Sensitization of imatinib-resistant CML cells to TRAIL-induced apoptosis is mediated through down-regulation of Bcr-Abl as well as c-FLIP

Soo-Jung PARK*†‡§, Mi-Ju KIM*†‡, Hak-Bong KIM*†‡, Chi-Dug KANG*†‡ and Sun-Hee KIM*†‡†

*Department of Biochemistry, Pusan National University School of Medicine, Yangsan 622-770, South Korea, †Pusan National University BK21 Medical Science Education Center, Yangsan 622-770, South Korea, ‡Research Center for Ischemic Tissue Regeneration, Pusan National University School of Medicine, Yangsan 622-770, South Korea, and §Division of Intractable Diseases, Center for Biomedical Sciences, National Institute of Health, Seoul 122-701, South Korea

INTRODUCTION

The constitutively activated Bcr-Abl tyrosine kinase has been defined as the pathogenic principle of Ph+ (Philadelphia chromosome-positive) human leukaemias [1,2]. Imatinib, in its mesylate salt, marketed as Glivec/Gleevec, and known as HL-60/Bcr-Abl and K562 cells, which are relatively resistant to Ph+ cell survival, including amplification of the Bcr-Abl gene, increased Bcr-Abl expression, up-regulation of Bcr-Abl kinase activity [10,11] and activation of alternative signalling pathways [4]. Although most cases of acquired imatinib resistance are associated with reactivation of Bcr-Abl activity, there are some in vitro studies showing imatinib resistance in CML cells with down-regulation or loss of Bcr-Abl [12–16]. It has been reported that loss of kinase-target-dependence and acquired Bcr-Abl-independent signalling characteristics were associated with imatinib resistance in some patients [17].

Resistance to imatinib is commonly associated with reactivation of Bcr-Abl signalling. However, Bcr-Abl-independent signalling pathways may be activated and contributed to imatinib resistance in some CML (chronic myelogenous leukaemia) patients. We had isolated three imatinib-resistant K562/R1, R2 and R3 variants with gradual loss of Bcr-Abl from K562 cells to develop effective therapeutic strategies for imatinib-resistant CML. Interestingly, we found that these cells became highly sensitive to TRAIL (tumour necrosis factor-related apoptosis-inducing factor) in comparison with K562 cells showing high resistance to TRAIL. Treatment of K562/R3 cells with TRAIL resulted in activation of TRAIL receptor pathway by including caspase 8 activation, Bid cleavage, cytochrome c release and caspase 3 activation. These results were accompanied by down-regulation of c-FLIP (cellular FLICE [FADD (Fas-associated death domain)-like interleukin 1β-converting enzyme]-inhibitory protein) in imatinib-resistant K562 variants compared with K562 cells. Overexpression of c-FLIP in K562/R3 cells acquired TRAIL resistance and conversely, c-FLIP-silenced K562 cells became sensitive to TRAIL. Moreover, Bcr-Abl-silenced K562 cells showed down-regulation of c-FLIP and the subsequent overcome of TRAIL resistance. Taken together, our results demonstrated for the first time that the loss of Bcr-Abl in imatinib-resistant cells led to the down-regulation of c-FLIP and subsequent increase of TRAIL sensitivity, suggesting that TRAIL could be an effective strategy for the treatment of imatinib-resistant CML with loss of Bcr-Abl.

Key words: Bcr-Abl, cellular FLICE [FADD (Fas-associated death domain)-like interleukin 1β-converting enzyme]-inhibitory protein (c-FLIP), death receptor 5 (DR5), imatinib resistance, tumour-necrosis factor-related apoptosis-inducing factor (TRAIL).

Abbreviations used: c-FLIP, cellular FLICE [FADD (Fas-associated death domain)-like interleukin 1β-converting enzyme]-inhibitory protein; CML, chronic myelogenous leukaemia; DR, death receptor; FADD, Fas-activated death domain; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PARP, poly(ADP-ribose) polymerase; Ph+, Philadelphia chromosome-positive; RT, reverse transcription; siRNA, short interfering RNA; STAT, signal transducer and activator of transcription; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing factor; TRAIL-R, TRAIL receptor; z-VAD-fmk, benzyloxy carbonyl-valyl-alanyl-yl-aspartyl-fluoromethane.

