

# Glucocorticoids inhibit the apoptotic actions of UV-C but not Fas ligand in hepatoma cells: direct evidence for a critical role of Bcl-x<sub>L</sub>

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Our laboratory has shown that glucocorticoids can inhibit apoptosis in rat hepatoma cells; however, the mechanisms are incompletely understood. To address this issue we sought to determine if glucocorticoid inhibition is effective when death is induced by stimuli that more selectively activate either the intrinsic (UV-C) or extrinsic (FasL) apoptotic pathways. Using flow cytometric analysis, we show that pretreatment of HTC cells with dexamethasone (Dex) inhibits UV-C- but not FasL-induced apoptosis. This inhibition requires Dex pretreatment and can be abrogated by the glucocorticoid antagonist RU486 indicating glucocorticoid receptor-mediated action. Dex increases anti-apoptotic Bcl-x<sub>L</sub> at both mRNA and protein levels. The Bcl-x<sub>L</sub> protein level remains elevated even after apoptosis induction with either UV-C or FasL although only UV-C-induced cell death is inhibited. Repression of Bcl-x<sub>L</sub> protein with siRNA abrogates the anti-apoptotic effect of glucocorticoids. Together these data provide direct evidence that Bcl-x<sub>L</sub> mediates glucocorticoid inhibition of UV-C induced apoptosis.

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Apoptosis is a highly regulated physiological process of cell death that is necessary for proper development and maintenance of an organism. This regulation is carried out in part by a balance in the synthesis and degradation of pro- and anti-apoptotic factors that determine cell fate. Disturbances in this process are the basis for various disease states including cancer, neurodegenerative diseases such as Alzheimer's and auto-immune diseases including lupus and diabetes. Mutations or changes in expression levels of pro- or anti-apoptotic proteins are also often associated with malignant transformation.<sup>1</sup> As multiple changes can exist in the apoptotic machinery in one type of malignant cell, cancer chemotherapy regimens often employ a combination of drugs each with a different mechanism of inducing cell death. One class of drugs widely used in cancer treatment is the glucocorticoids. They are used as direct cell death-inducers in lymphomas and leukemias.<sup>2</sup> Glucocorticoids are also often given in conjunction with other chemotherapies to ameliorate side effects of the primary drugs or to reduce edema. Although primarily recognized for their apoptotic stimulating actions on T lymphocytes, reports have suggested that glucocorticoids can inhibit apoptosis in several cell types including glioma and astrocytoma cell lines,<sup>3</sup> fibroblasts,<sup>4,5</sup> gastric cancer cell lines, mammary epithelial cells,<sup>6,7</sup> hepatoma cells<sup>8</sup> and glomerular endothelial cells.<sup>9</sup> Previous studies from our laboratory have shown that glucocorticoids can inhibit serum-deprivation induced apoptosis in hepatoma cells<sup>10</sup> but the mechanisms underlying this action are largely undefined. Specific inhibition of cancer cell apoptosis would obviously not be the desired

effect of an adjunct chemotherapy. Thus, fully understanding how glucocorticoids inhibit apoptosis in cancer cells is of the utmost importance. In particular, we wanted to further delineate the mechanisms of the inhibitory effects of glucocorticoids on apoptosis that has been induced via the extrinsic or intrinsic apoptotic pathway.

Current research on apoptotic signaling mechanisms primarily supports the idea of two caspase-dependent pathways leading to programmed cell death.<sup>11</sup> The first involves the death receptor or extrinsic pathway which is initiated by TNF-receptor family members that recruit adaptor and signaling molecules to assemble the death-inducing signaling complex (DISC). This complex leads to rapid activation of caspases 8 and/or 10, which then activate downstream effector caspases such as caspase 3, 6 and 7.<sup>12,13</sup> Caspase 8 can also cleave the pro-apoptotic protein Bid to form tBid and this truncated form of Bid translocates to the mitochondrial membrane to trigger the cell death process and may form an amplification loop for the death receptor pathway.<sup>14,15</sup> In contrast, the intrinsic pathway for apoptosis is activated by various cellular stress stimuli, including UV irradiation (for a review see Fumarola and Guidotti<sup>16</sup>). In this pathway, the mitochondria appear to be the primary target for the death-inducing stress response. These organelles undergo a loss of mitochondrial membrane potential ( $\Delta\Psi_{\text{mito}}$ ) and release several apoptotic proteins including cytochrome *c*, which participates in the formation of the apoptosome together with Apaf-1 to activate caspase 9. This pathway is subject to regulation by Bcl-2 family members.<sup>17–20</sup> This intrinsic path-

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**Abbreviations:**  $\Delta\Psi_{\text{mito}}$ , mitochondrial membrane potential; FasL, fas ligand; Dex, dexamethasone; AFU, arbitrary fluorescence units

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way converges at later stages of the apoptotic process with the extrinsic pathway on downstream effectors of cell death.<sup>21,22</sup>

Glucocorticoids are now recognized to inhibit apoptosis in some cells but the mechanism of inhibition induced by glucocorticoids has not been fully elucidated, although a role for the anti-apoptotic Bcl-2 family proteins has been suggested in mammary tumor cells and leukemia cells. For example increases in Bcl-x<sub>L</sub> mRNA occur in leukemia cells<sup>23</sup> and in mammary epithelial cells<sup>6</sup> treated with dexamethasone. Promoter analysis in heterologous expression systems suggest that glucocorticoid response elements exist in one of the Bcl-x gene's promoters.<sup>24</sup> Additionally, it has been recently shown that dexamethasone induces Bcl-x<sub>L</sub> transcription during fibrosarcoma tumor growth.<sup>25</sup> However, data has been largely correlative and do not provide direct evidence for Bcl-x<sub>L</sub>'s glucocorticoid regulated anti-apoptotic effects.

In the current investigation, we demonstrate that dexamethasone can protect the rat hepatoma cell line, HTC, from UV-C but not FasL-induced apoptosis. Dexamethasone pretreatment causes a receptor-dependent increase in both Bcl-x<sub>L</sub> mRNA and protein. Interestingly, the level of Bcl-x<sub>L</sub> protein remains elevated during treatment irrespective of the apoptosis inducer. Inhibition of the dexamethasone-induced increase in Bcl-x<sub>L</sub> protein by concurrent treatment with the antagonist RU486 abolishes the anti-apoptotic effect of glucocorticoids on UV-C induced cell death. In addition, when the endogenous levels of Bcl-x<sub>L</sub> are reduced by siRNA, the hepatoma cells are prone to spontaneous apoptosis without additional apoptotic stimuli and Dex has no anti-apoptotic effect on these cells. These data suggest that Bcl-x<sub>L</sub> is a primary component of the underlying mechanism of glucocorticoid-induced inhibition of apoptosis.

