Inhibition of apoptosis by downregulation of hBex1, a novel mechanism, contributes to the chemoresistance of Bcr/Abl+ leukemic cells

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Overexpression of multidrug resistance proteins (Mdrs) and enhanced antiapoptotic capability are two of the main mechanisms by which Bcr/Abl+ chronic myeloid leukemia cells acquire drug resistance; however, it has been shown that Mdr-1 expression provides minimal protection against cell apoptosis induced by chemotherapeutic drugs. The mechanism by which cells acquire an enhanced antiapoptosis capacity in the drug-resistant process needs to be further understood. Here, we identified human brain expressed X-linked 1 (hBex1) as a downstream target of the p75 neurotrophin receptor pathway in imatinib-resistant K562 cells by comparing the gene expression profiles with the parent K562 cells. Silencing hBex1 inhibited imatinib-induced cell apoptosis and overexpression of hBex1-sensitized cells to imatinib-induced apoptosis. Further investigation revealed that hBex1 associates with protocadherin 10 (PCDH10). Silencing of pcdh10 attenuated apoptosis induced by imatinib in hBex1 transfected cells, suggesting that, in addition to Mdr and Bcl-2 family members, reduced expression of hBex1 can also inhibit imatinib-induced apoptosis. These data provide evidence that expression of hBex1 in leukemic cells is a novel mechanism by which chemoresistance is achieved and suggests that hBex1 is a potential molecular target for the development of novel leukemic treatments.

Introduction

The Bcr/Abl fusion protein plays an important role in the blast crisis of chronic myeloid leukemia (CML). Activation of Bcr/Abl leads to both the malignant transformation of CML cells and resistance to various antitumor/apoptosis induction agents (1–3). Currently, inhibition of Bcr/Abl activity is regarded as one of the most effective treatments for Bcr/Abl+ CML (4,5). Imatinib (imatinib mesylate, STI571), a Bcr/Abl-specific inhibitor, can compete with adenosine triphosphate for the nucleotide-binding site in the catalytic group of Bcr/Abl and can inhibit catalytic activity of this kinase, making it unable to phosphorylate and activate downstream effectors, which results in the suppression of cell proliferation and increased apoptosis (6,7). Imatinib is one of the first batch of molecular-targeted drugs approved for the clinical treatment of CML and gastrointestinal stroma tumors (8,9). In recent years, it has also been determined that imatinib may kill tumor stem cells during the G0 phase (10). However, many CML patients treated with imatinib developed a recurrence of the disease, suggesting that acquired drug resistance of the leukemia cells to imatinib is a major concern that has yet to be overcome (11–13).

The development of resistance against imatinib in Bcr/Abl+ tumor cells is a complex process. In addition to the production of multidrug resistance proteins (Mdrs), namely adenosine triphosphate-binding cassette transport proteins (ABC), the enhanced antiapoptosis capacity of these cells plays an important role in the process of drug resistance (14,15). Overexpression of Bcl-2/Bcl-xL and inhibition of Bim expression by RNAi can inhibit the killing effect of imatinib on Bcr/Abl+ tumor cells (16,17). Additionally, activation of extracellular signal-regulated kinase 1/2 through RhoA and RASAP1 increased the drug resistance. Inhibition of mitogen-activated protein kinase (MAPK) activity promoted imatinib-induced cell apoptosis (18–20). Besides the Bcl-2 family and MAPK, it was unclear if the antiapoptotic capacity of leukemic cells could be increased via other mechanisms. Furthermore, overexpression of the ABC transporter, Mdr-1, is closely associated with acquired resistance of the majority of tumor cells, but Mdr-1 provides minimal protection against cell growth inhibition and apoptosis induced by imatinib (21). Previous studies have also reported the establishment of imatinib-resistant Bcr/Abl+ tumor cells that express Mdr-1, but do not exhibit changes in Bcl-2 family members and MAPK activity, indicating that molecules other than Bcl-2 and MAPK are involved in enhancing antiapoptotic capacity (22).

