Dexamethasone suppresses apoptosis in a human gastric cancer cell line through modulation of *bcl-x* gene expression

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Abstract Treatment of human gastric cancer TMK-1 cells with transcription and translation inhibitors rapidly triggered cell apoptosis. Along with cell apoptosis, the Bcl-x_S level was markedly upregulated suggesting a crucial role of this protein in promoting the apoptotic process. In the presence of dexamethasone, however, cell apoptosis was greatly attenuated as demonstrated by DNA histogram shift and DNA fragmentation. Studies using the glucocorticoid receptor antagonist RU486 indicated that attenuation of apoptosis was mediated through glucocorticoid receptors. Dexamethasone not only suppressed the apoptosis-associated upregulation of Bcl-x_S but also enhanced the basal level of Bcl-x_L in the cells. In addition, *bcl-x* mRNA stability was significantly extended in the presence of dexamethasone. These results indicate that dexamethasone exerted a protective effect and delayed apoptosis of TMK-1 cells by modulating *bcl-x* gene expression.

Key words: Dexamethasone; Apoptosis; Actinomycin D; *bcl-x*; mRNA stability

1. Introduction

Apoptosis plays an important role in cell proliferation, organ involution, tumor development, and as a cellular response to a variety of stress insults. Apoptosis is an active, irreversible process by which cells die as a result of activation of an internally encoded cell death program [1,2]. Much effort has recently gone into identifying the genes responsible for apoptosis induction and suppression [3]. Among them, the products of the *bcl-2* gene family regulate conserved cellular pathways for apoptosis of different cells in response to a variety of apoptotic stimuli. Some of the members of this gene family are cell death inhibitors, such as Bcl-2, Bcl-x_L, Mcl-1, and Bfl-1, whereas others are promoters of cell death, such as Bax, Bad, Bak, and Bcl-x_S [2,4,5]. These proteins can physically interact with each other to form homo- or heterodimers that affect the susceptibility of cells to apoptotic stimuli. It has been suggested that the apoptotic threshold of a cell may be the result of a dynamic balance between these positive and negative regulators of apoptosis [6]. The *bcl-x* gene is unique in that it encodes two proteins, Bcl-x_L and Bcl-x_S, by alternatively spliced transcripts [5]. Recent studies have indicated that overexpression of Bcl-2 or Bcl-x_L protects cells against apoptosis induced by a wide range of stimuli [2]. In contrast, the short form, Bcl-x_S, functions as a dominant negative inhibitor of Bcl-2 and Bcl-x_L, and promotes cell apoptosis [5,6]. It has been shown that overexpression of Bcl-x_S sensitized cultured cells or tumor to apoptosis [7,8]. Bax and Bcl-x_S expression have been shown to be induced at the onset of apoptosis in involuting mammary epithelial cells [9]. Our previous study also demonstrated that the apoptosis in TMK-1 cells triggered by the transcription inhibitor actinomycin D (ActD), the RNA polymerase II inhibitor 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB) or the translation inhibitor cycloheximide (CHX) is associated with rapidly increased expression of Bcl-x_S [10]. In this study, the effects of dexamethasone (Dex) on *bcl-x* gene expression and apoptosis in TMK-1 cells induced by transcription and translation inhibitors were studied. We showed that Dex efficiently suppresses cell apoptosis by stabilization of *bcl-x* mRNA, increasing the Bcl-x_L level, and inhibition of apoptosis-associated upregulation of Bcl-x_S expression.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). Culture plasticware was purchased from Costar (Cambridge, MA, USA). [α-^{32}P]dCTP (6000 Ci/mmol) was purchased from Dupont NEN (Boston, MA, USA). 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 *bcl-$x_{L/S}$* were obtained by RT-PCR from total RNA of TMK-1 cells. Antibodies against human Bcl-2, Bax, Bak, and Bcl-$x_{L/S}$ were purchased from Oncogene Sciences (Uniondale, NY, USA) and Santa Cruz Biotech (Santa Cruz, CA, USA). DRB, ActD and CHX were obtained from Sigma-Aldrich (St. Louis, MO, USA). RU486 was generously provided by Uclaf, France. All other chemicals used were purchased either from Sigma or from E. Merck (Darmstadt, Germany).