## Results

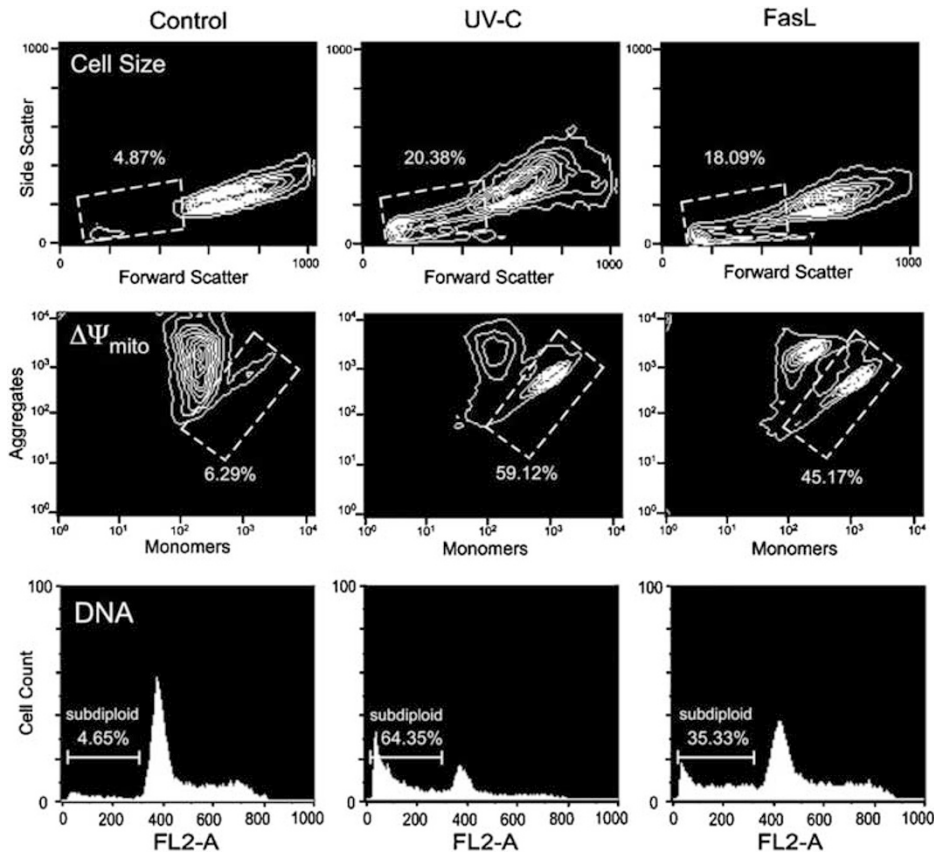
**UV-C irradiation or FasL induce apoptosis in HTC cells.** The rat hepatoma cell line HTC will undergo apoptosis induced by serum/growth factor deprivation<sup>10</sup> but the cell death response induced by other stimuli has not been extensively studied. Here, we examined the ability of other stimuli to induce apoptosis in HTC cells namely UV-C irradiation which signals primarily via the intrinsic or mitochondrial pathway<sup>26</sup> or FasL which activates the death receptor or extrinsic pathway.<sup>12</sup> HTC cells were left untreated, treated with either 50 ng/ml FasL or irradiated with 90 mJ/cm<sup>2</sup> UV-C light. After 24 h the cells were trypsinized, centrifuged, resuspended in media, loaded with the appropriate dye and then examined by flow cytometry.

Both UV-C and FasL treatments resulted in the appearance of populations of cells with classic apoptotic characteristics after 24 h (Figure 1). Untreated cells display a single population on a forward scatter *versus* side scatter plot indicating a normal cell size. Cells treated with either UV-C or FasL show cells of normal size and cells with decreased forward scatter indicating cell shrinkage, a hallmark of apoptotic cell death (Figure 1, first row). We also evaluated mitochondrial membrane potential depolarization (loss of  $\Delta\Psi_{\text{mito}}$ ), another indicator of apoptotic cell death, which can be monitored utilizing the mitochondrial membrane potential

sensitive dye JC-1. Mitochondria from normal cells take up the dye in aggregate form, which emits fluorescence in the orange range (~580 nm); however, when mitochondria become depolarized the dye reverts to a monomeric form which emits fluorescence in the green range (~520 nm). Untreated HTC cells show mainly a single population with high aggregate (orange) fluorescence indicating cells with functional mitochondria and normal  $\Delta\Psi_{\text{mito}}$ . Cells treated with either FasL or UV-C display the normal population but also a dramatic increase in a second population with decreased aggregate (red) but increased monomer (green) fluorescence indicating cells with mitochondria which show a loss of  $\Delta\Psi_{\text{mito}}$  (Figure 1, second row). Another classical hallmark of apoptosis is DNA degradation. Using ethanol fixation and the DNA intercalating dye propidium iodide, we analyzed the DNA degradation following apoptosis induction. Untreated HTC cells show a large G1/G0 peak (at approximately 400 arbitrary fluorescence units) with a smaller G2/M peak (approximately 800 AFU) and very few cells (less than 10%) in the subdiploid range (less than 400 AFU). Cells induced to undergo apoptosis with either FasL or UV-C show an increase in the number of cells with subdiploid DNA (Figure 1, third row). These results reveal that HTC cells undergo classical apoptosis at 24 h post-stimuli with these doses of FasL and UV-C.

### Dexamethasone pre-treatment protects HTC cells from UV-C induced but not FasL-induced apoptosis.

After establishing that HTC cells undergo classic apoptosis when induced by UV-C or FasL, we next examined if pretreatment with glucocorticoids could inhibit cell death. Previous studies from our lab have shown that HTC cells required a 24 h glucocorticoid pretreatment for maximal inhibition against serum deprivation induced apoptosis.<sup>10</sup> As the UV-C and FasL pathways could have different kinetics than serum deprivation cells were treated with or without 10 nM Dex for 4, 8, 16 or 24 h. The cells were then left untreated or treated with FasL 50 ng/ml or 90 mJ/cm<sup>2</sup> UV-C, which yields comparable induction of apoptosis and percentages of cells with apoptotic features were determined by flow cytometry as described in Figure 1. In addition, treated and non-treated cells were examined by confocal microscopy to determine if the cells had morphologic features of apoptosis. Consistent with the data in Figure 1 both apoptotic inducers alone caused an increase in the population of cells with loss of cell size, decreased  $\Delta\Psi_{\text{mito}}$ , and degraded DNA (Figure 2a). Dex pretreatment followed by induction of apoptosis with FasL did not prevent the appearance of apoptotic cell populations as determined by flow cytometry; specifically cell shrinkage, loss of  $\Delta\Psi_{\text{mito}}$ , and DNA degradation (Figure 2a). In contrast Dex pretreated cells followed by UV-C irradiation showed less cell shrinkage, changes in  $\Delta\Psi_{\text{mito}}$ , and DNA degradation with the greatest inhibition seen at pretreatment times of 16 and 24 h (Figure 2a). Visually FasL- and UV-C-treated cells displayed typical apoptotic morphology, namely shrinkage and blebbing of the plasma membrane as opposed to untreated cells and cells treated with Dex alone (Figure 2b). Pretreatment with Dex inhibits the appearance of apoptotic morphology in UV-C-treated cells, however, there is no inhibition in FasL-treated cells (Figure 2b).



