Dexamethasone-resistant Human Pre-B Leukemia 697 Cell Line Evolving Elevation of Intracellular Glutathione Level: An Additional Resistance Mechanism

Hitoshi Inoue,[1] Haruyuki Takemura,[1] Yasukazu Kawai,[1] Akira Yoshida,[1] Takanori Ueda[1, 3] and Toshiyuki Miyashita[2]

[1]First Department of Internal Medicine, Fukui Medical University, 23-3 Shimoaizuki, Matsuoka-cho, Yoshida-gun, Fukui 910-1193 and [2]National Children's Medical Research Center, 3-35-31 Taishido, Setagaya-ku, Tokyo 154-8509

Glucocorticoids remain among the most important drugs in the treatment of acute lymphoblastic leukemia (ALL). Although the mechanisms of glucocorticoid resistance have been studied in some T-cell leukemic cell lines, less work has been done with B-cell lines. We established a dexamethasone (DEX)-resistant human pre-B lineage leukemia cell line (697/DEX) and investigated the mechanism of resistance. 697/DEX was over 430-fold more resistant to DEX compared with the parental cells (697/Neo). Overexpression of Bcl-2 protein was not observed in 697/DEX, different from the mechanism of resistance in Bcl-2-virus-infected cells (697/Bcl-2). Although the expression of p-glycoprotein (Pgp) in 697/DEX was positive, its functional activity was not detected. The numbers of glucocorticoid receptors (GR) in 697/DEX and 697/Bcl-2 were significantly lower than those in 697/Neo. In addition, 697/DEX and 697/Bcl-2 had higher levels of glutathione (GSH) than 697/Neo. In the presence of L-buthionine-(S,R)-sulfoximine (BSO), an inhibitor of GSH synthesis, both 697/DEX and 697/Bcl-2 recovered their sensitivity to DEX. Interestingly, cell death by the depletion of GSH did not involve caspase-3/7 activation in 697/Bcl-2 and 697/DEX, different from 697/Neo, suggesting a death mechanism through caspase-independent programmed cell death or necrosis. In conclusion, DEX-resistance in 697/DEX was related not only to a GR decrease, but also to an increase in intracellular GSH level in the DEX-resistant B-cell leukemia cell line. Circumvention of DEX-resistance with BSO may offer an approach to overcoming resistance to chemotherapy in B-cell lineage ALL.

Key words: Dexamethasone (DEX) — 697 cells — Bcl-2 — Glutathione (GSH) — Caspase-3

Recent progress in combination chemotherapy for ALL has resulted in a high remission rate, reaching 60–80% in many multi-institutional trials.[1–3] However, the cure rates in most trials still remain low at 20–40%.[3] The non-curable cases are associated with the emergence of resistant clones of malignant cells.

Treatment of ALL has included glucocorticoids for almost 50 years.[1, 2, 4] Although 70–90% of ALL primarily respond to single use of glucocorticoids, many cases become resistant to glucocorticoids at relapse.[1] Moreover, PSL resistance is significantly higher in adults than in childhood.[5] Recently, glucocorticoids have been the subject of renewed interest. *In vitro* and *in vivo* responses of leukemic cells to glucocorticoids have highly predictive outcomes.[6] Kaspers *et al.* reported that *in vitro* resistance to PSL was significantly related to the short-term and long-term clinical response to chemotherapy[7] and *in vitro* resistance to PSL was related to the probability of disease-free survival after combination chemotherapy.[8] Given this, glucocorticoids still remain among the key drugs, as do vinca alkaloids and anthracyclines.

However, the mechanisms of resistance to glucocorticoids have not yet been precisely clarified, though several studies have been reported. Glucocorticoid resistance is most commonly linked to altered receptor number or function. Low expression of GRs was associated with a poor response to glucocorticoid-based therapies and a higher risk of relapse.[9, 10] As Pgp has been shown to transport DEX in experimental cell lines, increased expression of Pgp may be important for glucocorticoid resistance.[11] It has also been reported that overexpression of Bcl-2 is

related to resistance to glucocorticoid-induced apoptosis.[12, 13] An earlier study by Miyashita and Reed using 697/Bcl-2, which is a stable transfectant with a recombinant Bcl-2-containing retrovirus,[14] demonstrated that high levels of Bcl-2 protein protected human pre-B 697 cells from the acute cytotoxic effects of DEX. However, it remains unclear whether Bcl-2 also has a central role in the mechanism of DEX resistance in acquired-resistant cell lines. In addition, the mechanisms of glucocorticoid resistance have been studied *in vitro* in some T-cell leukemic cell lines, such as CEM.[15, 16] However, less work has been done with resistant B-cell leukemic cell lines.[13, 14]

In this study, we established a DEX-resistant human pre-B lineage leukemia cell line (697/DEX) from human pre-B lineage leukemia cells (697/Neo) and investigated the mechanism of resistance. We also investigated the difference of drug resistance mechanisms between the acquired DEX-resistant cell line and the Bcl-2-transfected cell line (697/Bcl-2). The possibility of elevation of the GSH redox pathway, which has received attention as a mechanism of clinical resistance in leukemia[17] and of relatively poor prognosis in ALL,[18] was also investigated.

MATERIALS AND METHODS

Cell lines 697/Neo and 697/Bcl-2 cell lines were established from a human pre-B leukemia cell line derived from child ALL.[14] These cells are infected with control retrovirus (697/Neo) and recombinant Bcl-2 retrovirus (697/Bcl-2), respectively.[14] The DEX-resistant cell line was obtained by exposure of 697/Neo to stepwise increasing concentrations (from 1 nM to 1 μM) of DEX. When its doubling time in the presence of 1 μM DEX was similar to that of parental 697/Neo in the absence of DEX, the cells were cloned by the limiting dilution method. The established 697/DEX was more than 430-fold resistant to DEX. 697/Neo, 697/Bcl-2 and 697/DEX were maintained by serial culture in RPMI-1640 medium (Nissui, Tokyo) containing 10% fetal bovine serum (FBS; Gibco BRL, Life Technologies, Rockville, MD) and kanamycin (Meiji Seika Co., Tokyo) at 37°C in humidified air with 5% CO_2.