2.2. Cell culture

The cultured human gastric carcinoma cell line TMK-1, derived from a poorly differentiated adenocarcinoma [11], was a generous gift from Dr. Eiichi Tahara (Hiroshima University, Japan). This cell line carries a mutated form, G to A point mutation at codon 173, of the *p53* gene (*mp53*) as described [12]. None of the *bcl-2* mRNA or protein was detected in the cells in previous studies [10,13]. In this study, TMK-1 cells were cultured at 37°C in DMEM medium 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

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Abbreviations: Dex, dexamethasone; ActD, actinomycin D; DRB, 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole; CHX, cycloheximide; GAPDH, glyceraldehyde 3-phosphodehydrogenase; PI, propidium iodide

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the cells was determined by counting the viable cells with trypan blue exclusion. Cells in the logarithmic growth phase were used in this study. The cells were seeded into culture dishes and allowed 24 h prior to treatment. ActD (100 ng/ml), DRB (25 μg/ml), CHX (2 μg/ml), dexamethasone (10^{-6} M), and RU486 (10^{-6} M) were added to the medium as a concentration stock in ethanol. Control cells were fed with medium containing the same amount of ethanol.

2.3. Analysis of DNA fragmentation

Treated or untreated TMK-1 cells were lysed with 0.5 ml lysis buffer (5 mM Tris-borate (pH 8.0), 0.25 ml Nonidet P-40, and 1 mM EDTA), followed by the addition of RNase (20 μg/ml), and incubated at 37°C for 1 h. Cells were further treated with proteinase K (300 μg/ml) for an additional 1 h, and DNA was then isolated. The DNA was resuspended in 10 mM Tris-Cl buffer (pH 8.0) containing 1 mM EDTA and quantified. The same amount of DNA of each sample was loaded and electrophoresed in a 1.5% agarose in Tris-borate buffer (pH 8.0) containing 1 mM EDTA.

2.4. Western blot analysis

TMK-1 cells were plated on 6 cm dishes at a density of 1×10^6 cells/dish for 24 h prior to Dex treatment. After another 48 h, the Dex treated or untreated cells were incubated with DRB (25 μg/ml), ActD (100 ng/ml) or CHX (2 μg/ml) for the indicated time periods. Whole cell extract preparation and SDS-PAGE were performed as described. Immunoblotting was performed using rabbit anti-human antibodies for Bcl-2, Bax, Bak, and Bcl-$X_{L/S}$. Signals were visualized with an enhanced chemoluminescence kit (ECL, Amersham, UK) followed by exposure to X-ray films.

2.5. Isolation of RNA and Northern blot analysis

Total RNA isolation and Northern blot analysis were performed essentially as described [14]. For Northern analysis, 30 μg of total RNA was size fractionated, transferred to nylon filters, hybridized, and autoradiographed. Autoradiography results were quantified and normalized with respect to glyceraldehyde 3-phosphodehydrogenase (GAPDH) mRNA as determined by hybridization analysis.

2.6. Flow cytometric analysis

Hypodiploid DNA was analyzed using the method of propidium iodide (PI) staining and flow cytometry as described [15]. Briefly, drug treated or untreated cells (1×10^6) were washed and resuspended in 1 ml hypotonic fluorochrome solution (50 μg/ml of PI in 0.1% sodium citrate plus 0.1% Triton X-100). Samples were placed at 4°C in the dark, and the PI fluorescence of individual nuclei was analyzed using a FACScan flow cytometer (Becton Dickinson, Lincoln Park, NJ). Cellular debris was excluded from analysis by raising the forward scatter threshold, and the DNA content of intact nuclei was registered on a logarithmic scale. Apoptotic cell nuclei containing hypodiploid DNA emitting fluorescence in channels 10–400 were enumerated as a percentage of the total population.