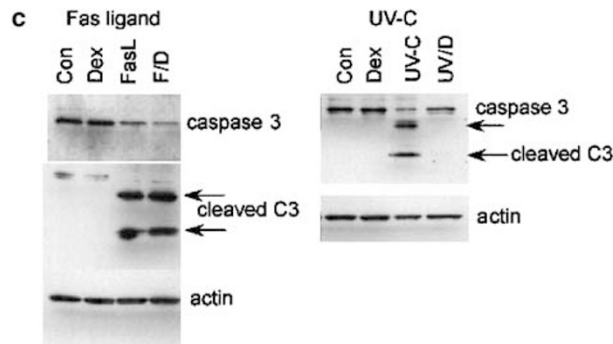
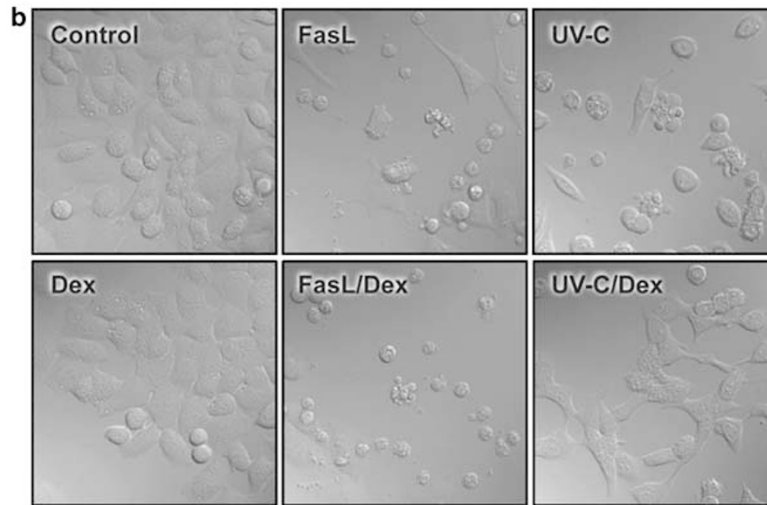
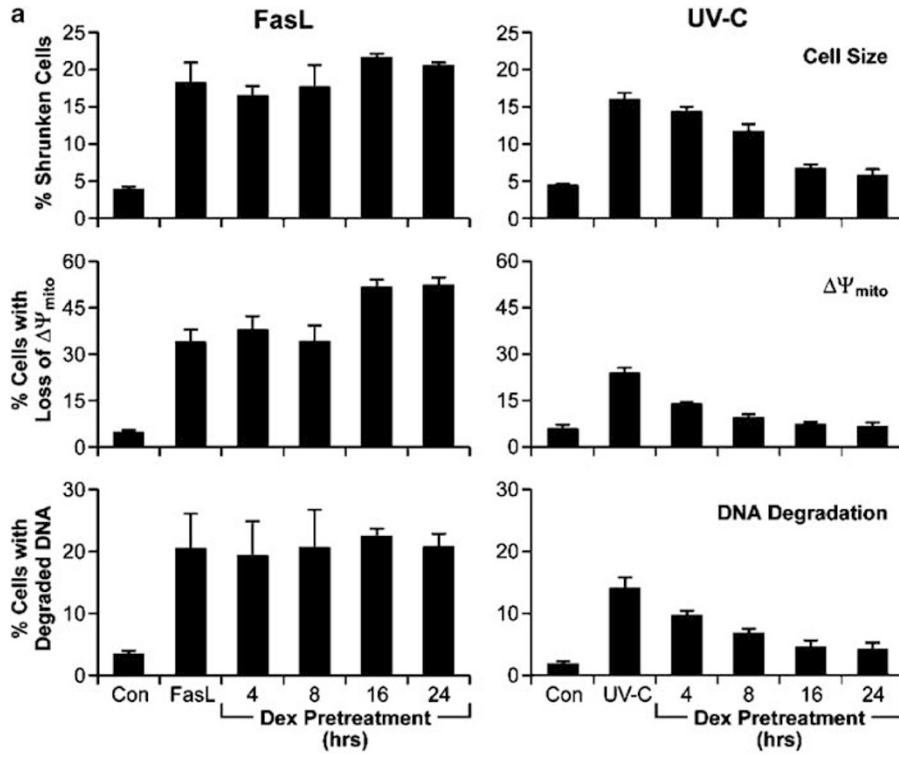
**Figure 1** UV-C or FasL induce features of apoptosis in HTC Cells. HTC cells were left untreated, treated with either 50 ng/ml FasL or irradiated with 90 mJ/cm<sup>2</sup> UV-C. After 24 h the cells were trypsinized, centrifuged, brought up in media (to a final concentration of 0.5 to 1 × 10<sup>6</sup> cells/ml) plus the appropriate dye and examined by flow cytometry (see Materials and Methods). Both UV-C and FasL treatments induce the appearance of populations of cells with apoptotic characteristics after 24 h. Shown in the figure are representative flow cytometry plots of three separate experiments: loss of cell size (forward scatter versus side scatter), loss of  $\Delta\Psi_{\text{mito}}$  (monomers versus aggregates), and DNA degradation (arbitrary fluorescence units versus cell number)

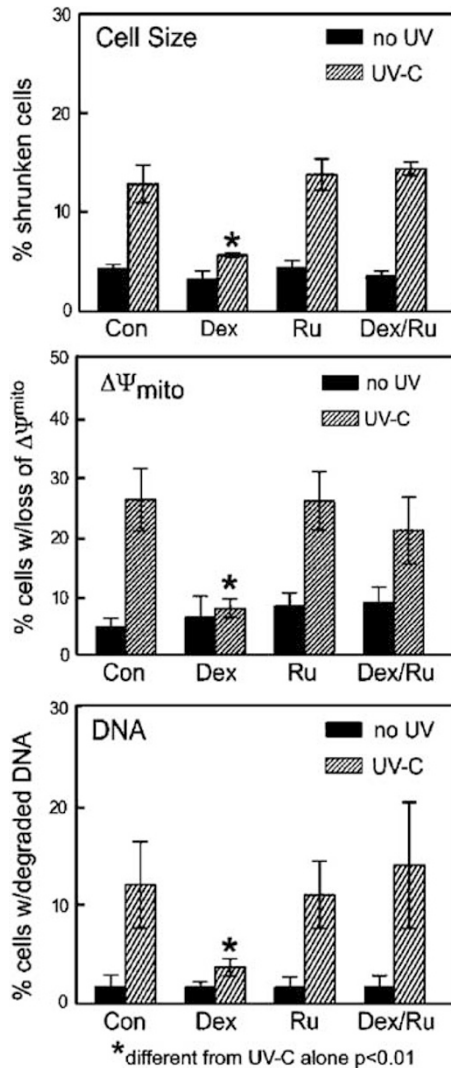
Western blot analysis demonstrates that caspase 3 is cleaved in the FasL- and UV-C-treated cells (Figure 2c). Pretreatment with Dex blocks caspase 3 cleavage in UV-C but not FasL treated cells (Figure 2c). These results suggest that glucocorticoid treatment is selectively affecting the signaling of the intrinsic apoptotic pathway. Based on knowledge of glucocorticoid receptor (GR) action and the requirement for long term steroid pretreatment suggests that receptor dependent gene induction is likely involved in the anti-apoptotic effect of glucocorticoid.

**Anti-apoptotic effects of Dex are blocked by RU486.** To determine if the anti-apoptotic effects of Dex treatment were GR-dependent the selective glucocorticoid antagonist,

RU486 was evaluated for its ability to block the action of GR. As Dex had no inhibitory effect on FasL-induced apoptosis the effects of pretreatment with Dex and RU486 were analyzed in UV-C treated cells. Consistent with previous findings the percentage of shrunken cells, cells with loss of  $\Delta\Psi_{\text{mito}}$  and cells with subdiploid DNA were increased by UV-C irradiation while either Dex or RU486 alone in unstimulated cells had no effect (Figure 3). The percentage of cells in these apoptotic populations after UV-C irradiation was decreased by Dex pretreatment (Figure 3). The anti-apoptotic effects of Dex pretreatment could be blocked by concurrent treatment with 100 nM RU486 indicating action via the glucocorticoid receptor (Figure 3). The fact that Dex pretreatment was anti-apoptotic only when

**Figure 2** Dex pretreatment inhibits UV-C-induced but not FasL-induced apoptosis. Cells were treated with FasL 50 ng/ml or 90 mJ/cm<sup>2</sup> UV-C and percentages of cells with apoptotic features were determined from at least three separate experiments or the cells were treated and visualized by microscopy in three experiments. (a) Cells were pretreated with Dex for 4, 8, 16 or 24 h before apoptosis induction. Both UV-C and FasL caused an increase in the population of cells with loss of cell size, and decreased  $\Delta\Psi_{\text{mito}}$ , and degraded DNA. Pretreatment with Dex for 16 and 24 h significantly decreased ( $P < 0.01$ ) these apoptotic populations in UV-C irradiated cells but had no inhibitory effect on FasL-treated cells. (b) Cells left untreated or treated with Dex alone show similar morphology. Cells treated with FasL or UV-C show apoptotic morphology namely cell shrinkage and membrane blebbing. Cells treated with Dex + FasL still show apoptotic morphology while cells treated with Dex + UV-C are protected. (c) Protein lysates of treated or untreated cells were analyzed by Western blot for the presence of cleaved caspase 3. Both FasL and UV-C treatment cause caspase 3 cleavage while Dex pretreatment prevented caspase 3 cleavage in UV-C but not FasL-treated cells





**Figure 3** Inhibition of UV-C-induced apoptosis by Dex blocked by co-administration of RU486. HTC cells were left untreated or treated with 10 nM Dex, 100 nM RU486, a glucocorticoid antagonist, or a combination of both 24 h before UV-C irradiation. UV-C caused an increase in the population of cells with loss of cell size, decreased  $\Delta\Psi_{mito}$ , and degraded DNA. Pretreatment with Dex significantly ( $P < 0.01$ ) decreased these apoptotic populations. RU486 alone had no effect. Co-treatment of Dex with RU486 inhibits Dex effects against UV-C. The results are averages  $\pm$  S.E.M. of at least three separate experiments

cell death was induced by the intrinsic pathway suggested the possibility of transcriptional regulation of Bcl-2 family members which are critical for this apoptosis pathway.