Anticancer drugs, reagents and chemicals DEX and BSO were obtained from Nacalai Tesque, Inc. (Kyoto). PSL was from Shionogi Co., Ltd. (Osaka). VCR, DNR and VP-16 were kindly supplied by Eli Lilly and Co. (Indianapolis, IN), Meiji Seika Co. (Tokyo) and Nippon Kayaku Co., Ltd. (Tokyo), respectively. MTX was purchased from Sigma (St. Louis, MO). PTX and calcein-AM were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). [3H]DEX (45 Ci mmol^{-1}) was purchased from Japan Daiichi Pure Chemicals (Tokyo). NADPH and GSH (reduced form) were obtained from Kohjin Co. (Tokyo). Anti-Bcl-2 monoclonal antibody (MoAb) (clone 124) was obtained from DAKO Japan Co., Ltd. (Kyoto). UIC2 was

obtained from Immunotec (Marseille, France) and MRPm6 was obtained from Kamiya Biomedical Co. (Seattle, WA). The fluorogenic substrate for caspase-3/7, Ac-DEVD-MCA, and caspase-3 family protease inhibitor, Ac-DEVD-CHO, were purchased from Peptide Institute, Inc. (Osaka). Other chemicals were obtained from usual commercial sources.

Cytotoxicity assay Cytotoxicity was determined after a 72 h incubation by the Trypan blue dye exclusion method. At selected time points, colony-forming assay was performed concurrently to compare survival with growth inhibition. The values are presented as means±SD of three separate experiments, with triplicate determinations. The resistance index (RI) was calculated by dividing the 50% inhibitory concentration (IC_{50}) of resistant cells by the IC_{50} of 697/Neo cells.

Measurement of Bcl-2 protein expression by flow cytometry For the immunodetection of the intracellular Bcl-2 protein, the paraformaldehyde/Triton (PFT) protocol was performed as described previously.[19, 20] Indirect immunofluorescence was carried out using anti-Bcl-2 MoAb (clone 124) and fluorescein isothiocyanate (FITC)-conjugated goat antimouse MoAb (BioErgonomics, Inc., St. Paul, MN). As a negative control for immunofluorescence, normal mouse serum at a final concentration of 1:400 was employed. The cells were analyzed using a Becton Dickinson FACScan (FACS Caliber) (San Jose, CA). The results are expressed as a percentage of positive cells compared to background fluorescence, for which mouse IgG was used in place of the MoAb, or mean fluorescence intensity (MFI; sample mean channel/control mean channel).

Detection of Pgp and MRP Pgp was analyzed by the method of Chaudhary *et al.*[21] with the Pgp monoclonal antibody PE-UIC2. MRP was analyzed by the method of Flens *et al.*[22] with the MRP monoclonal antibody m6 using a Becton Dickinson FACScan (FACS Caliber). As a negative control, we used the mouse IgG in place of MoAb.

DEX uptake and efflux assay The amounts of DEX accumulated by 697/Neo and 697/DEX were evaluated using [3H]DEX. Briefly, an aliquot of 2×10^6 cells were incubated with 1 μM of [3H]DEX with or without 10 μg/ml verapamil at 37°C for 90 min. After washing, scintillation liquid was added to the cell pellet, and the radioactivity was determined with a scintillation counter. Rhodamine 123 efflux assay was performed by the method of Webb *et al.*[23] Briefly, 5×10^5 resistant cells were incubated with 100 ng/ml of Rhodamine 123 with or without 10 μg/ml verapamil, an inhibitor of Pgp, for 30 min at 37°C. The cells were washed twice in PBS, resuspended in RPMI and efflux was allowed to proceed for 3 h at 37°C. The cells were then washed twice and analyzed on a Becton Dickinson FACScan (FACS Caliber). Calcein efflux assay was performed by using the method of Feller *et al.* with

some modification.[24, 25)] Briefly, 1×10^6 cells were incubated with 0.5 μM calcein-AM with or without 2 mM probenecid, an inhibitor of MRP, for 15 min at 37°C. After centrifugation, cells were resuspended in fresh medium, and the efflux of calcein was allowed to proceed for 90 min at 37°C. Intracellular calcein accumulation was determined by measuring calcein fluorescence through a 530 nm bandpass filter with excitation at 488 nm using a Becton Dickinson FACScan (FACS Caliber).

GR determination The number of specific GR was determined in terms of the binding of [^{3}H]DEX using a whole-cell assay[26)] with 5 different concentrations (1–50 nM) of [^{3}H]DEX in the presence (1–50 μM, respectively) and absence of unlabeled DEX at 37°C for 90 min. Specific binding was calculated as the number of binding sites per cell from the difference between the total and non-specific binding according to the method of Scatchard.[27)]

GSH measurement Total intracellular GSH content was measured by the Tietze method, as previously described, with minor modifications.[28)] Briefly, 5×10^6 cells were prepared, washed 2 times with ice-cold PBS, and suspended in 125 mM sodium phosphate buffer containing 6.3 mM EDTA, pH 7.5 (sodium phosphate buffer). The cells were lysed by sonicating four times at 130 W for 10 s at 4°C. Then 12% 5-sulfosalicylic acid was added to the lysates, and precipitation was allowed to occur for 2 h at 4°C. After centrifugation at 10 000g for 15 min, protein-free lysates were obtained. The reaction mixture for determination of the GSH content consisted of the lysates, 0.3 mM NADPH, 6 mM 5,5′-dithiobis-(2-nitrobenzoic acid), and 0.5 units of GSH reductase. The absorbance at 412 nm was monitored for 6 min using a plate reader (SPECTRA Max250) (Molecular Devices Co., Sunnyvale, CA). The GSH content was calculated from the change in the rate of absorbance on the basis of a standard curve.