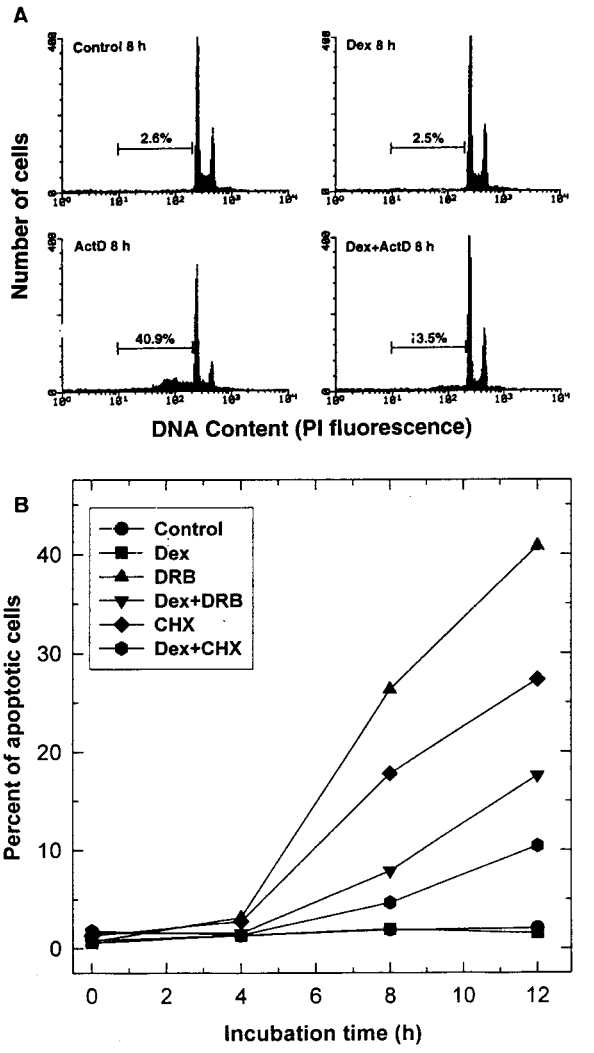

Fig. 1. Dex suppressed apoptosis triggered by transcription and translation inhibitors. TMK-1 cells (1×10^6) were plated into 6 cm culture dishes and incubated with or without Dex (10^{-6} M) for 48 h. The cells were then treated in the absence (control) or presence of (A) ActD; (B) DRB or CHX for the time indicated before being harvested and stained with PI. Hypodiploid DNA peaks corresponding to apoptotic nuclei were revealed by cytofluorometry as described in Section 2.

3. Results

3.1. Dex decreases the susceptibility of TMK-1 cells to apoptosis

To study the role of Dex on TMK-1 cell apoptosis, the effects of Dex on cell proliferation and morphology were first assessed. Although Dex inhibits cell proliferation in a number of cultured cells, the growth of TMK-1 cells was not influenced by 10^{-6} M Dex. No significant differences in cell viability and total cell number were observed in cells treated with or without Dex for 48 h although the cells were more flattened in the presence of Dex (data not shown). These observations indicate that Dex does not produce toxic effects on cell proliferation and morphology.

Treatment of TMK-1 cells with Dex for 48 h markedly decreased the susceptibility of the cells to apoptosis and the onset of apoptosis was also delayed. The observed apoptosis was characterized by chromatin condensation, nuclear fragmentation, and formation of apoptotic bodies under the fluorescent microscope as previously described [10]. In this study, cell apoptosis was assessed by DNA fragmentation and the manifestation of the broad hypodiploid DNA peak in flow cytometric analysis. Results shown in Fig. 1A indicate that the number of apoptotic cells in the TMK-1 population 8 h after ActD treatment was 40.9% but decreased to 13.5% when cells were coincubated with ActD and Dex. Dex alone had no effect on the background apoptotic cell population of the TMK-1 cells (Fig. 1A). Marked suppression of apoptosis by Dex was also demonstrated in apoptosis triggered by DRB and CHX (Fig. 1B). Results shown in Fig. 2 indicate that the absence or presence of hypodiploid peaks closely parallels the absence or presence of DNA ladder in variously treated cells. The relative intensity of DNA ladder was much evident in ActD treated cells than cells cocultured with Dex and ActD (Fig. 2, lanes 3 and 5). Furthermore, the protective effect of Dex was completely abolished by the presence of the gluco-