**Dex treatment causes an increase in Bcl-x<sub>L</sub> mRNA and protein.** To determine if Bcl-2 family members were involved in the anti-apoptotic effects of Dex, we initially examined HTC cells for changes in Bim, Bid, Bcl-2 and Bcl-x<sub>L</sub> mRNA after Dex treatment using real-time PCR. Bim and Bid mRNA were present but not affected by Dex treatment. Although the Bcl-2 mRNA was detected, it was at the very limit of the analysis and was not affected by Dex treatment (data not shown). In contrast, the mRNA for Bcl-x<sub>L</sub> was increased at 8 and 16 h after Dex treatment and remained

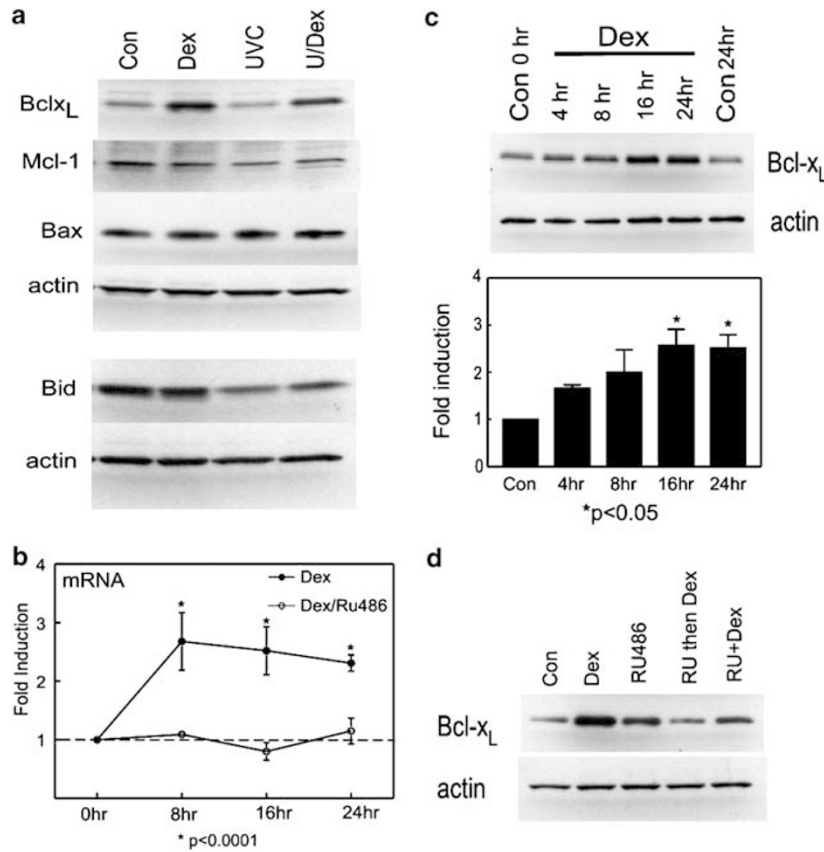
elevated more than twofold at 24 h. This induction of Bcl-x<sub>L</sub> mRNA was abrogated by co-treatment with RU486 (Figure 4b).

We further examined other Bcl-2 family members' protein levels in HTC cells. As shown in Figure 4a the anti-apoptotic protein Mcl-1 and the proapoptotic proteins Bax and Bid were not affected by Dex treatment, though UV-C caused a decrease in Mcl-1 and Bid proteins. However, reflecting the increase in mRNA seen in Figure 4a, Bcl-x<sub>L</sub> protein showed a Dex-induced increase (Figure 4a). Western blot analysis of whole cell lysates at 4, 8, 16 and 24 h after Dex treatment showed that Bcl-x<sub>L</sub> protein increased in a time dependent manner with the maximal induction being at 16 and 24 h (Figure 4c). This increase in protein could also be blocked by co-treatment of the cells with RU486 (Figure 4d).

**Dex-induced increase in Bcl-x<sub>L</sub> protein is sustained during apoptosis induction: Bcl-x<sub>L</sub> remains associated with mitochondria.** To determine whether the increase in Bcl-x<sub>L</sub> protein was responsible for the anti-apoptotic effects of Dex treatment, we examined the protein levels before and after UV-C treatment. While UV-C treatment alone decreased Bcl-x<sub>L</sub> protein levels, protein levels remained elevated during UV-C following Dex pretreatment (Figure 5a). Interestingly, the protein also remained elevated during FasL treatment although it apparently afforded no anti-apoptotic actions on the extrinsic pathway of apoptosis induced by FasL (Figure 5a). Together these results, in addition to our results demonstrating that co-treatment with RU486 can block the inhibitory effects of Dex on UV-C-induced apoptosis, suggest that the increase in Bcl-x<sub>L</sub> protein is responsible for the anti-apoptotic actions of glucocorticoid treatment when the apoptotic pathway is activated by UV-C.

Current research suggests that Bcl-2 family members exert their effects primarily at the mitochondria.<sup>19</sup> To determine if the Bcl-x<sub>L</sub> protein properly translocates in HTC cells, we examined whether the increased Bcl-x<sub>L</sub> protein was associated primarily with the cytosolic or mitochondrial compartments. HTC cells treated with or without Dex and then with or without UV-C irradiation were subjected to subcellular protein fractionation; a Dex-induced increase in Bcl-x<sub>L</sub> protein was detected by Western blot in both the cytosolic and mitochondrial fractions (Figure 5b). This increase in Bcl-x<sub>L</sub> was preserved in the mitochondrial fraction after UV-C irradiation while in the cytosolic fraction Bcl-x<sub>L</sub> protein was decreased (Figure 5b). Interestingly, Bcl-x<sub>L</sub> protein remaining elevated in the mitochondrial fraction even after UV-C irradiation supports the idea that the increase in Bcl-x<sub>L</sub> protein is correlated with the anti-apoptotic effects of Dex treatment.

Dex is a synthetic glucocorticoid that has been reported to bind using high doses to the drug metabolizing receptor PXR as well as the glucocorticoid receptor. As PXR actions have been reported to affect the expression of Bcl-2 family members,<sup>27</sup> we investigated whether our results could be recapitulated using the naturally occurring glucocorticoids cortisol and corticosterone which do not bind PXR. Using the same pretreatment times (24 h) and the same dose of UV-C irradiation (90 mJ/cm<sup>2</sup>) Bcl-x<sub>L</sub> protein levels were analyzed in cells treated with these hormones. These steroids also