1 Correspondence may be addressed to either of these authors (email kcdshbw@pusan.ac.kr or ksh7738@pusan.ac.kr respectively).
to TRAIL, TRAIL-induced apoptosis could be increased by co-treatment with imatinib [21]. TRAIL could also induce cell death in the Ph+ leukaemia cell lines that were refractory to the Bcr-Abl-specific tyrosine kinase inhibitor imatinib mesylate [22].

Previously, we isolated three imatinib-resistant variants from K562 cells that showed profound declines in Bcr-Abl levels and its tyrosine kinase activity [12,16]. In the present study, we demonstrated that TRAIL sensitized imatinib-resistant K562 cells to apoptosis via a caspase-dependent mitochondrial pathway. These imatinib-resistant variants exhibited the down-regulation of c-FLIP [cellular FLICE (FADD-like interleukin 1β-converting enzyme)-inhibitory protein], as well as up-regulation of DR5 and the activation of caspases, and consequently sensitized to TRAIL. We suggest the possibility that TRAIL may be an effective strategy in Bcr-Abl-negative imatinib-resistant CML cancer therapy.

**EXPERIMENTAL**

**Cell lines and cell cultures**

The human chronic myelocytic leukaemia K562 cells were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.) and cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum and penicillin/streptomycin (50 units/ml) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. To establish imatinib-resistant sublines, exponentially growing cells were exposed to increasing concentrations of imatinib (Novartis), starting with a concentration of 0.5 μM, and increasing gradually by 0.1 μM increments. After the cells acquired the ability to grow in the presence of a specific concentration of imatinib, the level of resistance was determined. A proportion of cells were then frozen, and the remaining cells were grown at the next highest drug level. In this way, three imatinib-resistant K562/R1, R2 and R3 cell populations were isolated and used for further studies.

**MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay for determining drug sensitivity**

Cells were seeded in 96-well plates at a concentration of 1 × 10⁴ cells/well and treated with increasing concentrations of TRAIL (R&D Systems) for 24 h. After treatment, the medium was incubated with 0.5 mg/ml MTT dye (Sigma) for 3 h, and the dark blue crystals that formed were dissolved in 0.1 M HCl in isopropanol alcohol (propan-2-ol), and the absorbance was measured at 570 nm using a spectrophotometer. Results are presented as a percentage of survival, taking the control as 100%.

**Annexin V analysis for determining apoptosis**

Cells were seeded in six-well plates at a concentration of 10⁶ cells/well and treated with increasing concentrations of TRAIL for 24 h. Cells were resuspended in 100 μl of staining solution containing annexin V–FITC (BD Biosciences) and propidium iodide (Sigma) in a Heps buffer. After incubation at room temperature (20°C) for 20 min, annexin V-positive cells were analysed by a FACSCalibur flow cytometer (Becton Dickinson). To determine that caspases are involved in apoptosis induced by TRAIL, caspase inhibitor z-VD-fmk (benzoylloxycarbonyl-valylalanyl-DL-aspartyl-fluoromethane) (R&D Systems) was used. The caspase inhibitor was dissolved in DMSO (Sigma); the final concentration of DMSO was 0.1%. Cells (5 × 10⁶ cells/well) were pre-incubated in the absence or presence of 20 μM of this inhibitor for 3 h at 37°C, treated with TRAIL for 24 h and then processed for the annexin V-binding assay described above.

**Flow cytometric analysis of TRAIL-Rs (TRAIL receptors)**

K562 and its imatinib-resistant variants (5 × 10⁶ cells/well) from the culture media were spun down at 500 g for 5 min, washed with PBS and resuspended in 500 μl of PBS. The cells were then incubated with 5 μl of goat IgG2a or anti-DR4 and anti-DR5 polyclonal goat antibody (1:100 dilution; R&D Systems) for 1 h respectively. After washing with PBS, FITC-conjugated rabbit anti-goat polyclonal antibody (1:200 dilution; Sigma) was added to the cell suspension and incubated for 1 h on ice followed by washing with PBS. After rinsing, the samples were analysed by flow cytometry using a FACSCalibur flow cytometer. The data were analysed using the CellQuest program.