GSH depletion assay GSH depletion was achieved by 24 h preincubation of the cells in RPMI-1640 supplemented with 15 μM BSO.[29)] Then, the cytotoxicity was determined after 72 h incubation with various concentrations of DEX. The IC$_{50}$ was determined by the Trypan blue dye exclusion method.

Caspase-3/7 enzyme activity For the detection of caspase-3/7 activity, we used Dimmeler's method.[30)] Briefly, 1×10^6 cells were lysed in buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM DTT, 10 mM Tris/HCl, pH 8) for 15 min at 4°C, followed by centrifugation (20 000g, 2 min). The caspase-3/7 activity was detected by measuring the fluorogenic MCA liberated from the caspase-3-like substrate, Ac-DEVD-MCA, in assay buffer (100 mM Hepes, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), pH 7.5, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM DTT) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. To confirm the inhibition of caspase-3/7, a control reaction was performed by incubating an induced sample with a caspase-3 family protease inhibitor, Ac-DEVD-CHO, before addition of the Ac-DEVD-MCA.

Statistical analysis Values are expressed as means±SD. Statistical differences were evaluated by using Student's t test, as for GR and GSH. $P<0.05$ was considered significant.

RESULTS

Establishment of DEX-resistant cell line and its cross-resistance to other antileukemia drugs The IC$_{50}$ values of 697/Neo, 697/DEX and 697/Bcl-2 to DEX were 23.3±5.7 nM, more than 10 μM, and more than 10 μM, respectively. Both 697/DEX and 697/Bcl-2 were over 430-fold more resistant to DEX than 697/Neo. A colony-forming assay with 697/DEX and 697/Bcl-2 also revealed over 400-fold resistance to DEX. The cytotoxicities of other antileukemic drugs to 697/DEX were compared with those to 697/Neo following incubation for 72 h in the presence of each drug. 697/DEX was over 140-fold more resistant to PSL, but showed weak resistance to DNR (2.4-fold) and no cross-resistance to VCR, PTX, VP-16 or MTX (Table I). A colony-forming assay in 697/DEX also revealed a 2.8-fold resistance to DNR.

The expression of Bcl-2 protein in 697/DEX To determine the expression of the Bcl-2 protein in 697/DEX compared with 697/Bcl-2 cells, we performed immunofluorescence analysis by flow cytometry. The expression of the Bcl-2 protein was at substantially the same level in 697/DEX as in 697/Neo (Fig. 1). The MFI of 697/Neo,

Table I. Cross Resistance to Antileukemic Drugs in 697/DEX

Drugs	IC$_{50}$ (nM)		
	697/Neo[a)]	697/DEX[a)]	Fold resistant
DEX	23.3±5.7	$10^5<$	430<
PSL	71.7±23.6	$10^5<$	140<
VCR	3.7±2.6	4.6±1.4	1.2
PTX	0.6±0.3	0.6±0.3	1.0
VP-16	86.6±2.9	89.3±4.0	1.0
DNR	2.8±1.2	6.8±0.6	2.4
MTX	88.3±2.9	73.3±7.6	0.8

Cytotoxicity was determined after 72 h incubation with various concentrations of each drug. The IC$_{50}$ was determined by the Trypan blue dye exclusion method.
a) The values are presented as means±SD of three separate experiments, with triplicate determination.
DEX, dexamethasone; PSL, predonisolone; VCR, vincristine; PTX, paclitaxel; VP-16, etoposide; DNR, daunorubicin; MTX, methotrexate.

697/DEX and 697/Bcl-2 were 15.6±1.0, 15.2±0.8, and 41.1±6.2, respectively. These data indicate that the over-expression of Bcl-2 protein was not associated with the resistance to DEX in 697/DEX, different from 697/Bcl-2.

DEX uptake and efflux assay The expression of efflux transporter molecules is one factor influencing drug resistance. According to [³H]DEX efflux assay, the amounts of DEX accumulated in 697/DEX with and without verapamil were 476.7±40.2 and 480.6±61.4 dpm, respectively. The expression of Pgp was positive in 697/DEX, but not in 697/Neo by direct immunofluorescence assay (Fig. 2). Interestingly, the Pgp efflux pump in 697/DEX did not work in Rhodamine 123 efflux assay using verapamil as a Pgp modulator (Fig. 3), suggesting that 697/DEX has a non-functional mutant of Pgp on its membrane. MRP was not expressed in 697/DEX by direct immunofluorescence assay (Fig. 4A), and also did not work in calcein efflux assay using probenecid as an MRP modulator (Fig. 4B).