In this study, we established an imatinib-resistant K562 cells (KR cells) that demonstrates high expression of Mdr-1 but does not change the expression levels of Bcl-2 family members. When analyzing the gene expression profile of KR cells, we identified human brain expressed X-linked 1 (hBex1), a downstream member of the p75 neurotrophin receptor (p75NTR) signaling cascade, which is involved in cell apoptosis during the process of cell resistance. Silencing of hBex1 inhibited cell apoptosis induced by imatinib, and re-expression of hBex1 increased imatinib-induced apoptosis. Further investigation demonstrated that hBex1 associates with protocadherin 10 (PCDH10), a member of the protocadherins, suggesting that, similar to Mdr-1, downregulation of hBex1 can inhibit imatinib-induced apoptosis. Thus, the apoptotic modulation in leukemia cells via hBex1 provides evidence for a novel mechanism of cell chemoresistance.

Materials and methods

Establishment of KR cells resistant to imatinib

K562 cells, purchased from American Type Culture Collection (Rockville, MD), were maintained in RPMI 1640 media containing 15% fetal bovine serum (FBS) (Gibco, Carlsbad, CA) at 37°C under an atmosphere of 5% CO2 and passaged at a ratio of 1:3 every 2 days. Imatinib-resistant cells were obtained in vitro from K562 cells that were cultured in RPMI 1640 media containing 15% FBS and a 0.5 μM concentration of imatinib. Upon acclimation of the cells to the culture conditions, the culture period was extended and was followed by increasing doses of imatinib until a terminal concentration of 2.2 μM was reached. The culture was then maintained in this condition.

Genech analysis

Total RNAs from synchronized KR cells, KR cells with stable expression of hBex1 and KR cells with stable expression of pEGFP blank vectors were extracted and purified using the RNasy mini kits (Qiagen, Hilden, Germany). Total RNA (10 μg) was converted into double-stranded cDNA by reverse transcription using the T7-T24 primer set and subjected to phenol–chloroform–isoamyl extraction. For cDNA conversion, the in vitro transcription MEGA-ScriptTM T7 kit (Ambion), along with biotinylated nucleotides, was used. The in vitro transcription product was then hybridized to an Affymetrix U133 plus 2.0 cDNA genechip and washed steps were performed as suggested by the manufacturer. Each hybridized Affymetrix genechip was

Abbreviations: ABC, adenosine triphosphate-binding cassette transport protein; ANOVA, analysis of variance; CML, chronic myeloid leukemia; FBS, fetal bovine serum; FCM, flow cytometry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; hBex1, human brain expressed X-linked 1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; Mdr, multidrug resistance protein; NF-kB, nuclear factor-kappa B; PCR, polymerase chain reaction; p75NTR, p75 neurotrophin receptor; PCDH10, protocadherin 10.

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scanned with an argon ion laser scanner at 570 nm. Initial absolute and comparative analysis of the resulting data images was performed with Affymetrix custom image analysis software (Genechip version 3.1). The differences between K562 cells and KR cells, hBex1 transfected KR cells (KR/hBex1) and pEGFP-C1 blank vector transfected KR cells (KR/pEGFP) were compared by univariate and multivariate analysis of variance (ANOVA) statistical analysis. The detection specificity of the genechip was 1/100 000 copies. Expressions of the target genes were judged to be increased if the value was >2.0 times that of the K562 cells or KR/pEGFP cells and to be decreased if it was <0.5 times that of the K562 cells or KR/pEGFP cells. The false gene information resulted, as per the references (23).

Real-time polymerase chain reaction

Total RNA was isolated from 1 × 10^6 cells using the RNeasy mini kit. cDNA was synthesized from total RNA by reverse transcription. The primers (sense: 5′-GATCGTCTGAGCAGGATATGGAGTCCA-3′; antisense: 5′-TCTGTCGGGATCTCTAGGCAAACTC-3′) were designed respectively in 25 μl SYBR green Mix (Invitrogen, Carlsbad, CA) for real-time polymerase chain reaction (PCR) assay. Lightcyle PCR conditions were 94°C for 2 min for predenaturing, then 35 cycles at 94°C for 15 s, 58°C for 30 s and 72°C for 30 s.