**Treatment of cells with neutralizing antibodies against DRs and determination of apoptosis**

To determine whether TRAIL-induced apoptosis occurs through the DRs, K562 and its imatinib-resistant variants were pre-treated with the neutralizing polyclonal goat antibodies raised against extracellular domains of DR4 and DR5 (20 μg/ml; R&D Systems) for 3 h before treatment with 10 and 50 ng/ml TRAIL respectively for 24 h. In control experiments, cells were treated with goat IgG2a before TRAIL treatment. Apoptosis was measured by annexin V analysis as described above.

**RT (reverse transcription)–PCR analysis**

RNA extraction and relative RT–PCR analysis were performed as described previously [23]. Briefly, total cellular RNA was extracted from cells harvested at the indicated times using TRI Reagent (Molecular Research Center). Then, 1 μg of total cellular RNA from K562 and its imatinib-resistant variants was reverse-transcribed using MMLV (Moloney murine leukaemia virus) reverse transcriptase (Invitrogen) with each dNTP and 1 μg of oligo(dT). Amplification of 2.5 μl of these cDNAs by PCR was performed using the following gene-specific primers: Bcr-Abl forward, 5’-GAAGGAAGGTGGTTCAGAACTTTCTCCC’; and reverse, 5’-GACCCCGAGCTTTTCACCTTTAGGT’; DR4 forward, 5’-CTGAGCAACGAGCTCGTGCCAC’; and reverse, 5’-AAGGACACGGACGACCTGTCACAT’; DR5 forward, 5’-CTGAAAGGACATCTGCTAGGG’; and reverse, 5’-CAGAGTCTGCATTACCTTACG’; FLIP₃ forward, 5’-GCTGAAGTCATCCACCAGT’; and reverse, 5’-CAGAAGACTGACAGGCAAT’; FLIP₄ forward, 5’-GCTGAAGTCATCCACAGT’; and reverse, 5’-TTGAGGTGTCATACATG’.

The resulting total cDNA was used in PCR performed in total volume of 30 μl using a TaKaRa Taq™ kit for 30 cycles of 94°C for denaturation for 60 s, 60°C for annealing for 60 s and 72°C for extension for 90 s, followed by a final extension at 72°C for 12 min. The amplified fragments were separated on a 1% agarose gel and visualized by ethidium bromide staining.

**Cell transfection**

For overexpression of c-FLIP₃, imatinib-resistant K562/R3 cells were transfected with pcDNA3.1 as a mock control or pcDNA3.1/FLIP₃ using FuGENE™ 6 (Roche) as described by the manufacturer. Transfection solutions consisted of 0.2 ml/well serum-free medium OptiMEM (Invitrogen) supplemented with 3 μl of FuGENE™ 6 and 2 μg of plasmid DNA were prepared according to the manufacturer’s instructions. After transfection, cells were incubated in antibiotic-free medium for 4 h, followed by replacement with fresh normal growth medium for a further incubation of 24–48 h before determination of the c-FLIP₃ levels.
The transfectants were treated with 10 and 50 ng/ml TRAIL for 24 h and annexin V analysis was performed as described above.

**siRNA (short interfering RNA) transfection**

The Bcr-Abl (5′-CAGAGUUCAAAAGCCCUUCdTdT-3′) and control scrambled (5′-CUUCCGAAAACUUUGAGCdTdT-3′) siRNAs were synthesized. Cells in the exponential phase of growth were plated in six-well plates at 5 × 10^5 cells/well, and then transfected with siRNA using Oligofectamine (Invitrogen) and OptiMEM I reduced serum medium, according to the manufacturer’s protocol. The concentrations of siRNAs were chosen based on dose–response studies. Silencing was examined 24–48 h after transfection.