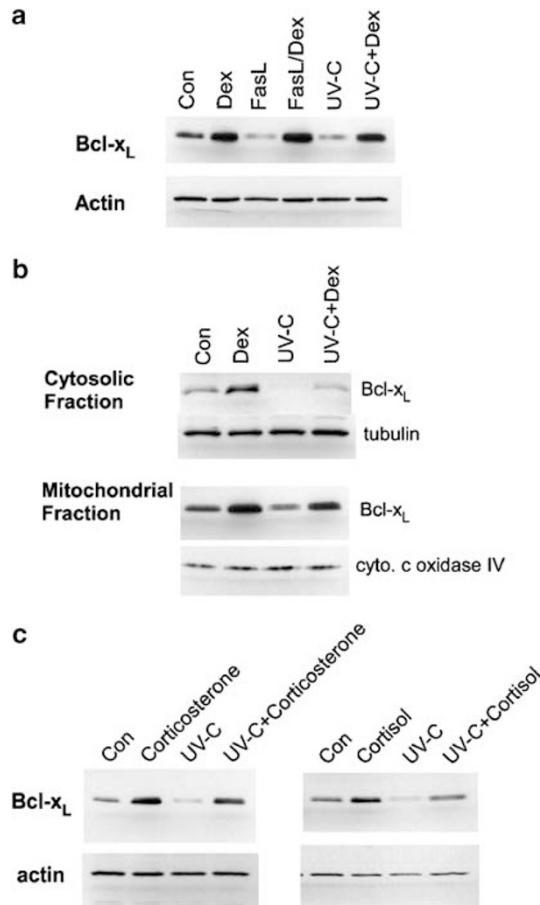
**Figure 4** Dex induces Bcl-x<sub>L</sub> mRNA and protein in HTC cells. (a) HTC cells were treated with or without Dex then left untreated or irradiated with UV-C. Protein lysates were analyzed by Western blotting for Bcl-x<sub>L</sub>, Mcl-1, Bax, Bid and actin (loading control). (b) HTC cells were treated with Dex or treated with Dex + RU486 for 0, 8, 16 and 24 h followed by total RNA isolation at each time point. Real-time PCR was performed with specific primers for rat Bcl-x<sub>L</sub>. The Bcl-x<sub>L</sub> mRNA increased by 8 h and remained elevated at 24 h. This increase was blocked by RU486. The results are averages ± S.E.M. of at least three separate experiments. (c) Cell lysates were assayed by western blot for presence of Bcl-x<sub>L</sub> protein 1. Representative Western blot of time course 2. Fold induction of Bcl-x<sub>L</sub> protein was determined by densitometry and plotted as averages ± S.E.M. of at least three experiments. (d) The Dex-induced increase in protein can be inhibited by co-administration of the antagonist RU486 and reduced to control levels by pre-treatment with RU486

induced an increase in Bcl-x<sub>L</sub> protein (Figure 5c). These results show that the increase in Bcl-x<sub>L</sub> is not restricted to the synthetic glucocorticoid, Dex, but can be regulated by the natural glucocorticoids corticosterone and cortisol found in rats and humans, respectively.

**siRNA knockdown of Bcl-x<sub>L</sub> protein.** Even though Dex increases Bcl-x<sub>L</sub> at the mRNA and protein level and these increases can be blocked by the antagonist RU486, there may be other apoptosis related genes affected by glucocorticoid receptor signaling. To test if the Dex-induced increase in Bcl-x<sub>L</sub> protein is the primary cause of the anti-apoptotic action of glucocorticoids on UV-C-induced apoptosis, we used small interfering RNA's (siRNA) to decrease transcription of the Bcl-x<sub>L</sub> gene and thus decrease the protein levels. Transfection of a random siRNA control (BLAST-searched to have no known homology to any gene sequence) did not interfere with the Dex induced increase in Bcl-x<sub>L</sub> protein. However, using siRNA sequences generated specifically for rat Bcl-x<sub>L</sub>, we decreased the induced levels of Bcl-x<sub>L</sub> protein as well as the endogenous levels (Figure 6). After transfecting siRNA to Bcl-x<sub>L</sub>, treating with or without Dex, we demonstrate that the

Dex-induced increase in protein is repressed (Figure 6). The data show this method can be used to repress Bcl-x<sub>L</sub> protein specifically in HTC cells thus allowing us to examine the consequences on the anti-apoptotic effects of Dex.

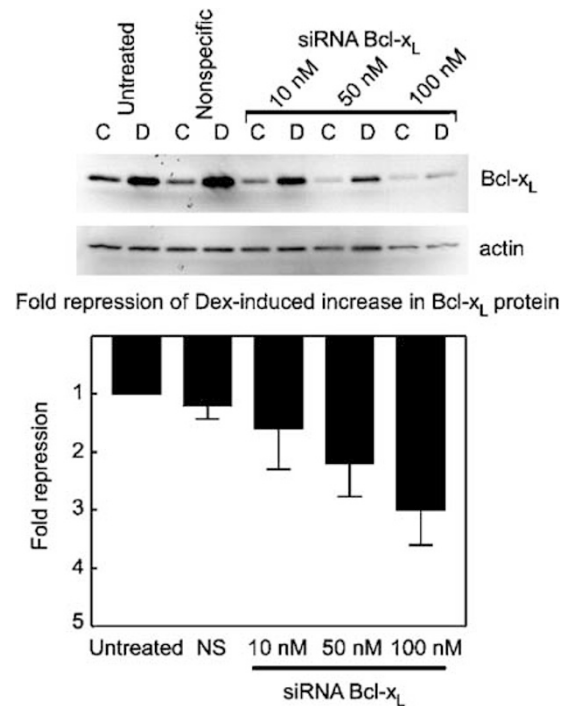
**Decreased Bcl-x<sub>L</sub> levels abrogate the anti-apoptotic effect of glucocorticoids.** Using this siRNA system to decrease the Bcl-x<sub>L</sub> protein in HTC cells, we returned to our original experimental paradigm to determine if Dex pretreatment was still anti-apoptotic in cells which had reduced levels of Bcl-x<sub>L</sub> as a result of siRNA treatment. Cells were transfected with 10, 20, 50 or 100 nM siRNA or non-specific RNA and incubated for 24 h, Dex treated for an additional 24 h, then UV-C irradiated. After a subsequent 24 h they were analyzed for apoptotic characteristics. *Apoptotic volume decrease.* Cells left untransfected or transfected with non-specific RNA display an increased population of shrunken cells after UV-C-irradiation (measured by forward scatter light as in Figure 1) and this population is decreased by pretreatment with Dex (Figure 7a). Cells transfected with siRNA to Bcl-x<sub>L</sub> show a dose-dependent relief of Dex-inhibition. Interestingly, the knock down of Bcl-x<sub>L</sub> to less than endogenous levels (which



**Figure 5** (a) Dex-induced increase in Bcl-x<sub>L</sub> protein is sustained during FasL or UV-C treatment. HTC cells were treated with Dex, FasL, UV-C, Dex + FasL or Dex + UV-C and lysates assayed for Bcl-x<sub>L</sub> protein by Western blot. Bcl-x<sub>L</sub> protein remains elevated in the Dex-pretreated cells regardless of apoptotic stimulus. Shown is a representative blot from at least three separate experiments. (b) Bcl-x<sub>L</sub> remains elevated in the mitochondrial compartment after UV-C treatment. HTC cells were pretreated with Dex and then UV-irradiated. A subcellular fractionation was performed to obtain cytosolic and mitochondrial fractions. Each fraction was assayed by western blot for Bcl-x<sub>L</sub>. Tubulin was used as loading control for cytosol and cytochrome c oxidase subunit IV used with the mitochondrial fraction. Dex caused an increase in Bcl-x<sub>L</sub> in both cytosolic and mitochondrial compartments. The protein levels remained elevated in the mitochondrial fraction even after UV-C irradiation. Shown are representative western blots from at least three separate experiments. (c) Effects of Dex can be recapitulated with naturally occurring glucocorticoids. HTC cells were pretreated with either 10 nM cortisol or corticosterone for 24 h and then UV-irradiated. 24 h postirradiation the cells were harvested for protein and assayed by Western blot for Bcl-x<sub>L</sub> protein. Both glucocorticoids caused an increase in Bcl-x<sub>L</sub>, which remained elevated even after UV-C irradiation

occurs with 50 and 100 nM siRNA, see Figure 6) causes the appearance of shrunken cells in the non-UV irradiated group (Figure 7a).