Commmunoprecipitation and western blot analysis

For immunoprecipitation experiments, 1 × 10^6 cells were lysed in 3-[3-(3-cholamidopropyl) dimethylammonio]propanesulfonate buffer pH 7.4, 50 mM Tris–HCl pH 7.4 buffer containing 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% 3-[3-(3-cholamidopropyl) dimethylammonio]propanesulfonate, 1 mM phe- nylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM NaF and 1 mM pepstatin) on ice for 10 min. Cold lysates were then centrifuged at 13 000 r.p.m. for 30 min at 4°C. Clarified lysates were incubated with 2 μg of one of the following antibodies at room temperature for 1 h (mouse antibody to Bcr-Abl and rabbit antibody to green fluorescent protein (GFP); Santa Cruz Biotechnology, Santa Cruz, CA) overnight (mouse antibody to HA-tag; Cell Signaling Technology, Danvers, MA) or normal IgG (control). The samples were subsequently precipitated with protein A/G-agarose beads (Santa Cruz) at 4°C overnight. The resulting immunocomplex was washed three times with ice-cold RIPA buffer, suspended in Laemmli sample buffer containing dithio- theoril, heated to 100°C for 5 min, centrifuged at 13 000 r.p.m. for 5 min and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. For western blot analysis, total cellular extracts were obtained by lysis of cells in a lysis buffer and a protease inhibitor cocktail. Protein concentrations of the cell lysates were determined by the Bradford method (Bio-Rad, Hercules, CA). An equal volume of 2× sodium dodecyl sulfate loading buffer was added, and the samples were boiled for 5 min. Protein samples (70 μg per lane) were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose filters (Amersham Biosciences, Piscataway, NJ). The filters were blocked with Tris–HCl buffer containing Tween-20 buffer (pH 7.6, 10 mM Tris–HCl buffer, 0.15 M NaCl and 0.05% [pH 7.4, 50 mM Tris–HCl buffer with 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% 3-[3-(3-cholamidopropyl) dimethylammonio]propanesulfonate, 1 mM phe- nylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM NaF and 1 mM pepstatin) for 15 s, at 58°C for 2 min, the samples were subjected to 35 cycles of 94°C for 30 s. PCR products were 2.0 times that of the seed nuclei and cytosolic extracts was quantified using the percentage of propidium iodide-stained nuclei in the sub-diploid peak by flow cytometry (FCM).

Screening and establishment of KR cells with stable expression of hBex1

KR cells at 90% confluence in six-well plates were transfected with 10 μg hBex1/pEGFP-C1 or pEGFP-C1 blank plasmid using Lipofectamine 2000 according to the manufacturer’s instructions. The cells were passaged at 48 h after transfection and 1000 μg/ml G418 (Clontech) was added to screen for resistant clones for 7 days. Expression of hBex1 was identified by western blot in screened cells.

Retrovirus production

The PT76 packaging cells (Clontech) were used to produce the recombinant retrovirus. Retrovirus production was transferred using Lipofectamine 2000 according to the manufacturer’s instructions. The supernatant containing recombinant retrovirus was harvested at 72 h after transfection by following by filtering through 0.45 μm filters with low protein binding (Millipore, Bellerica, MA) and then infecting KR cells. A 4 μg/ml polybrene (Sigma) was added simultaneously.

Soft agar growth assay

KR cells (1 × 10^5) were infected with hBex1/pLXSN or pLXSN retrovirus for 24 h (polybrene concentration was 4 μg/ml). The next day, the cells were washed three times with serum-free RPMI 1640 medium. The cells were adequately mixed with prewarmed RPMI 1640 medium containing 15% FBS, 0.4% agarose (Type 7A, Sigma) and 200 μl/ml gentamycin. Two milliliters of this mixture for each group was seeded into a 35 mm well that was precoated with 1 ml of 0.4% agarose and incubated for 24 h (polybrene concentration was 4 μg/ml). The next day, the cells were washed three times with serum-free RPMI 1640 medium. The cells were adequately mixed with prewarmed RPMI 1640 medium containing 15% FBS, 0.4% agarose (Type 7A, Sigma) and 200 μl/ml gentamycin. Two milliliters of this mixture for each group was seeded into a 35 mm well that was precoated with 1 ml of 0.4% agarose and incubated for 48 h at 4°C. After solidification, 0.5 ml of 0.4% agarose and 0.5 ml medium were laid on each well and incubated overnight at 37°C in a 5% CO2 incubator. The plate was placed in a sealed chamber and incubated at 37°C in a 5% CO2 incubator for 14 days. The number of colonies per cm^2 was counted under an inverted microscope.