**Western blot analysis**

Cells were washed in ice-cold PBS and extracted for 30 min with a buffer containing 50 mM Tris/HCl (pH 7.5), 140 mM NaCl, 5 mM EDTA, 5 mM Na_2_3_, 1% (v/v) Triton X-100, 1% (v/v) Nonidet P40, 1 mM EGTA and protease inhibitor cocktail. Lysates were cleared by centrifugation at 15,000 rev./min for 30 min in an Eppendorf centrifuge (F-45-24-11 rotor), and protein concentrations were determined using Bradford protein assay. Proteins were denatured in 2% SDS-containing sample buffer and the same total protein amount was transferred on to a nitrocellulose membrane (GE Healthcare). The membranes were probed with specific antibodies. The following primary antibodies were used: c-Ab (24-11), Bcl-X_s (S-18), PARP [poly(ADP-ribose) polymerase] (H-250), caspase 3 (H-277) and cytochrome c (H-104) antibodies were purchased from Santa Cruz Biotechnology. The anti-(mouse caspase 8) (1C12) monoclonal antibody was purchased from Cell Signaling Technology. The anti-(goat Bid) (AF860) antibody was purchased from R&D Systems. The anti-mouse c-FLIP (NF-6) monoclonal antibody was purchased from Axxora. Anti-β-actin (AC-74) antibody was purchased from Sigma–Aldrich. Immunocomplexes were detected using horseradish-peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat antibodies (GE Healthcare) followed by ECL® (enhanced chemiluminescence) detection (GE Healthcare).

**Isolation of cytosolic fraction for detecting cytochrome c**

K562 and K562/R3 cells (approx. 2 × 10^7 cells/well) were treated with 10 and 50 ng of TRAIL for 24 h. Cells were harvested by centrifugation at 600 g for 5 min at 4°C, washed twice in ice-cold PBS and then suspended in 5 vol. of ice-cold buffer (20 mM Heps, pH 7.2, 10 mM KCl, 1.5 mM MgCl_2_, 1 mM sodium EDTA, 1 mM sodium EGTA, 250 mM sucrose and protease inhibitor mixture). Cells were mechanically disrupted with a Dounce homogenizer (15 strokes) and centrifuged at 750 g for 5 min at 4°C. The supernatants were centrifuged for 15 min at 13,000 rev./min at 4°C (Type 90 Ti rotor; Beckman Coulter) to pellet the mitochondria. The supernatant which contained mitochondria was subjected to further centrifugation at 45,000 rev./min for 60 min at 4°C (Type 90 Ti rotor; Beckman Coulter) to pellet the mitochondrial fraction. Samples were dissolved in SDS sample buffer, heated to 95°C for 3 min and subjected to Western blot analysis for cytochrome c.

**RESULTS**

**TRAIL induces apoptosis and inhibits cell proliferation in imatinib-resistant K562 variants**

To overcome resistance to imatinib and develop a new potential therapeutic strategy against imatinib-resistant CML, we isolated three imatinib-resistant K562/R1, R2 and R3 variants from K562 cells, which showed approx. 42-, 50- and 63-fold resistance to imatinib compared with parental K562 cells respectively. Because Bcr-Abl is an essential target of imatinib for CML cells, we examined Bcr-Abl expression in K562 and its imatinib-resistant variants using RT–PCR and Western blot analysis (Figure 1A). Compared with Bcr-Abl-expressing K562 cells, K562/R1, R2 and R3 cells showed a gradual loss of Bcr-Abl at mRNA and protein levels, which removed the dependency of these cells on Bcr-Abl-mediated survival signalling, thus making these cells unresponsive to imatinib. Since it has been reported that K562 leukaemia cells are resistant to TRAIL-induced apoptosis [24], we determined changes of TRAIL sensitivity against imatinib-resistant variants. These cells were treated with 1, 10 and 50 ng/ml TRAIL for 24 h and apoptosis was measured by annexin V analysis (Figure 1B). The responsiveness to TRAIL-induced apoptosis showed a reverse correlation between imatinib and TRAIL sensitivity. K562/R3 cells exhibited the highest sensitivity to TRAIL-induced apoptosis, whereas K562 cells did not show responsiveness to TRAIL. Similar results were found using the MTT assay, with the highest growth inhibitory effect of TRAIL on K562/R3 cells (Figure 1C). We also determined whether imatinib can enhance susceptibility of imatinib-resistant variants to TRAIL-induced apoptosis. Co-treatment with imatinib enhanced TRAIL-induced apoptosis in K562 cells, but this potentiating effect was gradually reduced in K562/R1 and R2 cells, which have not lost Bcr-Abl completely. Moreover, imatinib did not potentiate TRAIL-induced apoptosis of K562/R3 cells with complete loss of Bcr-Abl, which is a major target for imatinib (Figure 1D). Our results suggest the possibility that acquiring the susceptibility to TRAIL-induced apoptosis of imatinib-resistant variants might be associated with the loss of Bcr-Abl.