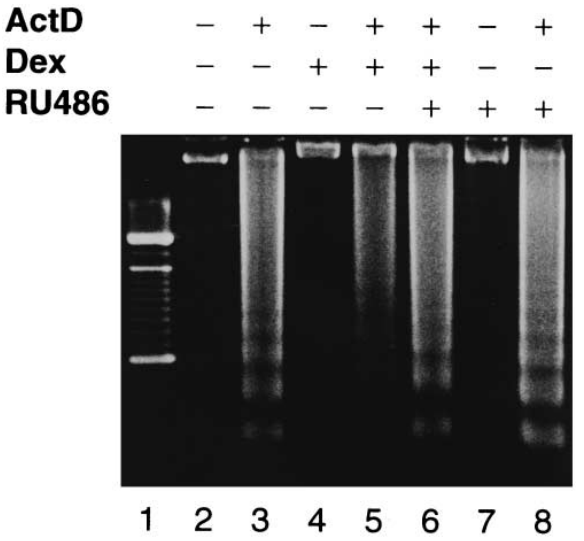

Fig. 2. Internucleosomal DNA fragmentation induced by ActD. TMK-1 cells (1×10^6) were plated into 6 cm culture dishes and incubated with or without Dex (10^{-6} M) for 48 h. The cells were then treated in the absence (lane 2) or presence of ActD, Dex, ActD plus Dex, ActD plus Dex plus RU486, RU486, or ActD plus RU486 (lanes 3–8, respectively); lane 1 is the 100 bp DNA ladder.

corticoid receptor (GR) antagonist RU486, which suggests a GR-mediated mechanism in the action of Dex (Fig. 2, lanes 5 and 6). DNA fragmentation in DRB or CHX treated cells was similarly modulated by Dex and RU486 (data not shown). Taken together, these results clearly demonstrate that glucocorticoid efficiently suppresses the apoptosis of TMK-1 cells triggered by transcription and translation inhibitors.

3.2. Dex suppresses the upregulation of Bcl-x_S in apoptotic cells

To study the effect of Dex on the level of Bcl-2 related proteins, we examined the alterations of Bcl-x, Bax, and Bak in the cells during apoptosis. Although TMK-1 cells express a relatively high level of Bcl-x_L, Bax, and Bak proteins, both *bcl-2* mRNA and protein were not detectable in the cells [10,13]. The changes in the Bcl-x, Bax, and Bak protein levels in response to ActD are summarized in Fig. 3. The basal level of Bcl-x_L protein was significantly increased after 48 h treatment with Dex (Fig. 3, lanes 1 and 6). Consistent with our earlier findings, the apoptosis triggered by ActD is associated with overexpression of Bcl-x_S in TMK-1 cells (Fig. 3, lanes 1–5). Interestingly, pretreatment of the cells with Dex abolished or delayed the increase of Bcl-x_S associated with ActD treatment (Fig. 3, lanes 6–10). In contrast to changes in Bcl-x_S and Bcl-x_L, the Bax and Bak proteins displayed few changes during ActD treatment in the cells. In a similar manner, pretreatment of Dex suppressed or delayed the overexpression of Bcl-x_S in the DRB or CHX treated cells (data not shown). These results demonstrate that the susceptibility of TMK-1 cells to apoptosis triggered by these inhibitors was associated with the relative level of Bcl-x_S in the cells. In addition, Dex attenuated cell apoptosis through increase in the expression of Bcl-x_L and blocked the pathway for Bcl-x_S overexpression.

