Alterations of Ceramide/Sphingosine 1-Phosphate Rheostat Involved in the Regulation of Resistance to Imatinib-induced Apoptosis in K562 Human Chronic Myeloid Leukemia Cells

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In this study, mechanisms of resistance to imatinib-induced apoptosis in human K562 cells were examined. Continuous exposure to stepwise increasing concentrations of imatinib resulted in the selection of K562/IMA-0.2 and -1 cells, which expressed ~2.3- and 19-fold resistance, respectively. Measurement of endogenous ceramides by high performance liquid chromatography/mass spectroscopy showed that treatment with imatinib increased the generation of ceramide, mainly C18-ceramide, which is generated by the human longevity assurance gene 1 (hLASS1), in sensitive, but not in resistant cells. Inhibition of hLASS1 by small interfering RNA partially prevented imatinib-induced cell death in sensitive cells. In reciprocal experiments, overexpression of hLASS1, and not hLASS6, in drug-resistant cells caused a marked increase in imatinib-induced C18-ceramide generation, and enhanced apoptosis. Interestingly, there were no defects in the levels of mRNA and enzyme activity levels of hLASS1 for ceramide generation in K562/IMA-1 cells. However, expression levels of sphingosine kinase-1 (SK1) and generation of sphingosine 1-phosphate (S1P) were increased significantly in K562/IMA-1 cells, channeling sphingoid bases to the sphingosine kinase pathway. The partial inhibition of SK1 expression by small interference RNA modulated S1P levels and increased sensitivity to imatinib-induced apoptosis in resistant cells. On the other hand, forced expression of SK1 in K562 cells increased the ratio between total S1P/C18-ceramide levels ~6-fold and prevented apoptosis significantly in response to imatinib. Additional data indicated a role for SK1/S1P signaling in the up-regulation of the Bcr-Abl expression at the post-transcriptional level, which suggested a possible mechanism for resistance to imatinib-mediated apoptosis. In conclusion, these data suggest a role for endogenous C18-ceramide synthesis mainly via hLASS1 in imatinib-induced apoptosis in sensitive cells, whereas in resistant cells, alterations of the balance between the levels of ceramide and S1P by overexpression of SK1 result in resistance to imatinib-induced apoptosis.

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disorder (1). There is an elevated but immature white blood cell count in CML. The natural history of CML follows a progression from a chronic phase to an accelerated phase or to a rapidly fatal blast crisis within 3–5 years in patients. Blood cells differentiate normally in the chronic phase but not in the blast phase (2).

CML is usually diagnosed by the presence of an abnormal Philadelphia (Ph) chromosome, which results from a translocation between the long arms of chromosomes 9 and 22. This exchange brings together two genes: the Bcr gene on chromosome 22 and the proto-oncogene Abl on chromosome 9 (3). The resulting hybrid gene, Bcr-Abl, encodes for a fusion protein with tyrosine kinase activity, which mediates signal transduction pathways, leading to uncontrolled growth.

One of the major advancements in the treatment of CML has been the development of imatinib (imatinib mesylate, STI571, Gleevec), which shows striking activity in the chronic and accelerated phases but has less activity in the blast phase of the disease (4). Imatinib directly associates with the ATP-binding site of the Bcr-Abl tyrosine kinase and prevents the kinase activity of the enzyme (5). Inhibition of Bcr-Abl by imatinib...
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RESULTS

Involvement of sphingolipids in the regulation of imatinib-induced cell death and/or resistance in human CML cells has not been examined previously. Therefore, in this study, possible roles of ceramide/S1P pathway in the mechanisms of resistance to imatinib in K562 cells were determined. The data presented here showed that treatment with imatinib results in increased generation of endogenous ceramides, mainly C18-ceramide, in the sensitive parental K562 cell line and not in its resistant subclones in response to imatinib. Additional data with molecular approaches showed that LASS1-dependent generation of C18-ceramide plays important roles in mediating imatinib-induced apoptosis in these cells. Importantly, in resistant cells, although C18-ceramide synthesis was functional, the balance between C18-ceramide/S1P levels by the overexpression of SK1 was altered. Moreover, the data presented here indicated that SK1/S1P play a role in the regulation of Bcr-Abl expression, which is mechanistically involved in the development of resistance to imatinib-mediated apoptosis.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—The Ph chromosome-positive K562 human CML cells were obtained from the German Collection of Microorganisms and Cell Cultures and maintained in RPMI 1640 growth medium containing 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen) at 37 °C in 5% CO2.

