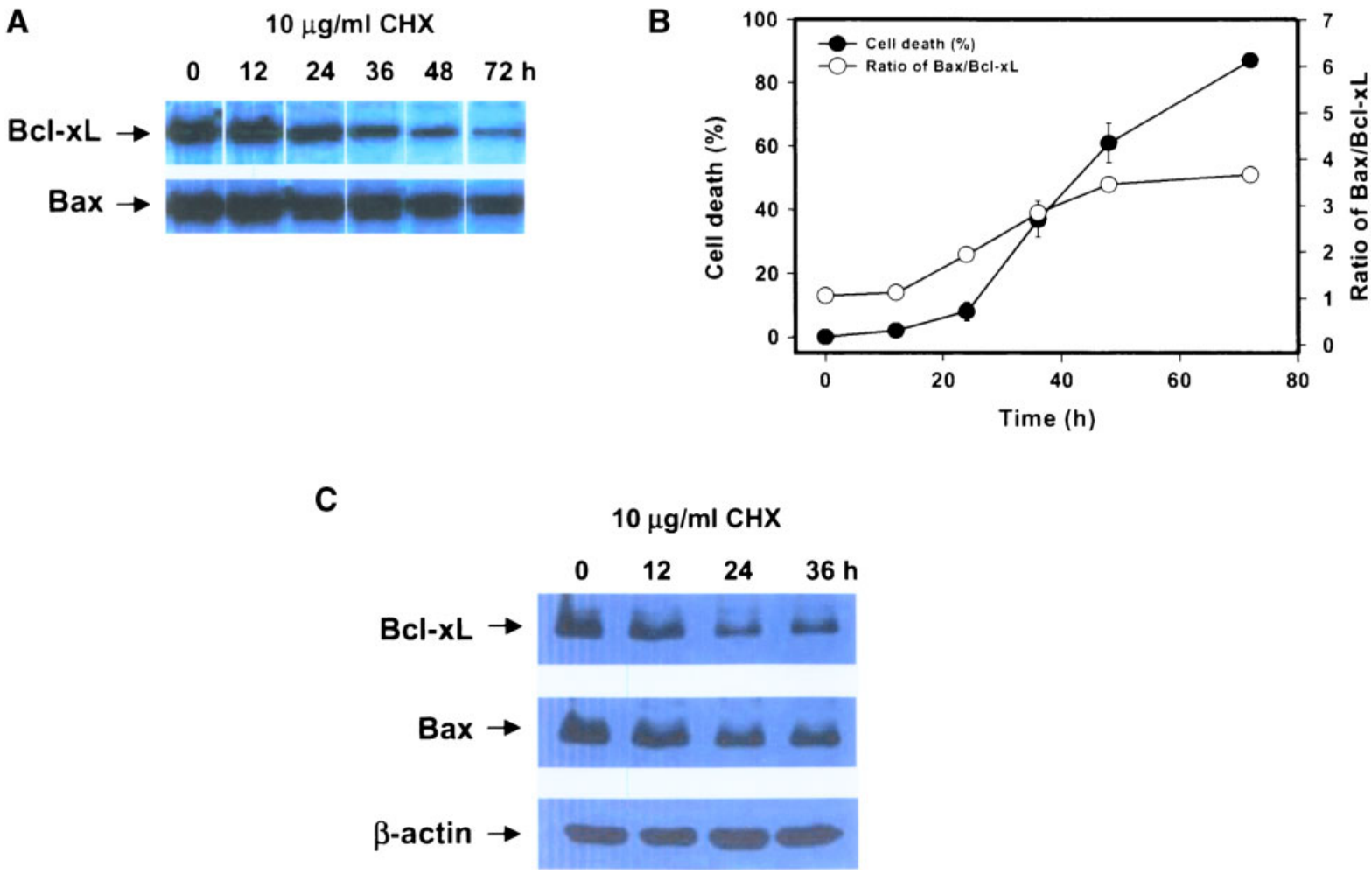

Fig. 6. Correlation between cell death and a decrease in Bcl-xL mRNA/protein levels by treatment with CHX in CT26 cells. **A**: CT26 cells were treated with 10 μg/ml CHX and collected at the indicated time points. mRNA levels of the Bcl-2 family were analyzed by RPA using multiple probes. **B**: Relative degrees of Bax and Bcl-xL mRNA were quantitated on a phosphoimager. The mRNA ratios of Bax/Bxl-xL are shown (right-side Y-axis). CT26 cell death was determined by Trypan Blue exclusion assay (B, left-side Y-axis). The apoptosis assay was performed in triplicate and data are shown as means ± SD. **C**: CT26 cells were treated with 10 μg/ml CHX for 0–36 h, and then total cellular proteins (40 μg) were resolved by SDS–PAGE and subjected to

survival in a number of carcinoma cells [Eliopoulos et al., 1996; Hess and Engelmann, 1996; Grell et al., 1999].

Recent studies have demonstrated that combination treatment with TNF family ligands and chemotherapeutic agents is an effective way of sensitizing cancer cells to apoptosis [Eliopoulos et al., 2000; Ghamande et al., 2001; Jazirehi et al., 2001; Mizutani et al., 2002; Munshi et al., 2002]. To investigate the molecular events underlying the synergistic effects mediated by chemotherapeutic agents, we examined the transcriptional regulation of apoptosis-related genes including the TNFR family and the Bcl-2 family members, after treatment with chemotherapeutic agents in mouse cell-lines.

Interestingly, we found that CHX dramatically increased CD40 mRNA expression in CT26 cells, and that dexamethasone significantly upregulated Fas mRNA in TA3HA cells (Fig. 1). In addition, anisomycin and emetine induced CD40 mRNA expression (Fig. 3A). This CHX-induced CD40 expression was observed in various human cancer cells. In this study, we examined the induction of CD40 mRNA in 27 human cancer cell lines established from the stomach, colon, lung, liver, and