**Loss of  $\Delta\Psi_{mito}$ .** Non-transfected cells or cells transfected with non-specific siRNA show a population of cells with mitochondria showing a loss of  $\Delta\Psi_{mito}$  after UV-C irradiation. The anti-apoptotic effects of Dex are observed in both non-transfected and non-specific RNA transfected cells (Figure 7b). Cells transfected with increasing amounts of siRNA to Bcl-x<sub>L</sub> show a dose-dependent inhibition of the



**Figure 6** siRNA knockdown of Bcl-x<sub>L</sub> protein. HTC cells were transfected with increasing amounts of siRNA to Bcl-x<sub>L</sub>, a non-specific RNA or left untransfected. 24 h later the cells were harvested and analyzed by Western blotting. The highest amount of siRNA (100 nM) decreased the level of induced as well as endogenous Bcl-x<sub>L</sub> protein. Fold repression was plotted as averages ± S.E.M. of at least three separate experiments

anti-apoptotic effects of Dex. As seen with cell shrinkage reduction of endogenous levels of Bcl-x<sub>L</sub> protein causes the loss of  $\Delta\Psi_{mito}$  in non-irradiated cells suggesting that constitutive expression of Bcl-x<sub>L</sub> has a function in maintaining cellular viability in these hepatoma cells.

**DNA degradation.** Non-transfected cells or cells transfected with non-specific siRNA show a population of cells with sub-diploid or degraded DNA after UV-C irradiation. The number of cells with degraded DNA is decreased by pretreatment with Dex (Figure 7c). Cells transfected with siRNA to Bcl-x<sub>L</sub> no longer show an anti-apoptotic effect of Dex pretreatment (Figure 7c). However, as with cell shrinkage and loss of  $\Delta\Psi_{mito}$ , knockdown of endogenous Bcl-x<sub>L</sub> protein causes DNA degradation without additional apoptotic stimulus. These results support the premise that the increase in Bcl-x<sub>L</sub> protein is primarily responsible for the anti-apoptotic actions of Dex. Interestingly, the appearance of apoptotic characteristics in non-UV-C-treated cells in which the endogenous Bcl-x<sub>L</sub> has been reduced suggests that this hepatoma cell line requires endogenous levels of Bcl-x<sub>L</sub> to maintain viability.

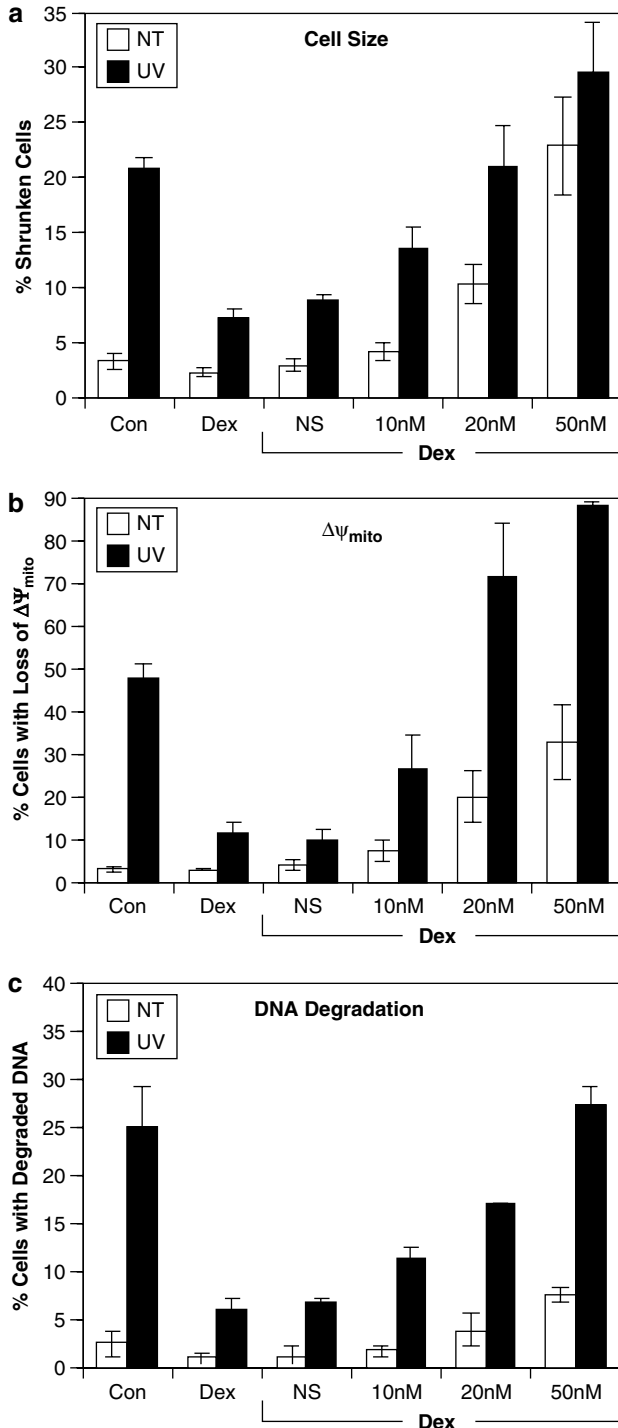
## Discussion

Dexamethasone acting via the glucocorticoid receptor can have many, quite often opposing effects depending on the cell type, state of differentiation or phase of the cell cycle. Glucocorticoids can induce apoptosis in thymocytes and other cells of lymphoid origin; where in other cell types these

hormones can be anti-apoptotic. This seeming contradiction can be at least partially explained by the fact that the glucocorticoid receptor has many proposed mechanisms of action such as direct transcriptional activation by specific responsive elements or more indirect repressive mechanisms via interference with other transcription factors such as AP-1 and NFκB. It is interesting to note that glucocorticoids have even been reported to have opposing effects on the same gene depending on the cell type. For example the Bcl-x gene

was reported to be downregulated by dexamethasone in rat thymocytes which die in response to Dex,<sup>28</sup> but upregulated in mouse fibrosarcoma cells.<sup>25</sup> Interestingly, the mouse Bcl-x gene has been reported to have five different promoters which exhibit a tissue-specific pattern of promoter usage.<sup>29</sup> This is a possible explanation of the differing effects of glucocorticoids on apoptosis in cells of lymphoid and non-lymphoid origin. While the induction of the Bcl-x<sub>L</sub> mRNA in mammary epithelial and fibrosarcoma cells has been reported there are several other examples of anti-apoptotic actions of glucocorticoids that do not involve the regulation of Bcl-x<sub>L</sub> such as the upregulation of serum and GC-inducible protein kinase-1 (SGK-1)<sup>30</sup> and mitogen-activated protein kinase phosphatase-1 (MKP-1).<sup>31</sup> In addition, dexamethasone alone<sup>32</sup> or in conjunction with TNF-alpha can induce the cellular inhibitor of apoptosis-2 (c-IAP2) gene.<sup>33</sup> Thus the precise mechanism for the anti-apoptotic effects of glucocorticoids remains unclear.