GR determination The roles of the GRs were evaluated in these cell lines. Radioligand binding studies were performed to determine the density of the binding sites of 697/Neo, 697/DEX and 697/Bcl-2. The binding site numbers of 697/Neo and 697/DEX amounted to 11 050±437 and 3390±786 sites per cell, respectively ($P=0.0001$). These results showed that the GRs in 697/DEX were 3-fold lower than those in 697/Neo. In addition, the binding site numbers of 697/Bcl-2 were also significantly reduced to 6280±830 sites per cell ($P=0.001$).
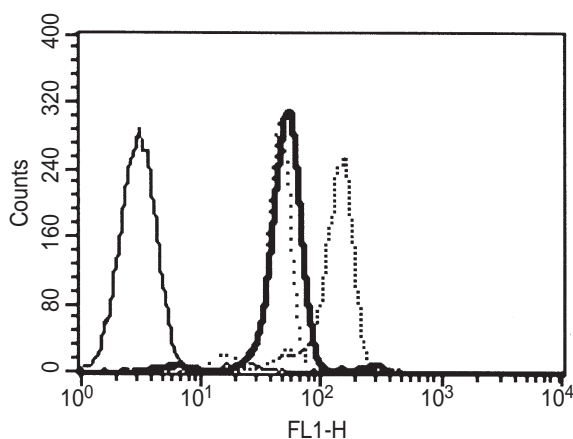
Measurement of GSH and depletion by BSO In an attempt to define further the mechanisms of resistance, we investigated whether the amount of anti-oxidative substances increased to enhance the redox state of 697/DEX. We measured the intracellular levels of total GSH. The GSH contents of 697/Neo, 697/DEX and 697/Bcl-2 were 241.8±31.4, 346.5±39.2 and 323.3±38.2 nmol/min/mg
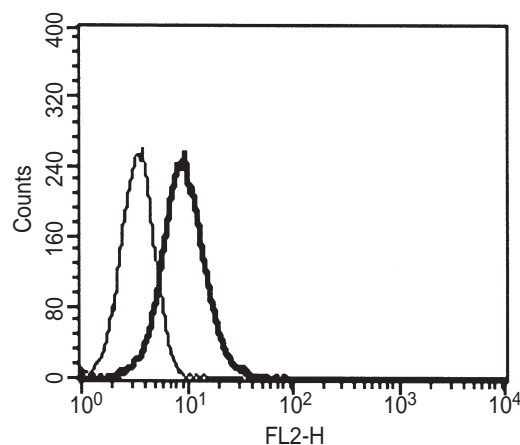

Fig. 2. Pgp expression in 697/DEX. Cells (1×10^6) were treated with PE-UIC2 in complete medium (PBS containing 2% FCS and 0.1% sodium azide) for 30 min on ice, washed twice with complete medium, and resuspended in complete medium. As a negative control, we used mouse IgG in place of MoAb. The cells were analyzed using a FACscan (Becton Dickinson). Thin solid line, without PE-UIC2; thick solid line, with PE-UIC2.
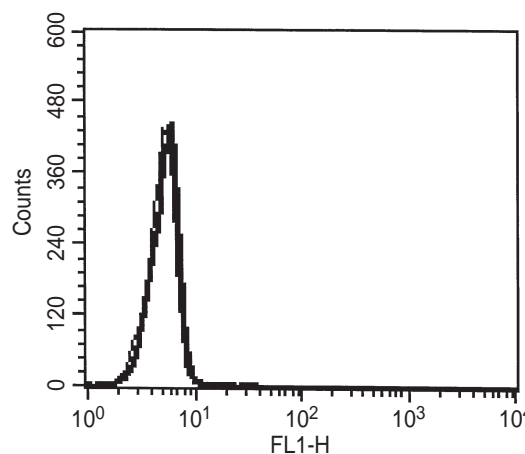

Fig. 1. Immunodetection of Bcl-2 in 697/Neo and 697/DEX. Cells (2×10^6) per sample were resuspended in 2% paraformaldehyde in PBS. After 10 min on ice, 1% Triton X-100 was added, and 10 min later the cells were washed twice in cold PBS. Indirect immunofluorescence assay was then carried out using anti-Bcl-2 MoAb (clone 124). FITC-conjugated goat antimouse MoAb was added for 30 min at 4°C. The cells were analyzed using a FACScan flow cytometer. The x axis shows log fluorescence intensity and the y axis shows relative cell number. —— negative control, —— 697/Neo, ---- 697/DEX, ……… 697/Bcl-2.

Fig. 3. Pgp-mediated efflux pump function in 697/DEX. Cells (5×10^5) from 697/DEX were incubated with 100 ng/ml of Rhodamine 123 with or without 10 μg/ml verapamil for 30 min at 37°C. The cells were washed twice in PBS and resuspended in RPMI and efflux was allowed to occur for 3 h at 37°C. The cells were then washed twice and analyzed on a FACscan (Becton Dickinson). Thin solid line, without verapamil; thick solid line, with verapamil.

585

protein, respectively. 697/DEX and 697/Bcl-2 showed reproducibly higher levels of GSH than the parental 697/Neo (P=0.005, P=0.016, respectively vs. 697/Neo). These data suggested that the GSH redox cycle was more active in 697/DEX and 697/Bcl-2 than in 697/Neo. On addition of BSO, both 697/DEX and 697/Bcl-2 recovered sensitivity to DEX (Fig. 5). We have previously confirmed that GSH depletion was achieved by 24 h preincubation of the cells in RPMI-1640 supplemented with 15 μM BSO.

Caspase-3/7 enzyme activity   Apoptotic cell death is usually known to be associated with activation of cysteine proteases, mainly caspase-3. As shown in Fig. 6A, the caspase-3/7 activity with BSO and DEX treatment was increased 6-fold compared with BSO treatment at 24 h of culture in 697/Neo. However, the activation of caspase-3/7 was not detected in 697/DEX or 697/Bcl-2. Preincubation with the caspase-3 family protease inhibitor, Ac-DEVD-MCA, had no effect. With BSO treatment, both 697/DEX and 697/Bcl-2 recovered sensitivity to DEX, but caspase-3/7 was not activated during 24 h of culture (Fig. 6A). Furthermore, we also did not detect the activation of caspase-3/7 during 48 h culture with BSO and DEX treatment in 697/DEX and 697/Bcl-2 (Fig. 6B).

DISCUSSION

In this study, we established a DEX-resistant cell line, 697/DEX. This cell line showed no cross-resistance to other anti-leukemic drugs including vinca alkaloids,

except for weak resistance to DNR (2.4-fold). Since the mechanisms of glucocorticoid resistance have been mainly studied with T-cell leukemia cell lines, such as CEM-C1 *in vitro* so far,[17, 18] our cell line should be useful for studies on the glucocorticoid resistance mechanism of B-cell leukemia cell lines.