**TRAIL-induced apoptosis in imatinib-resistant cells is mediated through caspase activation and a mitochondrial pathway**

To identify the mechanism of TRAIL-induced apoptosis in imatinib-resistant K562 variants with loss of Bcr-Abl, we investigated the changes in the intracellular proteins related to the TRAIL pathway. After treatment with TRAIL (10 and 50 ng/ml), activation of caspase 8 and 3, truncation of Bid, degradation of PARP and cytoplasmic release of cytochrome c were observed in K562/R3 cells, but not in K562 cells (Figures 2A and 2B).

To confirm further whether activation of caspases contributes to the sensitization of K562/R3 cells to TRAIL-induced apoptosis, the cells were treated with 20 μM of a cell-permeable caspase inhibitor, z-VAD-fmk for 3 h before addition of TRAIL. Results presented in Figure 2(C) show clearly that z-VAD-fmk treatment in K562/R3 cells efficiently blocked apoptosis induced by TRAIL.

A previous study showed that blockade of the Bcr-Abl kinase activity induces apoptosis of CML cells by suppressing STAT (signal transducer and activator of transcription) 5-dependent Bcl-X_s expression [25], which is identified as a key modulator of TRAIL sensitivity [26]. We therefore determined whether the levels of STAT5 and Bcl-X_s in both K562 and K562/R3 cells were modulated by TRAIL (Figure 2D). The expression of both Bcl-X_s and STAT5 in K562/R3 cells was significantly down-regulated compared with those in K562 cells, and the level of both proteins was reduced further in TRAIL-treated K562/R3 cells, but not in K562 cells, indicating that down-regulation of STAT5-dependent Bcl-X_s in K562/R3 cells with loss of Bcr-Abl might accelerate in part TRAIL-induced apoptosis in imatinib-resistant cells.
S.-J. Park and others

Figure 1  TRAIL induces apoptosis and inhibits cell growth in imatinib-resistant K562 variants

(A) The level of Bcr-Abl in K562 and its imatinib-resistant K562/R1, R2 and R3 cells was determined by RT–PCR (upper panel) and Western blot analysis (lower panel). The levels of β-actin served as the loading control. (B) These cells were also treated without or with 1, 10 and 50 ng/ml TRAIL for 24 h, and the percentage of apoptotic cells was measured by annexin V binding followed by flow cytometry. (C) Inhibition of cell growth by TRAIL was determined using the MTT assay after these cells were treated without or with 1, 10 and 50 ng/ml TRAIL for 24 h. (D) These cells were exposed to co-treatment of imatinib (1 μM) and TRAIL (10 ng/ml) for 24 h, and the percentage of apoptotic cells was determined by annexin V analysis. Data are the means ± S.E.M. of triplicate determinations from three independent experiments. Molecular masses are indicated in kDa.

Up-regulation of DR5 is involved in sensitizing imatinib-resistant cells to TRAIL

Since the TRAIL pathway could be activated by the treatment with TRAIL in imatinib-resistant K562/R3 cells, but not in K562 cells, some upstream components of the TRAIL pathway which can activate or inhibit the whole pathway might be modulated in imatinib-resistant K562 variants to increase the susceptibility to TRAIL. Two TRAIL-Rs, DR4 and DR5, bind to TRAIL, which leads to activation of TRAIL-mediated apoptosis. We therefore investigated the mRNA levels and cell-surface expression of DR4 and DR5 in imatinib-resistant K562 variants using RT–PCR and flow cytometric analysis (Figures 3A and 3B respectively). Both mRNA levels and cell-surface expression of DR5 in K562/R1, R2 and R3 cells were significantly up-regulated, but those of DR4 in imatinib-resistant variants were rather down-regulated compared with K562 cells, especially in K562/R3 cells. To confirm further the relative importance of DR4 or DR5 for apoptosis induction by TRAIL in imatinib-resistant cells, we examined the effect of neutralizing antibodies against DR4 and DR5 on TRAIL-induced apoptosis of both K562 and K562/R3 cells. As shown in Figure 3(C), the anti-DR5-neutralizing antibody showed a significant reduction of TRAIL-induced apoptosis in K562/R3 cells, whereas the anti-DR4-neutralizing antibody had a less inhibitory effect on TRAIL-induced apoptosis in the cells. Although reduction of DR4 levels in imatinib-resistant cells might decrease the effect of anti-DR4 antibody, the predominant inhibitory effect of anti-DR5 antibody appeared to result at least in part from a higher basal level of DR5 compared with that of DR4. This result indicated that the increase of DR5 plays an important role in DR-mediated TRAIL-induced apoptosis in imatinib-resistant cells.