In the present study, we wished to further delineate the mechanism of the anti-apoptotic effect of Dex on the rat hepatoma cell line, HTC. We show in conjunction with our previously reported studies that Dex can protect HTC cells from apoptosis induced by stimuli that affect primarily the intrinsic or mitochondrial pathway (UV-irradiation) but not from apoptosis induced solely via the extrinsic pathway (FasL). We also demonstrate that the anti-apoptotic protein Bcl-x<sub>L</sub> is upregulated at the mRNA and protein level by glucocorticoids in a receptor and time dependent manner, which coincides with pretreatment times necessary for anti-apoptotic actions of glucocorticoids. Interestingly, the Bcl-x<sub>L</sub> protein remains elevated in the mitochondrial compartment even during UV-treatment. When this increase in Bcl-x<sub>L</sub> is abrogated by co-treatment with RU486 or gene knockdown with siRNA there is no longer an inhibition of UV-C-induced apoptosis providing direct evidence for its role in mediating the anti-apoptotic effects of glucocorticoids. These results are also in agreement with current research which states that Bcl-x<sub>L</sub> exerts its protective effects at the mitochondrial membrane and so would be expected to act on the intrinsic apoptotic pathway (for review Chan and Yu<sup>34</sup>). In contrast, the extrinsic or death receptor pathway can bypass mitochondrial signaling through direct activation of caspase 3 by caspase 8<sup>13,15</sup> thus it is not surprising that increased levels of Bcl-x<sub>L</sub> would not provide significant protection in this scenario. Interestingly, our results differ from Oh *et al*<sup>35</sup> who recently reported that primary



**Figure 7** Knockdown of Bcl-x<sub>L</sub>: effects on cell shrinkage, changes in  $\Delta\Psi_{mito}$ , DNA degradation. HTC cells were transfected with 10, 20, 50 or 100 nM siRNA to Bcl-x<sub>L</sub> or a non-specific RNA. At 24 h later they were treated with Dex for 24 h followed by UV-C irradiation. After a final 24 h incubation the cells were harvested and assayed by flow cytometry. Results from at least three different experiments were averaged and displayed as percentage of shrunken cells for untreated, Dex treated, UV-C irradiated and Dex + UV-C. (a) UV-C causes the appearance of a population of shrunken cells. This population is decreased by pretreatment with Dex. Knockdown of Bcl-x<sub>L</sub> protein dose dependently abrogates the effect of Dex. (b) UV-C causes the appearance of a population of cells with depolarized  $\Delta\Psi_{mito}$ . This population is decreased by pretreatment with Dex and this decrease is abrogated by knockdown of Bcl-x<sub>L</sub> protein. (c) UV-C causes the appearance of a population of cells with degraded (subdiploid) DNA. Pretreatment with Dex inhibits this population. Knockdown of Bcl-x<sub>L</sub> protein abrogates this effect



hepatocytes could be protected from Fas ligand induced apoptosis by large doses of dexamethasone (5  $\mu$ M) via upregulation of cFLIP. We attempted to reproduce their results in our hepatoma cells, however, we found no anti-apoptotic effects of Dex either with physiological doses (Figure 2) or the higher dose used by Oh *et al* (data not shown). It would be of great interest to see if their results could be recapitulated with naturally occurring glucocorticoids since high doses of Dex are known to activate PXR. Additionally we did not detect any cFLIP protein in HTC cells (data not shown). This finding plus the fact of differing cell types – normal *versus* cancer cell – provides a possible explanation of the differences and does not negate the significance of our finding that a widely used class of pharmaceuticals can inhibit apoptosis in certain cancer cells. Indeed there is a clinical study by Herr *et al*<sup>36</sup> reporting glucocorticoid's protective effect on solid tumors. Another clinical study by Sui *et al*<sup>37</sup> demonstrates that glucocorticoids interfere with paclitaxel treatment of breast and ovarian xenograft tumors. There is a study reporting that the non-steroidal antibiotic rifampicin can protect Jurkat cells from Fas ligand induced apoptosis presumably via the glucocorticoid receptor.<sup>38</sup> Whether rifampicin is actually working through the GR is controversial,<sup>39</sup> however Jurkat cells are reported to be type II cells which require a mitochondrial amplification loop via Bid for effective death receptor killing. Upregulation of Bcl-x<sub>L</sub> (which they report as well as upregulation of cFLIP and Bcl-2) in this instance might be expected to protect Jurkat cells from Fas ligand-induced apoptosis.

Another interesting finding in this study was that suppression of the endogenous levels of Bcl-x<sub>L</sub> in these cells via siRNA knockdown sensitized them to cell death whereas transfection of a nonspecific RNA had no significant effect on viability. This data suggests that these hepatoma cells must utilize increased endogenous levels of Bcl-x<sub>L</sub> as a growth or survival advantage during transformation. This scenario for cancer cells has been described by Takehara *et al*<sup>40</sup> in HepG2 cells where repression of Bcl-x<sub>L</sub> protein sensitized them to stress-induced apoptosis.

The fact that glucocorticoids are a highly prescribed class of pharmaceuticals, understanding the effects of these hormones on apoptosis is vitally important for proper treatment of inflammatory disease and cancer. Not only are glucocorticoids given as chemotherapeutics in leukemias – where they are well-known to induce apoptosis – they are also given as adjunct therapy in other types of cancers to relieve side effects of chemotherapy. There is emerging evidence that glucocorticoids can have inhibitory effects on treatment of non-lymphoma/leukemic cancers.<sup>36</sup> In view of the data presented here and elsewhere we suggest their use as adjunct therapy be re-evaluated.

## Materials and Methods

**Cell Culture and steroid treatment.** The HTC rat hepatoma cell line (originally derived from the Morris 7288c hepatoma) was the generous gift of Dr Stoney Simons (NIDDK, NIH). HTC cells were cultured in DMEM-H medium containing 5% heat-inactivated fetal calf serum, 2 mM glutamine, 75 U/ml streptomycin, and 100 U/ml penicillin at 37°C, 5% CO<sub>2</sub> atmosphere. The cells were maintained in T75 tissue culture flasks and passaged every other day at a 1 : 5 dilution. The day before treatment the cells were split 1 : 40 into six-well plates in

complete media and returned to the incubator. The following day the cells were left untreated or treated with 10 nM glucocorticoid by dilution of a sterile 10<sup>-5</sup> M stock solution of dexamethasone (Dex) in dH<sub>2</sub>O or 10<sup>-5</sup> M stock solution of cortisol or corticosterone in EtOH 100% (all steroids from Steraloids, NH) and 24 h later the cells were induced to undergo apoptosis with either UV-C or FasL.

**Apoptosis induction by UV-C.** HTC cells in 3 ml complete media were irradiated in six-well tissue culture plates or 35 mm glass bottom culture dishes (MatTek, Ashland, MA, USA). The tops of the tissue culture plates were removed to prevent shielding by the polystyrene. The plates containing the cells were placed in a Stratalinker (Stratagene) set for a 90 mJ/cm<sup>2</sup> dose of UV-C radiation which was achieved after 80 s. Postirradiation the samples were returned to 37°C, 5% CO<sub>2</sub> atmosphere for further incubation.

**Apoptosis induction with FasL.** HTC cells in 3 ml complete media were treated with 50 ng/ml FasL (Kamiya Biomedical, Seattle, WA, USA) and returned to 37°C, 5% CO<sub>2</sub> atmosphere. Both UV- and FasL-treated cells were analyzed after 24 h incubation. HTC cells at this dose of Fas ligand undergo apoptosis at a similar rate to the dose of UV-C described previously.

**Microscopy.** HTC cells were plated in 35 mm glass bottom culture dishes (MatTek, Ashland, MA, USA) and treated as described above. After 24 h of either UV-C, FasL, Dex, UV-C + Dex, FasL + Dex or no treatment HTC cells were visualized with a Zeiss 510 LSM-UV confocal microscope using DIC optics and a  $\times$  40 oil objective. Multiple pictures were taken of each treatment group from three separate experiments and representative pictures were used in the final figure.

**Whole cell protein extraction.** Following experimental treatment cells were suspended in buffer containing 20 mM TRIS, 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100 and protease inhibitors (Complete Mini protease cocktail, Roche, IN, USA) by pipeting. The cells were sonicated for 10 s on ice. A 5  $\mu$ l sample of each lysate was assayed in a Beckman DU650 spectrophotometer for protein concentration using Biorad Protein Assay Reagent (Biorad Laboratories, CA, USA). Laemmli loading buffer containing glycerol, SDS, and bromphenol blue was added to each sample at a 1 : 1 ratio and then boiled for 5 min. The samples were stored at -80°C until electrophoresis and Western blot analysis for Bcl-x<sub>L</sub> protein.