Nuclear protein extraction and nuclear factor-kappa B determination

Nuclear protein extraction was performed according to the manufacturer’s specifications (Pierce Biotechnology, Rockford, IL). Briefly, the cells were harvested by centrifugation and the cell pellet was vortexed vigorously for 15 s, followed by transfer to an Eppendorf tube. A volume of 200 μl of the ice-cold cytoplasmic extraction reagent I containing a protease inhibitor cocktail (Roche, Mannheim, Germany) was then added to the tube. The tube was vortexed vigorously for 15 s and incubated on ice for 10 min, followed by the addition of 11 μl of cytoplasmic extraction reagent II. The sample was vortexed vigorously for 5 s and incubated on ice for 1 min. After incubation, the tube was once again vortexed for 5 s and centrifuged for 1 min at 13 000 r.p.m. The supernatant containing the cytosolic extract was transferred to tubes. The remaining insoluble pellet was resuspended in 100 μl nuclear extraction reagent containing a protease inhibitor cocktail. The tube was vortexed vigorously for 15 s. The tube was incubated on ice for 40 min and vortexed for 15 s at 10 min intervals. After incubation, the tubes were centrifuged at 13 000 r.p.m. for 10 min. The supernatant was removed and mixed with prewarmed Opti-MEM I (Gibco) and allowed to stand at room temperature for 5 min. The diluted Lipofectamine 2000 was then mixed well with the diluted plasmid DNA and incubated at room temperature for 20 min. The complexes were added to the seeded 35 mm plates and incubated at 37°C in a 5% CO2 incubator.

Flow cytometry assay

To start, 1×10^6 cells were washed twice with 10 mM phosphate-buffered saline (pH 7.4) by centrifugation and fixed in 70% ethanol at 4°C for 18 h. After additional washing, cells were digested with RNase (50 mg/ml) and stained with propidium iodide (100 mg/ml) for 30 min. Cell apoptosis was measured using the percentage of propidium iodide-stained nuclei in the sub-diploid peak by flow cytometry (FCM).

Plasmid construction

hBex1/pLXSN plasmid construction: a pair of specific primers (sense: 5′- GATCGTCTGAGCAGGATATGGAGTCCA-3′; antisense: 5′-TCTGTCGGGATCTCTAGGCAAACTC-3′) were designed and used to amplify the coding region of hBex1. PCR was performed using the cDNAs from K562 cells as templates under standard conditions. After pre-denaturing 94°C for 2 min, the samples were subjected to 35 cycles of 94°C for 15 s, at 58°C for 30 s and at 72°C for 30 s. PCR products were separated by electrophoresis in a 1% agarose gel and target fragments were recovered using a DNA recovery kit (Qiagen). The recovered fragments were digested with BamH I/Xho I restriction enzymes and subcloned into BamH I/
equilibrated at room temperature for 15 min, followed by the addition of 30 μl binding buffer and 10 μg nuclear proteins and the addition of a lysis buffer to achieve a final volume of 50 μl. The plate was incubated for 1 h at room temperature. It was washed three times with 1 × wash buffer. Antibody primary antibodies (anti-p65, anti-p50, anti-p52, anti-Rel-B and anti-Rel-C, 100 μl) at a 1:1000 dilution were added to each well, and the plate was incubated for 1 h at room temperature. The plate was washed three times with 1 × wash buffer. Horseradish peroxidase-labeled secondary antibody (100 μl) at a 1:2000 dilution was added to each well, and the plate was incubated for 1 h at room temperature. The plate was washed three times with 1 × wash buffer. After the addition of a reaction terminating solution, the absorbance was read under a microplate reader at 450 nm.

Rhodamine 123 retention test
The cells were mixed with 2 μl Rhodamine 123 dye (Sigma) and incubated at 37°C under an atmosphere of 5% CO2 for 4 h followed by washing twice with cold phosphate-buffered saline (pH 7.4, 10 mM) by centrifugation and FCM assay.