Prolonged survival of B-lineage ALL cells is accompanied by the expression of the Bcl-2 protein.[13, 31] It has been shown that Bcl-2 protects cancer cells from apoptosis induced by a variety of anticancer drugs,[14] although the precise mechanism of the Bcl-2-induced multidrug resistance is unknown. High levels of the Bcl-2 protein have been shown to protect 697 cells from the acute cytotoxic effects of DEX, using Bcl-2-virus-infected cells.[14] Our results demonstrated that the expression of Bcl-2 was not up-regulated at the protein level in 697/DEX compared with 697/Neo (Fig. 1). The results were the same using western blotting analysis (data not shown). These findings suggest that the level of Bcl-2 did not play an important role in the resistance to DEX in 697/DEX, different from the artificial Bcl-2-virus-transfected cell line, 697/Bcl-2.

Increased expression of Pgp has been recognized as the most consistent change in multidrug resistance.[32] Human Pgp has been shown to transport DEX in an experimental cell line.[11] Our results showed that the Pgp in 697/DEX is expressed on the membrane (Fig. 2). Interestingly, the Pgp efflux pump in 697/DEX did not work in [³H]DEX efflux assay and Rhodamine 123 efflux assay (Fig. 3). Pallis *et al.* reported that Pgp plays a drug efflux-independent role
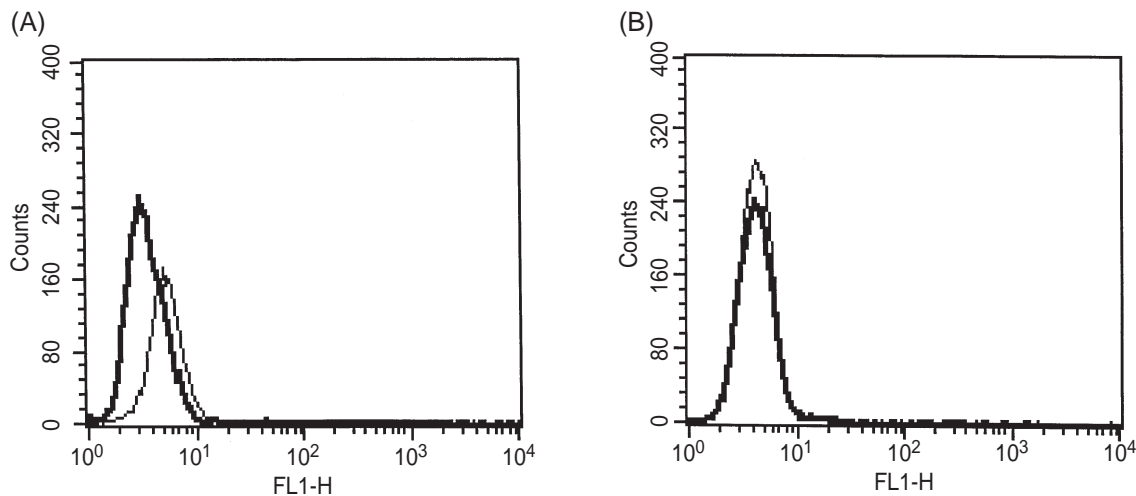

Fig. 4.   A. MRP expression in 697/DEX. Cells (1×10^6) were incubated with the MRP monoclonal antibody MRPm6 for 60 min on ice, washed twice and analyzed using a FACscan (Becton Dickinson). As a negative control, we used mouse IgG in place of the MoAb. Thin solid line, without MRPm6; thick solid line, with MRPm6. B. MRP-mediated efflux pump function in 697/DEX. Cells (1×10^6) were incubated with 0.5 μM calcein-AM with or without 2 mM probenecid for 15 min at 37°C. After centrifugation, cells were resuspended in fresh medium, and the efflux of calcein was followed for 90 min at 37°C. Intracellular calcein accumulation was measured in terms of the absorbance through a 530 nm bandpass filter with excitation at 488 nm using a FACscan (Becton Dickinson). Thin solid line, without probenecid; thick solid line, with probenecid.
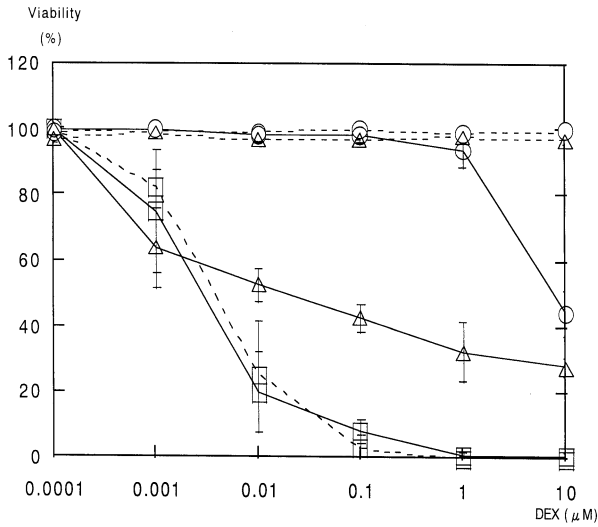
Fig. 5. Sensitization with BSO in 697/Neo, 697/DEX and 697/Bcl-2. After treatment with 15 μM BSO for 24 h, the cells were incubated with DEX at various concentrations for a further 72 h. Cell growth inhibition was determined by the Trypan blue dye exclusion method. The values are presented as means±SD of three separate experiments. □ Neo, ○ DEX, △ Bcl-2, ---- −BSO, —— +BSO.

in augmenting cell survival and is associated with the modulation of a sphingomyelin-ceramide apoptotic pathway.[33] It remains possible that among the Pgp-positive resistant cells in the presence of lower concentrations of DEX, another mutation affords cells with potent resistance via another mechanism, but with loss of Pgp function. The mechanisms of the non-efflux function in 697/DEX are still under investigation in our laboratory.