**Subcellular Fractionation to separate mitochondrial and cytosolic proteins.** Following experimental treatment cells were subjected to subcellular fractionation using a Mitochondrial/Cytosol Fractionation Kit (Biovision, Mountain View, CA, USA) following the manufacturer's recommended protocol. Treated and untreated cells ( $5 \times 10^7$ ) were collected by centrifugation at 600  $\times$  g for 5 min at 4°C. Cells were then washed with ice-cold PBS and centrifuged as above. The supernatant was discarded and the cells were resuspended in 1 ml 1  $\times$  cytosol extraction buffer with DTT and protease inhibitors (included in the kit) and incubated on ice for 10 min. The cells were then homogenized with an ice-cold dounce tissue grinder (50 passes). The homogenate was centrifuged at 700  $\times$  g for 10 min at 4°C and supernatant collected (pellet discarded). The supernatant was then centrifuged at 10 000  $\times$  g for 30 min at 4°C. The supernatant was collected as cytosolic fraction and the pellet resuspended in mitochondrial extraction buffer and vortexed for 10 s and collected as mitochondrial fraction. The protein concentration of each fraction was determined by the Biorad method described previously. After the protein concentration was determined the samples were mixed 1 : 1 with Laemmli loading buffer and boiled for 5 min. The samples were then stored at -80°C until assayed for Bcl-x<sub>L</sub> by Western blot.

**Western blot analysis.** Whole cell, mitochondrial or cytosolic protein lysates, 40  $\mu$ g/sample were separated on 4–20% polyacrylamide tris/glycine gels (Invitrogen, Grand Island, NY, USA) and transferred to nitrocellulose. After 2 h of blocking non-specific interactions with Tris-buffered saline (TBS) plus 0.5% Tween 20 with 10% Carnation nonfat-dry milk the membranes were incubated with primary antibodies to Bcl-x<sub>L</sub>, caspase 3 (Cell Signaling, Beverly, MA, USA) Mcl-1 (Trevigen, Gaithersburg, MD, USA) or Bid (Stratagene, La Jolla, CA, USA) and subsequently incubated with the appropriate secondary antibody (Amersham, Piscataway, NJ, USA), following the manufacturer's recommended protocol and visualized on film with the ECL chemiluminescent system (Amersham). The membranes were then stripped with buffer containing 2% SDS, 60 mM TRIS and  $\beta$ -mercaptoethanol at 55°C for 30 min, washed and reprobed with a monoclonal actin antibody (Chemicon, Temecula, CA, USA) or an  $\alpha$ -tubulin antibody (Sigma-Aldrich, MO,

USA) as a control for equal loading. Cytochrome oxidase subunit IV antibody (Molecular Probes, Eugene, OR, USA) was used for mitochondrial fraction control.

**Determination of change in cell size.** Cell size and changes in the light scattering properties of the cells were determined by flow cytometry with a Becton Dickinson FACSsort cytometer equipped with CellQuest software.<sup>41,42</sup> In each sample 10 000 cells were examined by exciting the cells with a 488 nm argon laser and by determining their distribution on a forward-scatter versus side-scatter dot plot. Light scattered in the forward direction is proportional to cell size, while light scattered at a 90° angle (side scatter) is proportional to cell granularity. As a cell shrinks or loses cell volume, a decrease in the amount of forward-scattered light is observed along with a change in side-scattered light. Propidium iodide (10 μg/ml) was added as a determinant of viability. This dye does not enter cells with an intact plasma membrane. Therefore, gating on propidium iodide negative cells defines the viable population. Only these cells were used in analyzing cell size. The resulting data were converted to contour plots for presentation.

**Mitochondrial membrane potential.** Changes in the mitochondrial membrane potential ( $\Delta\psi_{\text{mito}}$ ) were measured by flow cytometry using the mitochondrial potential dye, JC-1 (Molecular Probes, OR, USA).<sup>42,43</sup> At 30 min prior to analysis, JC-1 was added to 1 ml of cells (while vortexing) to a final concentration of 10 μM and incubated at 37°C, 5% CO<sub>2</sub> atmosphere. JC-1 has variable emission depending on the state of the mitochondrial membrane potential. JC-1 forms aggregates in mitochondria with normal  $\Delta\psi_{\text{mito}}$  and emits fluorescence in the orange range (~580 nm). A decrease in the  $\Delta\psi_{\text{mito}}$  results in a reduction in orange fluorescence with a concurrent gain in green fluorescence (~520 nm) as the dye shifts to a monomeric state. Loss of the dye in the mitochondria can be monitored through the increase in green fluorescence. Ten thousand cells per sample were analyzed and monomer versus aggregate fluorescence plots were used for subsequent analysis. Contour plots (aggregates versus monomers) were used for presentation.

**DNA analysis.** Cells were prepared for analysis of DNA content by slow addition of ice-cold 70% ethanol to 1 × 10<sup>6</sup> cells (final volume 5 ml) and stored overnight at 4°C. Following fixation the cells were pelleted, washed one time with PBS, then stained with 1 ml of 20 μg/ml propidium iodide, containing 1000 U of RNase One (Promega) in 1 × PBS for 20 min. Cells were analyzed by flow cytometry on a FACSsort cytometer (Becton-Dickinson) by gating on an area versus width dot plot to exclude debris and aggregates, and 10 000 cells were examined on 585 nm fluorescence/area histogram.<sup>41</sup> The percent of cells with degraded DNA was determined by the number of cells with subdiploid DNA divided by the total number of cells examined under each experimental condition.

**Real-time PCR analysis.** HTC cells were either treated for 24 h with 10 nM Dex, 10 nM Dex plus 100 nM RU486 or left untreated. Total cellular RNA was isolated using RNeasy columns (QIAGEN, CA, USA). Isolated RNA content was determined at 260 nm and samples were stored at -70°C until further use. Equal amounts of RNA for each sample were reverse-transcribed using an oligo d(T)<sub>16</sub> primer (ABI, CA, USA) and TaqMan<sup>®</sup> Reverse Transcription Reagents (ABI, CA, USA). The cDNA was quantitated in a PCR reaction with Taq polymerase for 40 cycles with a denaturing step at 95°C for 15 s and an annealing/extension step at 60°C for 60 s performed on an ABI 7900 real-time PCR machine. The forward primer for rBcl-x<sub>L</sub> was GGGCACTGTGCGTGGAA and the reverse primer was TGCCATCCAACTTGCAAT. The TaqMan TAMRA probe used was FAM-AACAGGACATTTTGTGGATCTCTACGGAA-TAMRA. From the obtained C<sub>T</sub> values a  $\Delta C_T$  value for rBcl-x<sub>L</sub> - rCyclophilin B (Assay-on-demand predeveloped primer/probe set, ABI) was calculated. The fold upregulation was calculated by comparing the  $\Delta C_T$  of untreated cells to that of Dex or Dex plus RU486 treated HTC cells.

**siRNA downregulation of Bcl-x<sub>L</sub> protein.** siRNA specific for rat Bcl-x<sub>L</sub> was generated by Dharmacon Inc. (Lafayette, CO, USA). The target sequence was AAAGCAUUCAGUGAUCUAACA, the sense sequence was AGCAUUCAGUGAUCUAACAAdTdT, and the antisense was UGUUAGAUCACUGAUGCudTdT. A non-specific negative control RNA (BLAST searches verified that it contained no homology to any known motifs) was also obtained from Ambion (Austin, TX, USA). A confluent T75 flask of HTC cells was split 1:20 into six-well plates. The next day the media was changed to OptiMem (Invitrogen-Gibco, CA, USA) with no additives and the cells transfected

with siRNA for Bcl-x<sub>L</sub>, negative control or no RNA using Metafectene (Biontex, Munich, Germany). After 4 h the cells were returned to complete media. The third day the cells were treated with hormone and the fourth day UV-C irradiated (always one plate of cells left non-treated for controls). On the fifth day the cells were harvested for analysis by flow cytometry and western blotting as previously described.

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