Caspase activity
A volume of 100 μl (2000 cells/ml) of cells were seeded in each well of a 96-well plate and 10 μl per well imatinib at a final concentration of 2.2 μM was added and cultured at 37°C under an atmosphere of 5% CO2 for 24 h, followed by adding 100 μl caspase-Glo® substrate mixtures (Promega, Madison, WI) per well for 1 h to allow evaluation of the fluorescence intensity.

Proliferation assay
Cells at a 50% density were seeded in 50 ml flask, washed three times with serum-free RPMI 1640 medium and incubated overnight in a serum-free medium, followed by incubation in RPMI 1640 medium containing 15% FBS for cell synchronization. The synchronized cells (2000 cells per well) were seeded into a 96-well plate and incubated at 37°C under an atmosphere of 5% CO2 for 24 h, followed by the addition of 20 μl per well of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxymethyl-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt and an electron coupling reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxymethyl-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] solution (Promega) and adequate mixing. The absorbance (A) of each well was measured at 0 and 2 h by using a microculture plate reader at a test wavelength of 490 nm. The proliferation rate was expressed as ΔA = A t - A 0 .

Results
KR cell resistant to imatinib is related to Mdr-1 and independent of Bcl-2 family members
To explore the molecular mechanism by which Bcr/Ablα+ cells develop resistance to imatinib, we established imatinib-resistant Bcr/Ablα− leukemia K562 cells (KR) which were also slightly resistant to doxorubicin. After >6 months of induction, the maintenance concentration of imatinib reached 2.2 μM (Figure 1a). PCR/DNA sequencing analysis showed that transcription of Bcr/Abl messenger RNA in KR cells was not significantly increased, no mutation was found in the exons, and western blot analysis revealed that there was no significant increase in Bcr/Abl protein in K562 cells (Figure 1b), suggesting that KR cells had no Bcr/Abl mutations and no increase in Bcr/Abl protein levels. This demonstrated that the imatinib resistance of KR cells is independent of Bcr/Abl. To further analyze the ABC transporter family members change during the development of resistance in KR cells, we used Affymetrix U133 plus 2.0 cDNA expression genechip detection and found that only ABCB1 (Mdr-1) was significantly increased, whereas ABCG2 slightly declined; the other ABC transporter family members displayed no significant change (Figure 1c). Western blotting for ABCB1 and ABCG2 showed results that were consistent with the genechip findings (Figure 1d), suggesting that drug resistance in KR cells is primarily related to the excessive expression of Mdr-1. However, since increased antiapoptosis cell capability is an important event in the resistance process and high expression of Mdr-1 has been shown to provide weak protection from imatinib-induced K562 cell proliferation inhibition and cell apoptosis (21) and inhibition of Mdr-1 activity by Verapamil did not fully restore sensitivity to imatinib (data not shown), it is possible that genes other than Mdr-1 maybe involved in the inhibition of KR cell apoptosis. It is known that Bcl-2 family members participate in drug resistance by blocking apoptosis induced by using a microculture plate reader at a test wavelength of 490 nm. The proliferation rate was expressed as ΔA = A t - A 0 .

Fig. 1. Resistance of KR cells to imatinib involved Mdr-1 expression, but was independent of the pro-survival proteins of the Bcl-2 family. (a) Imatinib resistance in KR cells. KR cells were resistant to imatinib and were also slightly resistant to doxorubicin compared with parent K562 cells as revealed by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxymethyl-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. (b) Detection of Bcr/Abl protein in KR and K562 cells. Bcr/Abl protein was detected in lysates from KR and K562 cells by western blot analysis. GAPDH levels were used as a loading control. (c) Alteration of ABC transporter family messenger RNA (mRNA) in KR cells. ABCB1 messenger RNA was increased and ABCG2 messenger RNA was decreased in KR cells versus K562 cells as measured by genechip analysis. The results were validated by real-time reverse transcription–PCR. The messenger RNA level of each gene was normalized to the GAPDH messenger RNA levels. (d) Detection of ABCB1, ABCG2 and the pro-survival proteins of the Bcl-2 family in KR and K562 cells. ABCB1 (Mdr-1), ABCG2 and three main pro-survival proteins of the Bcl-2 family (Bcl-2, Bcl-xl and Mcl-1) were detected in lysates from KR and K562 cells by western blot analysis. GAPDH levels were used as a loading control.
by imatinib; therefore, we examined the pro-survival proteins of the Bcl-2 family. Western blot analysis revealed that Bcl-2, Bcl-xL, and Mcl-1 were not significantly changed in KR cells (Figure 1d), suggesting that blocking of KR cell imatinib-induced apoptosis maybe independent of the pro-survival proteins of the Bcl-2 family.