3.3. Dex increases the stability of bcl-x mRNA

The steady-state levels of *bcl-x* mRNA were investigated by

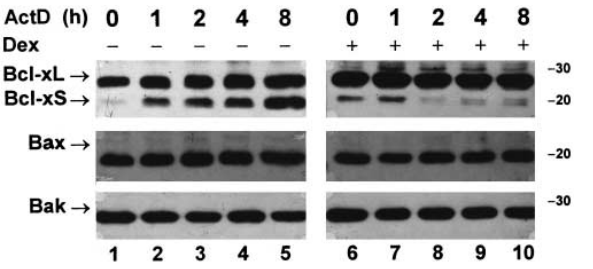

Fig. 3. Effects of ActD and Dex on Bcl-$x_{L/S}$, Bax, and Bak expression. TMK-1 cells were pretreated in the absence (lanes 1–5) or presence (lanes 6–10) of Dex for 48 h and ActD was then added for the time indicated. Whole extract (30 μg) of cells were analyzed by immunoblotting as described in Section 2. The immunoblots shown here represent the typical result from several independent experiments.

Northern blot analysis in the first 8 h after ActD treatment. Since it was shown that over 99% of total RNA synthesis was blocked in ActD treated (50 ng/ml) TMK-1 cells, the relative intensity of the *bcl-x* mRNA levels can be used to calculate the stability of *bcl-x* mRNA [10]. As shown in Fig. 4A, a single *bcl-x* mRNA band about 2.7 kbp was detected in TMK-1 cells which declined rapidly with time upon ActD treatment. When ActD was added to Dex treated cells, the turnover rate of *bcl-x* mRNA was significantly decreased compared to that observed when ActD was added to Dex un-

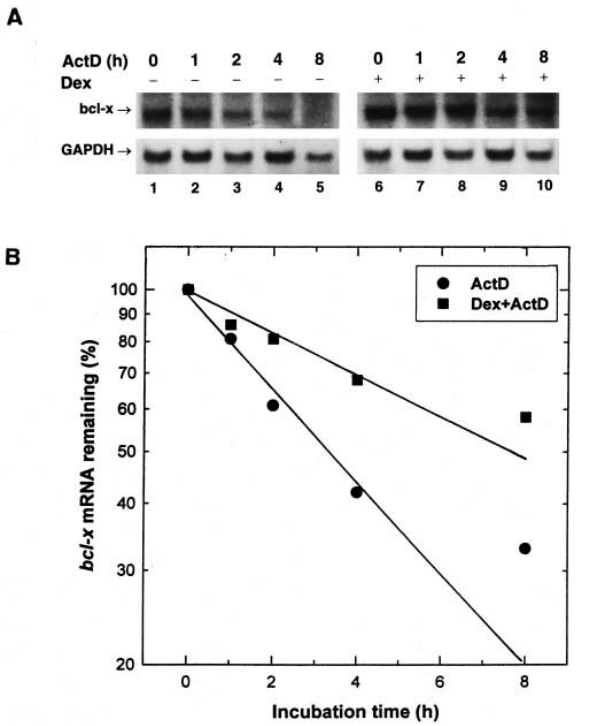

Fig. 4. Effects of ActD and Dex on *bcl-x* mRNA levels. TMK-1 cells were pretreated in the absence (lanes 1–5) or presence (lanes 6–10) of Dex for 48 h and ActD was then added for the time indicated. A: Total RNA (30 μg) from each sample was prepared, fractionated, transblotted, hybridized, and autoradiographed. B: Densitometric quantitation of the *bcl-x* mRNA level in each sample using the level of GAPDH transcript as the normalization control. The results are the average of two separate experiments.