Previous studies have shown that glucocorticoid-resistance is most commonly linked to altered receptor number or function.[10] In contrast, Nicklas *et al.* indicated that resistance to glucocorticoids *in vivo* is not due to an alteration of the GR.[34] Our results showed that the numbers of GR in 697/DEX and 697/Bcl-2 were fewer than those in 697/Neo. Although 697/DEX may decrease the number of down-regulated GR on exposure to DEX, the number of receptors in the 697/Bcl-2 cells was also decreased without exposure to DEX. Miyashita *et al.* reported that the S49.1 and WEHI7.2 murine T-lymphoid cell lines infected with a recombinant retrovirus encoding human Bcl-2 had a marked resistance to DEX-mediated cell death and apoptosis without lowering of the GR level.[35] On the other hand, Bcl-2 has recently been demonstrated to regulate the expression of hormone receptors, such as estrogen recep-
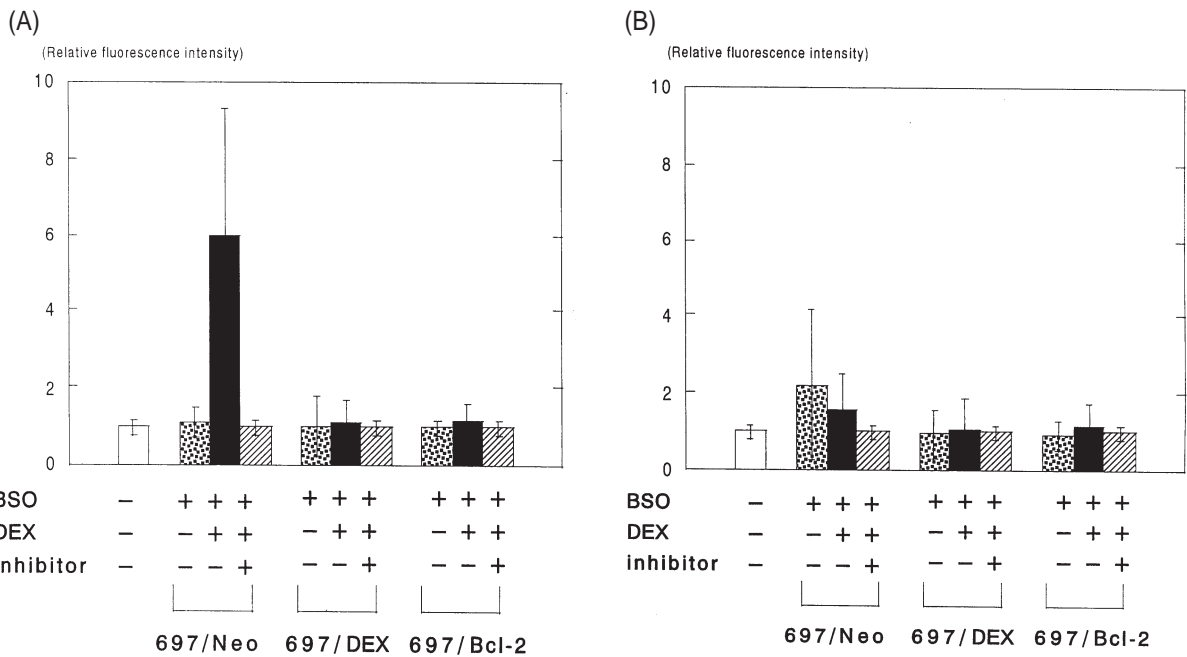

Fig. 6. Caspase-3/7 activation in 697/Neo, 697/DEX and 697/Bcl-2. After preincubation with 15 μM BSO for 24 h, cells were treated with 10 μM DEX for a further 24 h (A) or 48 h (B). Caspase-3/7 activity was detected in the resulting supernatants by measuring the proteolytic cleavage of the fluorogenic substrate, Ac-DEVD-MCA, using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. To confirm the inhibition of caspase-3/7, a control reaction was performed by incubating an activated sample with a caspase inhibitor, Ac-DEVD-CHO, before addition of the Ac-DEVD-MCA. Open bars, control; dotted bars, +BSO; black bars, +BSO+DEX; oblique bars, +BSO+DEX+inhibitor. Relative fluorescence intensity is sample fluorescence intensity/control fluorescence intensity based on five separate experiments.

587

tor, in breast carcinoma cells.[36] Bcl-2 may decrease the GR numbers. Since the correlation between sensitivity to glucocorticoids and the GR number is still controversial, further study is needed.

Recently, it has been noted that elevation of GSH in the GSH redox pathway is associated with acquired drug resistance.[29] For example, GSH levels in ALL on relapse were more than 2-fold higher than those at onset.[17] In the present study, we showed that 697/DEX and 697/Bcl-2 had higher levels of GSH than the parental 697/Neo. Moreover, we showed that BSO treatment, which is a specific inhibitor of GSH synthesis, could restore susceptibility to DEX in 697/DEX (Fig. 5). These results suggest that the intracellular GSH level is one of the important factors determining DEX resistance. As one possible mechanism, GSH can combine with anticancer drugs to form less toxic and more water-soluble GSH conjugates. The GSH conjugates of anticancer drugs can be exported from the cells by MRP and the glutathione-S conjugate export pump (GS-X pump).[37] However, we could not detect MRP expression and function in 697/DEX (Fig. 4). Iwata *et al.* reported that GSH plays crucial roles in the regulation of lymphocyte proliferation. Under GSH-depleted conditions, the inability to neutralize oxidative stress results in lymphoid cell death.[38] We speculate that the elevation of GSH in 697/DEX maintains the intracellular redox balance against oxidative stress induced by DEX. Further studies are required to elucidate the activity of enzymes responsible for GSH redox homeostasis, such as glutathione synthase, glutathione reductase and glutathione peroxidase.