hBex1 in KR cells is downregulated and re-expression of hBex1 promoted the sensitivity of KR cells to imatinib

To confirm the molecular mechanism involved in the inhibition of imatinib-induced apoptosis, we analyzed gene expression differences in K562 cells and KR cells and found hBex1, a downstream target of the p75NTR pathway, underwent the most notable transcriptional 'silencing' in KR cells. Compared with K562 cells, hBex1 decreased by >1000 times in KR cells and subsequently demonstrated no significant increase after tens of passage with or without the presence of imatinib (P > 0.05, ANOVA). KR cells were treated with 0–10 μM of the putative demethylation reagent, 5′-Aza-2′-deoxycytosine, for 48 h. Real-time PCR revealed no significant increase in the number of hBex1 copies in the KR cells after 5′-Aza-2′-deoxycytosine treatment (P > 0.05), suggesting that downregulation of hBex1 transcription in KR cells has no effect on the methylation of its promoter (Figure 2a). To investigate the impact of hBex1 expression on KR cells, we transfected KR cells with hBex1-pEGFP (KR/hBex1) and empty vector pEGFP plasmids (KR/pEGFP, respectively. We found that 24 h after transfection, hBex1 transfected KR cells demonstrated no significant change in cell apoptosis; however, 24–48 h after adding a maintenance dose of imatinib, FCM demonstrated that apoptosis of hBex1 transfected cells increased significantly (Figure 2b). Similarly, the growth of cell colonies of KR cells infected with hBex1-pLXSN recombinant retrovirus in soft agarose was also lower than that of the control group (P < 0.05, ANOVA) (Figure 2c). These results suggested that overexpression of hBex1 increased the sensitivity of cells to imatinib and that KR cells may prevent imatinib-induced apoptosis through the silencing of hBex1.

hBex1 activated JNK independent of Bcr/Abl pathway

The experiments described above prove that re-expression of hBex1 promotes the KR cell sensitivity to imatinib. Thus, since imatinib is a specific inhibitor of Bcr/Abl, it remained to be determined whether hBex1 is a downstream molecule in the Bcr/Abl pathway.
Materials and Methods. The results revealed that p50, p65, p52, Rel-B and Rel-C levels did not significantly increase in hBex1 transfected cells compared with the control group (Figure 3a). We subsequently analyzed the amino acid sequence and found that, although phosphorylation at Ser102 existed in hBex1 (http://www.expasy.ch/uniprot/Q9HBB7#general), there was no clear Bcr/Abl tyrosine phosphorylation site in its sequence. Coimmunoprecipitation experiments showed that hBex1 and Bcr/Abl could not be coprecipitated with the anti-Bcr-Abl and anti-GFP antibodies, respectively (Figure 3b). Because the Bcr/Abl pathway can activate Akt, MAPK and NF-κB, we also evaluated the impact of hBex1 expression on the activity of Akt, MAPK and NF-κB in cells. Western blot analysis demonstrated that the JNK of the MAPK pathway was obviously activated, whereas Akt and p38 showed no significant change in K562 cells with stable expression of hBex1 (Figure 3c). An enzyme-linked immunosorbent assay revealed that stable expression of hBex1 in KR cells and K562 cells did not significantly increase p50, p65, p52 and Rel-B and Rel-C levels in nuclear proteins (P > 0.05, ANOVA) (Figure 3d).

In summary, downregulation of the hBex1 transcription was not imatinib specific, but was associated with an enhanced antiapoptosis capacity in the process of cell resistance; therefore, hBex1 is not a downstream target of the Bcr-Abl pathway. It can activate MAPK, but does not affect NF-κB activity in Bcr/Abl+ cells.