Down-regulation of c-FLIP associated with loss of Bcr-Abl is involved in sensitizing imatinib-resistant cells to TRAIL

Recently, it has been reported that c-FLIP potently controls TRAIL responses, and K562 cells specifically lose their resistance to TRAIL by down-regulation of c-FLIP [27]. We therefore compared the mRNA and protein levels of c-FLIP between...
K562 and imatinib-resistant K562/R1, R2 and R3 cells using RT–PCR and Western blot analysis respectively. The mRNA level of c-FLIP\textsubscript{L,S} in K562/R1, R2 and R3 cells was gradually down-regulated compared with that of K562 cells (Figure 4A, left-hand panel). In accordance with the mRNA results, the protein levels of c-FLIP\textsubscript{L,S} were reduced in K562/R1, R2 and R3 cells compared with those of K562 cells (Figure 4A, right-hand panel). Moreover, after treatment with TRAIL (10 and 50 ng/ml), mRNA levels of c-FLIP\textsubscript{L,S} were decreased in K562/R3 cells, but not in K562 cells, although c-FLIP\textsubscript{L,S} proteins of K562/R3 cells were not detected regardless of TRAIL treatment (Figure 4B). However, the expression of c-FLIP at the transcriptional level did not exactly reflect the protein levels. This discrepancy may result from the low mRNA stability of c-FLIP, which has been described previously [28,29].