Next, we investigated whether the apoptotic signal pathway with the depletion of GSH was associated with the caspase-3/7 activation in these cell lines. Our results showed that caspase-3/7 was activated during apoptosis in

697/Neo under depletion of GSH with DEX treatment for 24 h. However, in 697/DEX and 697/Bcl-2, caspase-3/7 was not activated under the same conditions (Fig. 6A). Even with 48 h incubation, we could not detect the activation of caspase-3/7, although the cell growth was already suppressed (Fig. 6B). We also could not detect the activation of caspase-6 during 48 h incubation of 697/DEX and 697/Bcl-2 (data not shown). Our study suggested that the cell death mechanisms of 697/DEX and 697/Bcl-2 by GSH-depletion were different from that of 697/Neo. When 697/DEX was cultured in BSO and DEX for 48 h, we found 60% of the intracellular nuclei to be positive by propidium iodide staining. Iwata *et al.* reported that the lymphoid cells died by an apoptotic mechanism under GSH-depleted conditions.[38] We speculate that 697/DEX and 697/Bcl-2 might die through caspase-independent programmed cell death or the necrotic pathway.

In conclusion, DEX-resistance in 697/DEX is associated not only with GR reduction but also raised intracellular GSH levels. This is the first report to show that elevation of GSH plays an important role in the acquired DEX-resistance of B-cell lines. In the clinical field, measurement of GSH may be useful to predict DEX-resistance. In addition, the fact that DEX-resistance may be reversed with BSO may offer strategies for overcoming resistance to glucocorticoids in B-cell lineage ALL.

REFERENCES

1) Kaspers, G. J., Pieters, R., Klumper, E., De Waal, F. and Veerman, A. J. Glucocorticoid resistance in childhood leukemia. *Leuk. Lymphoma*, **13**, 187–201 (1994).

2) Federico, M. H., Martins, V. R., Pozzi, D. H., Llacer, P. E. and Brentani, M. M. Glucocorticoid receptors in acute leukemia. *Braz. J. Med. Biol. Res.*, **19**, 167–172 (1986).

3) Ueda, T., Miyawaki, S., Asou, N., Kuraishi, Y., Hiraoka, A., Kuriyama, K., Minami, S., Ohshima, T., Ino, T., Tamura, J., Kanamaru, A., Nishikawa, K., Tanimoto, M., Oh, H., Saito, K., Nagata, K., Naoe, T., Yamada, O., Urasaki, Y., Sakura, T. and Ohno, R. Response-oriented individualized induction therapy with six drugs followed by four courses of intensive consolidation, 1 year maintenance and intensification therapy: the ALL90 study of the Japan Adult Leukemia Study Group. *Int. J. Hematol.*, **68**, 279–289 (1998).

4) Gaynon, P. S. and Carrel, A. L. Glucocorticosteroid therapy in childhood acute lymphoblastic leukemia. *Adv. Exp. Med. Biol.*, **457**, 593–605 (1999).

5) Maung, Z. T., Reid, M. M., Matheson, E., Taylor, P. R., Proctor, S. J. and Hall, A. G. Corticosteroid resistance is increased in lymphoblasts from adults compared with children: preliminary results of *in vitro* drug sensitivity study in adults with acute lymphoblastic leukaemia. *Br. J. Haematol.*, **91**, 93–100 (1995).

6) Dordelmann, M., Reiter, A., Borkhardt, A., Ludwig, W. D., Gotz, N., Viehmann, S., Gadner, H., Riehm, H. and Schrappe, M. Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. *Blood*, **94**, 1209–1217 (1999).

7) Kaspers, G. J., Pieters, R., Van, Z. C., Van, W. E., Van, D. D., Van, D. B. A. and Veerman, A. J. Prednisolone resis-

tance in childhood acute lymphoblastic leukemia: *vitro-vivo* correlations and cross-resistance to other drugs. *Blood*, **92**, 259–266 (1998).

8) Kaspers, G. J., Veerman, A. J., Pieters, R., Van, Z. C., Smets, L. A., Van, W. E., Van, D. D. and Van, D. B. A. *In vitro* cellular drug resistance and prognosis in newly diagnosed childhood acute lymphoblastic leukemia. *Blood*, **90**, 2723–2729 (1997).

9) Moalli, P. A. and Rosen, S. T. Glucocorticoid receptors and resistance to glucocorticoids in hematologic malignancies. *Leuk. Lymphoma*, **15**, 363–374 (1994).

10) Costlow, M. E., Pui, C. H. and Dahl, G. V. Glucocorticoid receptors in childhood acute lymphocytic leukemia. *Cancer Res.*, **42**, 4801–4806 (1982).

11) van Kalken, C. K., Broxterman, H. J., Pinedo, H. M., Feller, N., Dekker, H., Lankelma, J. and Giaccone, G. Cortisol is transported by the multidrug resistance gene product P-glycoprotein. *Br. J. Cancer*, **67**, 284–289 (1993).

12) Dole, M., Nunez, G., Merchant, A. K., Maybaum, J., Rode, C. K., Bloch, C. A. and Castle, V. P. Bcl-2 inhibits chemotherapy-induced apoptosis in neuroblastoma. *Cancer Res.*, **54**, 3253–3259 (1994).

13) Alnemri, E. S., Fernandes, T. F., Haldar, S., Croce, C. M. and Litwack, G. Involvement of BCL-2 in glucocorticoid-induced apoptosis of human pre-B-leukemias. *Cancer Res.*, **52**, 491–495 (1992).

14) Miyashita, T. and Reed, J. C. Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. *Blood*, **81**, 151–157 (1993).

15) Harmon, J. M. and Thompson, E. B. Isolation and characterization of dexamethasone-resistant mutants from human lymphoid cell line CEM-C7. *Mol. Cell. Biol.*, **6**, 512–521 (1981).

16) Geley, S., Hartmann, B. L., Hala, M., Strasser-Wozak, E. M., Kapelari, K. and Kofler, R. Resistance to glucocorticoid-induced apoptosis in human T-cell acute lymphoblastic leukemia CEM-C1 cells is due to insufficient glucocorticoid receptor expression. *Cancer Res.*, **56**, 5033–5038 (1996).