In the presence of imatinib, hBex1 activates caspase 3/7 via the nonclassical pathway

To analyze the molecular mechanism by which hBex1 induces cell apoptosis, we carried out Rhodamine 123 efflux experiments, which examined whether hBex1 induces apoptosis through inhibiting the function of Mdr-1. Results demonstrated that transient and stable transfection of KR cells with hBex1 also resulted in no obvious change in the Rhodamine 123 efflux capacity (Figure 4a), suggesting that hBex1 does not affect Mdr-1 function. The above experiments prove that hBex1 can enhance JNK activity and that activation of the JNK pathway can trigger apoptosis. We subsequently examined the impact of the JNK pathway on cell apoptosis. However, inhibition of JNK with SP600125, a putative JNK-specific inhibitor, decreased KR cell proliferation and increased apoptosis (Figure 4b), suggesting that JNK maintains the survival of KR cells. To determine whether imatinib can affect the Bcl-2 pro-apoptotic proteins, we also examined whether hBex1 induces apoptosis through the endogenous or exogenous pathway (24). Western blot analysis demonstrated that Bad, Bim, Bid, Bmf and smac/diablo also showed no significant change in hBex1 transfected KR cells in the presence or absence of imatinib (Figure 4c). The luciferase assay showed that, in the presence of imatinib, caspase 3/7 was activated in hBex1 transfected KR cells, whereas caspases 8 and 9 showed no significant changes (Figure 4d). These results suggest that the caspase 8 and endogenous apoptosis pathways are not involved in hBex1-induced apoptosis; in the presence of imatinib, hBex1 activates caspase 3/7 via the nonclassical pathway.

**hBex1 induced apoptosis through PCDH10**

To screen for target proteins involved in hBex1-induced apoptosis, we used U133 plus 2.0 genecip to analyze the major alteration of global gene expression in KR cells. hBex1 transfected KR cells. The results demonstrated that pcdh10, as a member of cadherin superfamily, was significantly decreased in hBex1-silenced KR cells, while its expression in hBex1 transfected KR cells was significantly increased (Figure 5a). When 293T cells were cotransfected with pcdh10/pCMV-Myc and hBex1/pCMV-HA plasmids, we found that PCDH10 could be coimmunoprecipitated by hBex1 (Figure 5b), suggesting that hBex1 interacts with PCDH10. Transfection of K562 cells, HL-60 cells and colon cancer SW480 cells with pcdh10/pDNA 3.1(+), significantly inhibited the proliferation of these cells (Figure 5c). Interestingly,

**Fig. 3. hBex1 activated JNK independent of NF-κB pathway.** (a) hBex1 was downregulated in vincristine (VCR)-resistance cells. The messenger RNA level of hBex1 from vincristine-resistant K562 cells (K562/VCR) was downregulated compared with that from K562 cells showed by semiquantitative reverse transcription-PCR. β-actin levels were used as a loading control. (b) Analysis of hBex1/Bcr-Abl binding capacity in several cell lines. hBex1 was immunoprecipitated by anti-GFP antibody from hBex1 transfected cells (KR/hBex1), blank vector transfected cells (KR/pEGFP) or KR cells. No Bcr-Abl could be detected in hBex1 immunoprecipitates by western blot. (c) Analysis of MAP and Akt kinase activation in several cell lines. Total and phosphorylated extracellular signal-regulated kinase 1/2 (Erk1/2), JNK, p38 and Akt were detected in lysates from KR and K562 cells by western blot analysis. The results revealed that hBex1 activated JNK. (d) Quantitative analysis of nuclear NF-κB. Nuclear NF-κB was determined by an enzyme-linked immunosorbent assay, as described in the Materials and Methods. The results revealed that p50, p65, p52, Rel-B and Rel-C levels did not significantly increase in hBex1 transfected KR and K562 cells compared with expression in mock and blank K562 cells.
pcdh10, silenced by shRNAi, inhibited imatinib-induced apoptosis in KR cells (Figure 5d). These results suggest that PCDH10 promotes cell apoptosis and that KR cells inhibit apoptosis and promote the growth of resistant cells by downregulating PCDH10 through hBex1.

Discussion

Our research on the resistance of Bcr/Abl⁺ tumor cells to imatinib demonstrated that, in addition to drug efflux by overexpression of Mdr-1, silencing of hBex1 can strengthen the antiapoptosis capacity of the cells. Additionally, re-expression of hBex1 both increased PCDH10 and partially recovered sensitivity to imatinib, suggesting that the hBex1/PCDH10 pathway is a novel mechanism by which cells develop drug resistance.