treated cells (Fig. 4A). In addition, when cells were pretreated with Dex, the steady-state level of *bcl-x* mRNA was upregulated to about 1.5-fold over the control level in Dex untreated cells (Fig. 4A, lanes 1 and 6). The *bcl-x* mRNA exhibited an about 2-fold increase in stability after exposure to Dex as the apparent half-lives of *bcl-x* mRNA increased from about 3.5 h to 8 h (Fig. 4B). The *bcl-x* mRNA bands shown in Fig. 4 consist of *bcl-x$_L$* and *bcl-x$_S$* transcripts since the difference in size between *bcl-x$_L$* and *bcl-x$_S$* transcripts is too small for them to be distinguished. Nevertheless, the *bcl-x$_L$* transcript should mainly account for the *bcl-x* mRNA signals because of the much greater abundance of basal Bcl-x$_L$ than of Bcl-x$_S$ proteins (Fig. 3, lanes 1 and 6) and there is no transcription in the presence of ActD. These results indicate that Dex also modulates *bcl-x* gene expression at the posttranscriptional level.

4. Discussion

Glucocorticoids play a fundamental role in normal development and physiological control of animals by modulating proliferation of various normal and malignant cells [16]. Glucocorticoids have been employed as potent anti-inflammatory agents for years, yet the mechanisms remain to be elucidated. The glucocorticoids bind specifically to the glucocorticoid receptor and modulate gene expression such as activation or inhibition of transcription of specific genes. Glucocorticoids have been shown to induce irreversible G1 arrest and cell death in a human lymphoblastic leukemic T-cell line (CEM-C7) [17]. Clinically, they have been used in the treatment of lymphoid leukemia and lymphoma. The mechanism underlying this effect is the induction of cell apoptosis [18]. In rodent thymocytes, glucocorticoid-induced apoptosis is a model system for research of programmed cell death [19]. In this study, however, we show that Dex decreases the susceptibility of TMK-1 cells to transcription and translation inhibitor induced apoptosis. Our results are in accordance with the finding that glucocorticoids are known to prolong the life-time and inhibit apoptosis of neutrophils [20,21]. In M1 myeloid leukemic cells and human mammary luminal epithelial cells, treatment with glucocorticoids also resulted in a decrease in the susceptibility of the cells to apoptosis inducing agents [22,23]. The result that the GR antagonist RU486 completely abolished the suppressive effect of Dex on DNA fragmentation confirms that the protective effect of Dex to the cells is mediated through a GR dependent pathway (Fig. 2). Consistent with studies in neutrophils and leukemia cells, however, Dex delayed, rather than blocked, apoptosis which resulted in prolonged TMK-1 cell survival [20–23].

The susceptibility of TMK-1 cells to apoptosis in treatment with transcription and translation inhibitors may be due to the absence of Bcl-2 and the expression of Bcl-x$_S$ protein in the cells [10,13]. In this study, treatment of TMK-1 cells with Dex inhibited or delayed endogenous Bcl-x$_S$ overexpression observed during cell apoptosis (Fig. 3). Since the increased expression of Bcl-x$_S$ was shown to be associated with the onset of cell apoptosis [9,10], our results demonstrate that the suppression of TMK-1 cell apoptosis by Dex is mediated through inhibition of Bcl-x$_S$ overexpression. Dex was also shown to downregulate *bcl-x$_S$* gene expression in rat thymocytes which led to the suggestion that Bcl-x$_S$ may favor the survival of thymocytes [24]. Consistent with the increased expression of Bcl-x$_L$ protein (Fig. 3), significant increases in steady-state *bcl-x* mRNA levels were also shown in Dex treated cells (Fig. 4). The enhanced bcl-x$_L$ mRNA stability and protein levels may represent a part of the mechanism underlying the protective effect of Dex. Stabilization of mRNA transcripts is common for various genes which are regulated by glucocorticoids [25,26]. Suppression of susceptibility of M1 myeloid cells to apoptosis by Dex was shown to be associated with an enhanced steady-state level of *bcl-x$_L$* mRNA [22].

In conclusion, the expression of the *bcl-x* gene in TMK-1 cells is regulated at both posttranscriptional and translational levels by Dex. Results shown in this study indicate that modulation of *bcl-x* gene expression is related to the susceptibility of TMK-1 cells to apoptosis. The anti-apoptotic effects of glucocorticoids in cells such as TMK-1 may thus be related to the modulation of *bcl-x* gene expression in the cells.

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