In order to determine whether down-regulation of c-FLIP plays a critical role for increased susceptibility to TRAIL-induced apoptosis in imatinib-resistant cells, K562/R3 cells were transiently transfected with pcDNA3.1/FLIP\textsubscript{L} as c-FLIP\textsubscript{L} expression plasmid or pcDNA3.1 as mock control. The restoration of c-FLIP protein after transient transfection with pcDNA3.1/FLIP\textsubscript{L} was confirmed in K562/R3 cells (Figure 5A, inset). The transfectants were then treated with TRAIL (10 and 50 ng/ml) for 24 h, and then annexin V analysis was performed to determine apoptosis (Figure 5A). TRAIL-induced apoptosis was profoundly decreased in the K562/R3-FLIP\textsubscript{L} cells. These results strongly suggest that down-regulation of c-FLIP expression in imatinib-resistant cells contributes to the increased susceptibility to TRAIL. Conversely, K562 cells were transfected with siRNA targeting c-FLIP\textsubscript{L}, and silencing of c-FLIP\textsubscript{L} was examined 24–48 h after transfection. The transfectant was then treated with TRAIL (10 and 50 ng/ml) for 24 h (Figure 5B). The protein level of c-FLIP\textsubscript{L} in K562 cells transfected with c-FLIP\textsubscript{L} siRNA was significantly reduced compared with that in the cells transfected with scrambled siRNA (Figure 5B, inset), and down-regulation of c-FLIP\textsubscript{L} by siRNA sensitized TRAIL-resistant K562 cells to TRAIL-induced apoptosis (Figure 5B). These results suggest that down-regulation of c-FLIP contributes to the increased susceptibility to TRAIL of imatinib-resistant K562 cells.
Because the expression of c-FLIP is down-regulated in imatinib-resistant K562 variants with loss of Bcr-Abl kinase, we examined whether suppression of Bcr-Abl in K562 cells resulted in increased susceptibility to TRAIL-induced apoptosis and down-regulation of c-FLIP. As expected, after transfection with Bcr-Abl siRNA, K562 cells were sensitized to TRAIL-induced apoptosis (Figure 6A). This result was followed by suppression of c-FLIP expression (Figure 6B, left-hand panel). In addition, imatinib inhibited the expression of c-FLIP as well as Bcr-Abl and increased the expression of DR5 in K562 cells, dose-dependently (Figure 6B, right-hand panel). Our results strongly suggest that loss of Bcr-Abl in imatinib-resistant K562 variants leads to down-regulation of c-FLIP and up-regulation of DR5, following the increased sensitivity to TRAIL-induced apoptosis [8,9]. Although most cases of acquired imatinib resistance have been known to be associated with reactivation of Bcr-Abl, other Bcr-Abl-independent mechanisms are associated with some cases of resistance [13–16]. The phenomenon of Bcr-Abl-independent resistance is probably more frequent than generally appreciated and has a considerable clinical importance. It has been reported that cells from patients with imatinib-resistant Bcr-Abl mutation-negative CML exhibit an attenuated Bcr-Abl tyrosine kinase activity and resistance to apoptosis in response to imatinib [30]. However, the mechanism of this Bcr-Abl-independent resistance is poorly understood. The present paper reports that three imatinib-resistant K562/R1, R2 and R3 variants with gradual loss of Bcr-Abl kinase, which lost the dependency on Bcr-Abl-mediated survival signalling, became sensitive to TRAIL probably due to down-regulation of c-FLIP as well as up-regulation of DR5. Our results suggest the possibility that TRAIL may be an effective therapeutic strategy for CML patients with imatinib-resistance due to Bcr-Abl loss. Since imatinib-resistant K562/R1, R2 and R3 variants showed a gradual increase in TRAIL sensitivity and corresponding changes of TRAIL-R signalling molecules such as activation of caspase 8 and truncation of Bid, which were positively correlated with a degree of imatinib resistance, we were prompted to study whether it could be mediated by modulation of regulating molecules for the TRAIL-induced apoptotic signalling pathway. Up-regulation of DRs such as DR4 or DR5 on the cell surface may either enhance the biological activity of TRAIL-insensitive cells or sensitize TRAIL-resistant cells. Our results showed that the mRNA level and cell-surface expression of DR5 in K562/R1, R2 and R3 cells were gradually up-regulated compared with those in K562 cells and further treatment of neutralizing anti-DR5 antibody resulted in a significant reduction of TRAIL-induced apoptosis. In contrast, the levels of mRNA and cell-surface expression of DR4 in these resistant cells were decreased rather gradually compared with K562 cells. Although reduction of DR4 levels in imatinib-resistant cells might cancel the increased effect of TRAIL obtained from an increased level of DR5, this effect appeared to predominate over the cancelling effect from down-regulation of DR4, since the basal level of DR5 was much higher than that of DR4. Therefore up-regulation of DR5 might contribute to the increased susceptibility of the imatinib-resistant cells to TRAIL-induced apoptosis.

It has been reported that c-FLIP is an apoptosis-inhibitory protein homologous with caspase 8 that lacks catalytic activity and has been shown to effectively inhibit TRAIL-mediated apoptosis, and down-regulation of c-FLIP corresponds to the differentiation-mediated sensitization of K562 cells to TRAIL [27]. We found that the expression of c-FLIP in K562/R1, R2 and R3 variants was gradually down-regulated. Since it has been known that c-FLIP potently blocks TRAIL-mediated cell death by interfering with caspase 8 activation [31,32], down-regulation of c-FLIP might affect TRAIL-sensitivity in the imatinib-resistant variants. This hypothesis was confirmed by demonstrating that over-expression of c-FLIP in K562/R3 cells by transfection with pcDNA3.1/c-FLIP resulted in acquisition of resistance to TRAIL and, conversely, suppression of c-FLIP in K562 cells by transfection with c-FLIP siRNA resulted in sensitization of the cells to TRAIL. However, the relationship between Bcr-Abl activity and c-FLIP expression is not clear. Our data showed that imatinib inhibited the expression of c-FLIP as well as Bcr-Abl in K562 cells, and suppression of Bcr-Abl in K562 cells by transfection with siRNA targeting Bcr-Abl resulted in down-regulation of both c-FLIP and Bcr-Abl. In addition, during the treatment with imatinib, the levels of c-FLIP as well as Bcr-Abl were down-regulated in K562 cells. Therefore it could be suggested that the