blood after treatment with CHX by semi-quantitative RT-PCR. Significant increases (more than threefold increases) of CD40 mRNA were detected in 20 of the 27 cell lines, including five cell lines that showed strong induction (more than tenfold increases). Only two among the cell lines, which constitutively expressed CD40 mRNA, showed reduced CD40 expression levels (Table I). Although, it is documented that CHX can increase the mRNA levels of immediate early genes [Lau and Nathans, 1987; Wilson and Treisman, 1988; Edwards and Mahadevan, 1992], the present study is the first to report that CHX specifically induces CD40 mRNA expression in human and mouse carcinoma cells.

Previously, CHX has been found to sensitize many of cells to apoptosis induced by the activation of TNF family receptors including CD40 [Hess and Engelmann, 1996; Eliopoulos et al., 2000; LeBlanc et al., 2002]. CHX can also induce apoptosis directly in various cells by itself [Lemaire et al., 1999; Tang et al., 1999; Higami et al., 2000]. However, the mechanism of CHX action has not been firmly established. The mechanism whereby CHX induces CD40 mRNA expression may involve the stimulation of the transcription and/or the stabilization of mRNAs. Previous studies have shown that protein synthesis inhibitors, such as CHX, anisomycin, and emetine, can induce the activation of the transcription factor, NF-κB, or the activation of the p38 MAP kinase signal pathway [Zinck et al., 1995; Newton et al., 1996; Hazzalin et al., 1998; Krzesz et al., 1999]. Consistently, we also observed that SN50, an NF-κB inhibitor, significantly blocked CHX or anisomycin induced-CD40 mRNA expression. In addition, CFPD, a p38 MAP kinase inhibitor, also suppressed the CD40 mRNA expression induced by CHX or anisomycin (Fig. 3). These findings suggest that the apoptosis induced by CHX is mediated, at least in part, by both the p38 MAP kinase signaling pathway and an NF-κB-dependent system.