Bex is a member of the cell death precursor (p75NTR-associated cell death executor) molecule in the downstream p75NTR/TrkA/B pathway, belonging to tumor necrosis factor receptor superfamily members. Currently, five highly homologous members have been...
found, namely Bex1 (TCEAL8), Bex2, Bex3, Bex4 (TCEAL7) and Bex5. Bex is primarily expressed in nerve cells. Bex1 and Bex2 are also expressed in the hematological system and other tumor cells (25–27). Fischer and Quentmeier (28,29) found that Bex1 and Bex2 are the markers of acute myeloid leukemia with mixed lineage leukemia rearrangements. Bex is a signal transduction mediator whose function is not yet fully understood. Most studies suggest that Bex functions as a tumor suppressor gene. Foltz et al. observed that class I Hox1 and hBex2 were silenced by extensive promoter methylation in the tissue and cell lines of brain glioma and found that expression levels of both hBex1 and hBex2 increased after demethylation. Re-expression of hBex1 or hBex2 led to cell sensitivity to chemotherapeutic drugs and increased apoptosis. Furthermore, tumor formation was delayed in animal glioma cells that expressed hBex1 and hBex2 (30). Chien (26) found that hBex1 expression increased JNK activity, we found that the inhibition of JNK inhibited cell proliferation and increased apoptosis, suggesting that JNK is not involved in imatinib-induced apoptosis of KR cells. Western blot analysis revealed that imatinib activated caspase-3 activity independent of the endogenous apoptosis pathway in KR cells. As a candidate tumor suppressor gene, hBex1's function is inseparable with apoptosis; however, the mechanism of apoptosis induced by hBex1 remains unclear. It was first found that ectopic expression of Bex can enhance the NGF-induced apoptosis of HEK293 through activating caspases 2 and 3 cascades (32). Because hBex1 exists downstream in the p75NTR pathway, p75NTR can induce apoptosis through the activation of the JNK pathway in nerve cells (33). Though hBex1 expression increased JNK activity, we found that the inhibition of JNK inhibited cell proliferation and increased apoptosis, suggesting that JNK is not involved in imatinib-induced apoptosis of KR cells. Western blot analysis revealed that imatinib activated caspase 3 activity independent of the endogenous apoptosis pathway in KR cells transfected with hBex1, while caspase 2 (data not shown) and -8 demonstrated no significant change in the exogenous pathway, suggesting that apoptosis induced by hBex1 has unique characteristics. To screen and identify hBex1-induced apoptosis, we compared the changes in the gene expression profile before and after transfection of KR cells with hBex1 and identified the cadherin superfamily protocadherin member, PCDH10. Protocadherin has six series of extracellular domains, a transmembrane region, and the unique cytoplasmic tail CM2 domain. Compared with other cadherins, PCDH10 does not bind with the β-catenin/T cell factor pathway to affect the cytoskeleton (34,35). Protocadherin has weak cell adhesion between cells and its function needs to be further clarified. PCDH10 maybe a candidate tumor suppressor gene. Qian found that pcdh10 was down-regulated...
by promoter methylation in nasopharyngeal carcinoma tissues and leukemic cells. Inhibiting methylation or overexpression of PCDH10 significantly inhibited colony formation and proliferation of cultured cells in vitro (35). Uemura (36) found pcdh10−/− mice presented deficiencies in axon transportation. In this study, our findings suggest that PCDH10 may also participate in cell apoptosis, which maybe involve the unique CM2 domain of PCDH10 in the cytoplasm. Furthermore, hBex1 interacts with PCDH10 as revealed using coimmunoprecipitation experiments. The role of PCDH10’s participation in apoptosis needs to be further clarified.

In summary, our research demonstrates that hBex1 silencing in Bcr/Abl−/− K562 cells, in addition to resulting in the high expression of Mrd-1 protein, can also inhibit imatinib-induced apoptosis. Re-expression of hBex1 recruited PCDH10 and partially recovered sensitivity to imatinib. To our knowledge, our study marks the first report on the role of hBex1pcdh10 silencing in the development of drug resistance in Bcr/Abl+ cells and subsequently, inhibition of imatinib-induced apoptosis.

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References


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