**DISCUSSION**

Imatinib, which targets the Bcr-Abl protein, has been known as a very effective targeted drug for CML patients. However, during the treatment of CML with imatinib, some patients have acquired increased sensitivity to TRAIL[5,6] and the therapy may be an effective therapeutic strategy for CML patients with imatinib-resistance due to Bcr-Abl loss. Since imatinib-resistant K562/R1, R2 and R3 variants showed a gradual increase in TRAIL sensitivity and corresponding changes of TRAIL-R signalling molecules such as activation of caspase 8 and truncation of Bid, which were positively correlated with a degree of imatinib resistance, we were prompted to study whether it could be mediated by modulation of regulating molecules for the TRAIL-induced apoptotic signalling pathway. Up-regulation of DRs such as DR4 or DR5 on the cell surface may either enhance the biological activity of TRAIL-insensitive cells or sensitize TRAIL-resistant cells. Our results showed that the mRNA level and cell-surface expression of DR5 in K562/R1, R2 and R3 cells were gradually up-regulated compared with those in K562 cells and further treatment of neutralizing anti-DR5 antibody resulted in a significant reduction of TRAIL-induced apoptosis.
TRAIL-induced apoptosis in imatinib-resistant cells

Figure 4  Down-modulation of c-FLIP mRNA and protein levels in imatinib-resistant K562 variants

(A) The mRNA and protein levels of c-FLIP in K562 and its imatinib-resistant K562/R1, R2 and R3 cells were determined by RT–PCR (left-hand panel) and Western blot analysis (right-hand panel) respectively. (B) The altered levels of c-FLIP mRNA and its protein in K562 and K562/R3 cells treated with or without TRAIL (10 and 50 ng/ml) for 24 h were determined by RT–PCR (left-hand panel) and Western blot analysis (right-hand panel) respectively. Sizes and molecular masses are indicated in bp and kDa respectively.

Figure 5  Altered susceptibility to TRAIL-induced apoptosis is closely correlated with the expression of c-FLIP in imatinib-resistant K562 variants

(A) K562/R3 cells were transiently transfected with pcDNA3.1/c-FLIP, or pcDNA3.1 as mock control. The transfectants were treated with TRAIL (10 and 50 ng/ml) for 24 h and annexin V analysis was performed. (B) K562 cells were transfected with c-FLIP-targeted siRNA or scrambled siRNA as control. The transfectants were treated with TRAIL and annexin V analysis was performed. Results are means ± S.E.M. of triplicate determinations from three independent experiments.

Loss of Bcr-Abl in imatinib-resistant K562 variants led to down-regulation of c-FLIP.

In conclusion, our results strongly suggest that, during the selection process of imatinib-resistant variants after treatment with imatinib, the decreased activity and level of Bcr-Abl are followed by down-regulation of c-FLIP and up-regulation of DR5. These molecular changes appear to affect the susceptibility of imatinib-resistant variants to TRAIL. Therefore TRAIL would be an effective strategy for treatment of imatinib-resistant CML cancer patients with loss of Bcr-Abl.

© The Authors Journal compilation © 2009 Biochemical Society
masses are indicated in kDa.

Figure 6 Suppression of Bcr-Abl renders K562 cells susceptible to TRAIL-induced apoptosis

(A) K562 cells were transfected with Bcr-Abl-targeted siRNA or scrambled siRNA as control. The transfectants were treated with TRAIL (10 and 50 ng/ml) and annexin V analysis was performed. Results are means ± S.E.M. of triplicate determinations from three independent experiments. (B) The altered levels of Bcr-Abl, FLIPL or DR5 in K562 cells transfected with Bcr-Abl siRNA (left-hand panel) or K562 cells treated with imatinib (0.1, 0.5 and 1 μM for 24 h) (right-hand panel) were determined by Western blot analysis. The levels of β-actin served as the loading control. Molecular masses are indicated in kDa.

REFERENCES


FUNDING

This work was supported by the Medical Science and Engineering Research Center program of the Ministry of Science and Technology/Korea Science and Engineering Foundation, South Korea [grant number R13-2005-003 (to S.-H. K.)].

© The Authors. Journal compilation © 2009 Biochemical Society
TRAIL-induced apoptosis in imatinib-resistant cells


31 Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J. L., Schrter, M., Burns, K., Maltmann, C. et al. (1997) Inhibition of death receptor signals by cellular FLIP. Nature 388, 190–195


Received 27 October 2008/S February 2009; accepted 9 February 2009
Published as BJ Immediate Publication 9 February 2009, doi:10.1042/BJ20082131