On the other hand, CHX has also been shown to reduce the degradation of certain mRNAs, and thus, may increase mRNA levels [Wilson and Treisman, 1988; Edwards and Mahadevan, 1992]. However, when CT26 cells were co-treated with CHX and actinomycin D, a transcription inhibitor, CD40 mRNA expression augmentation was significantly suppressed (data not shown). Hence, the induction of CD40 mRNA by CHX probably results from the increased synthesis of CD40 mRNA.

Our results also show that CHX specifically downregulates Bcl-xL mRNA in a time- and dose-dependent manner (Fig. 5) in CT26 cells. This decreased Bcl-xL mRNA levels result in a relative increase in the Bax mRNA/Bcl-xL mRNA ratio, which correlates with CT26 apoptosis (Fig. 6). CHX appeared to affect Bak mRNA expression weakly because we observed a little variation in the Bak mRNA levels although Bcl-xL mRNA levels decreased consistently in CHX-treated cells.

The Bcl-2 family proteins play a key role in the regulation of apoptosis. Some members of this family, including Bax, Bak, Bid, and Bik, function as proapoptotic factors, and others, including Bcl-2, Bcl-xL, Mcl-1, and A1, function as antiapoptotic proteins [Gross et al., 1999; Antonsson, 2001]. Bcl-2 family proteins, including Bcl-xL and Bax, are capable of physically interacting with each other to form complex networks of homo- and hetero-dimers via molecular regions called the Bcl-2 homology (BH) domains. While there are different ways in which Bcl-2 members may function, those physical interactions between anti- and

proapoptotic proteins have been suggested to play a pivotal role in the determination of the cell fate [Gross et al., 1999; Antonsson, 2001].

A previous study showed that CHX treatment converts Fas-resistant thyroid carcinoma cells to Fas-sensitive cells, and it was suggested that there might be a short-lived anti-apoptotic molecule(s) that can block caspase-8 activation within Fas-resistant thyroid carcinoma cells [Mitsiades et al., 2000]. In addition, a recent study demonstrated that several chemotherapeutic agents, such as etoposide and camptothecin, induce the expression of Bak, a pro-apoptotic Bax homolog, and thereby sensitize Bax-deficient human colon cancer cells to TRAIL-mediated apoptosis [LeBlanc et al., 2002]. Therefore, we suggest that Bcl-2 family members, such as Bcl-xL, Bak, and Bax, are regulated by chemotherapeutic agents at both the transcriptional and translational levels, which would explain the altered sensitivities of cancer cells to TNF family ligands.

Moreover, the results of the present study suggest that chemotherapeutic agents may sensitize cancer cells to treatment with TNF family ligands in at least two ways at the transcriptional level. First, chemotherapeutic agents regulate the gene expressions of TNF family receptors, target molecules of the treated ligands. Second, chemotherapeutic agents regulate Bcl-2 family gene expression, thus reducing the apoptotic threshold in response to TNF family ligands. In addition, chemotherapeutic agents and the TNF family ligands may synergistically mediate apoptosis by sharing common intracellular signaling pathways, such as the NF-κB-mediated and the p38 MAP kinase pathways, which are known to be critical signal transduction systems in death receptors-induced apoptosis [Ashkenazi and Dixit, 1998; Herr and Debatin, 2001; Budd, 2002].

In our experimental system, we observed that altered apoptotic gene expression by treatment with chemotherapeutic agents was both cell type and chemotherapeutic agent dependent. For this reason, information about cell type-specific differences in response to chemotherapeutic agents is a prerequisite for choosing the appropriate chemotherapeutic TNF family ligands partner. Although the present study requires elucidation at the protein level, it does go some way toward explaining the mechanisms underlying the synergistic effects of chemotherapeutic agents when used in combination therapies with TNF family ligands.

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