17) Maung, Z. T., Hogarth, L., Reid, M. M., Proctor, S. J., Hamilton, P. J. and Hall, A. G. Raised intracellular glutathione levels correlate with *in vitro* resistance to cytotoxic drugs in leukaemic cells from patients with acute lymphoblastic leukaemia. *Leukemia*, **8**, 1487–1491 (1994).

18) Kearns, P., Pieters, R., Rottier, M. M. A., Veerman, A. J. P., Schmiegalow, K., Pearson, A. D. J. and Hall, A. G. Glutathione in childhood acute leukaemias. *Adv. Exp. Med. Biol.*, **457**, 211–216 (1999).

19) Aiello, A., Delia, D., Borrello, M. G., Biassoni, D., Giardini, R., Fontanella, E., Pezzella, F., Pulford, K., Pierotti, M. and Della, P. G. Flow cytometric detection of the mitochondrial BCL-2 protein in normal and neoplastic human lymphoid cells. *Cytometry*, **13**, 502–509 (1992).

20) Tosi, P., Visani, G., Ottaviani, E., Manfroi, S. and Tura, S. Biological and clinical significance of *in vitro* prednisolone resistance in adult acute lymphoblastic leukaemia. *Eur. J. Haematol.*, **57**, 134–141 (1996).

21) Chaudhary, P. M., Mechetner, E. B. and Roninson, I. B. Expression and activity of the multidrug resistance P-glycoprotein in human peripheral blood lymphocytes. *Blood*, **80**, 2735–2739 (1992).

22) Flens, M. J., Izquierdo, M. A., Scheffer, G. L., Fritz, J. M., Meijer, C. J., Scheper, R. J. and Zaman, G. J. Immunochemical detection of the multidrug resistance-associated protein MRP in human multidrug-resistant tumor cells by monoclonal antibodies. *Cancer Res.*, **54**, 4557–4563 (1994).

23) Webb, M., Raphael, C. L., Asbahr, H., Erber, W. N. and Meyer, B. F. The detection of rhodamine 123 efflux at low levels of drug resistance. *Br. J. Haematol.*, **93**, 650–655 (1996).

24) Takemura, H., Urasaki, Y., Yoshida, A., Fukushima, T. and Ueda, T. Simultaneous treatment with 1-β-D-arabinofuranosylcytosine and daunorubicin induces cross-resistance to both drugs due to a combination-specific mechanism in HL60 cells. *Cancer Res.*, **61**, 172–177 (2001).

25) Feller, N., Broxterman, H. J., Wahrer, D. C. and Pinedo, H. M. ATP-dependent efflux of calcein by the multidrug resistance protein (MRP): no inhibition by intracellular glutathione depletion. *FEBS Lett.*, **368**, 385–388 (1995).

26) Smets, L. A., van der Klooster, P. and Otte, A. Glucocorticoid receptors of normal and leukemic cells: role of proliferation conditions. *Leuk. Res.*, **9**, 199–207 (1985).

27) Scatchard, G. Attraction of proteins for small molecules and ions. *Ann. NY Acad. Sci.*, **51**, 660–672 (1949).

28) Tietze, F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.*, **27**, 502–522 (1969).

29) Lai, G. M., Moscow, J. A., Alvarez, M. G., Fojo, A. T. and Bates, S. E. Contribution of glutathione and glutathione-dependent enzymes in the reversal of adriamycin resistance in colon carcinoma cell lines. *Int. J. Cancer*, **49**, 688–695 (1991).

30) Dimmeler, S., Haendeler, J., Nehls, M. and Zeiher, A. M. Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. *J. Exp. Med.*, **185**, 601–607 (1997).

31) Smets, L. A., Van den Berg, J., Acton, D., Top, B., Van, R. H. and Verwijs, J. M. BCL-2 expression and mitochondrial activity in leukemic cells with different sensitivity to glucocorticoid-induced apoptosis. *Blood*, **84**, 1613–1619 (1994).

32) Aszalos, A. and Ross, D. D. Biochemical and clinical aspects of efflux pump related resistance to anti-cancer drugs. *Anticancer Res.*, **18**, 2937–2944 (1998).

33) Pallis, M. and Russell, N. P-glycoprotein plays a drug-efflux-independent role in augmenting cell survival in acute myeloblastic leukemia and is associated with modulation of a sphingomyelin-ceramide apoptotic pathway. *Blood*, **95**, 2897–2904 (2000).

34) Nicklas, B. E., Rosemary, B. E. and John, A. C. *In vivo* resistance to glucocorticoid-induced apoptosis in rat thy-

mocytes with normal steroid receptor function *in vitro*. *Endocrinology*, **138**, 810–818 (1997).

35) Miyashita, T. and Reed, J. C. bcl-2 gene transfer increases relative resistance of S49.1 and WEHI7.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticoids and multiple chemotherapeutic drugs. *Cancer Res.*, **52**, 5407–5411 (1992).

36) Leek, R. D., Kaklamanis, L., Pezzella, F., Gatter, K. C. and Harris, A. L. bcl-2 in normal human breast and carcinoma, association with oestrogen receptor-positive, epidermal growth factor receptor-negative tumours and *in situ* can-

cer. *Br. J. Cancer*, **69**, 135–139 (1994).

37) Zhang, K., Mack, P. and Wong, K. P. Glutathione-related mechanisms in cellular resistance to anticancer drugs. *Int. J. Oncol.*, **12**, 871–882 (1998).

38) Iwata, S., Hori, T., Sato, N., Hirota, K., Sasada, T., Mitsui, A., Hirakawa, T. and Yodoi, J. Adult T cell leukemia (ATL)-derived factor/human thioredoxin prevents apoptosis of lymphoid cells induced by L-cystine and glutathione depletion: possible involvement of thiol-mediated redox regulation in apoptosis caused by pro-oxidant state. *J. Immunol.*, **158**, 3108–3117 (1997).