Selection of Imatinib-resistant K562 Cells—Cells maintained in liquid cultures were exposed to stepwise increasing concentrations of imatinib, which was kindly provided by Novartis, Switzerland, starting with a concentration of 0.05 μM. Subpopulations of cells that were able to grow in the presence of 0.2 and 1 μM imatinib, were then selected, and referred to as K562/IMA-0.2 and -1 cells, respectively. Then, the inhibitory concentration 50 (IC50) values of imatinib, which inhibited the growth of the cell population by 50%, were determined and compared with control sensitive parental cells as described below.

Measurement of Growth by MTT or Trypan Blue Exclusion Assays—The IC50 values of imatinib that inhibited cell growth by 50% were determined from cell survival plots obtained by MTT or trypan blue exclusion assays (31). In short, cells (2 × 104 cells/well) were plated into 96-well plates containing 100 μl of the growth medium in the absence or presence of increasing concentrations of imatinib at 37 °C in 5% CO2 for 72 h. They were then treated with 5 μl of MTT (5 mg/ml) for 4 h. After lysing the cells in 50 μl of the lysis buffer, the plates were read in a microplate reader (Dynatech) at 570 nm. After that, the IC50 concentrations of the compound were determined from cell survival plots as described (31). Triplicate wells were used for each treatment.

For trypan blue exclusion analysis (31), cells (5 × 104 cells/well) were grown in 6-well plates with 2 ml of media in the absence or presence of increasing concentrations of imatinib for 48 h. Then, cells were counted using a hemocytometer in the presence of trypan blue solution at a 1:1 ratio (v/v) (Sigma), as described by the manufacturer.

Cell Cycle Analysis—Cell cycle profiles were examined by flow cytometry using propidium iodine labeling as described previously (31, 32), and sub-G0/G1 cell populations were considered apoptotic.

Detection of the Loss of Mitochondrial Membrane Potential—The collapse of an electrochemical gradient across the mitochondrial membrane during apoptosis was measured using a JC-1 mitochondrial membrane potential detection kit (Cell Technology) by flow cytometry as described previously (32). This kit uses a unique cationic dye, JC-1, to signal the loss of the mitochondrial membrane potential. In healthy cells, the dye accumulates in the mitochondria as aggregates, which become fluorescent red. In apoptotic cells, the mitochondrial potential collapses, and the JC-1 cannot accumulate within the mito-
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Chondria and remains in the cytoplasm as a green fluorescent monomeric form. These different forms of JC-1 were then detected by flow cytometry as described by the manufacturer.

Measurement of Caspase-3 Activity—Caspase-3 activity was determined using the caspase-3 colorimetric assay (R&D Systems) as described by the manufacturer. Briefly, after lysis, cell extracts were used for caspase-3 activity by the addition of a caspase-3-specific peptide that was conjugated to the fluorescent reporter molecule 7-amino-4-trifluoromethoxy coumarin. In this assay, cleavage of the peptide by caspase-3 releases the fluorochrome that emits fluorescence at 505 nm, after excitation at 400 nm wavelength. The levels of caspase-3 enzymatic activity are directly proportional to the fluorescence signal detected using a fluorescent microplate reader. Caspase-3 activity levels were normalized to total protein amounts for each sample.

Measurement of Ceramide Levels by HPLC/MS—The cellular levels of endogenous ceramides and sphingomyelin were measured using high performance liquid chromatography/mass spectrometry (HPLC/MS) as described previously (32). In short, after cells were collected by centrifugation, lipids were extracted directly from cell pellets, and the levels of sphingolipids and inorganic phosphate levels in the same extracts were measured as described (32).

Analysis of the Endogenous Ceramide Synthase and Sphingosine Kinase Activities by HPLC/MS—Endogenous enzyme activities of (dihydro)ceramide synthase and sphingosine kinase for the generation of ceramide and S1P were measured by HPLC/MS after pulsing the cells with 17C-dihydrosphingosine, which contains 17-carbons, whereas its natural analogue contains 18-carbons, as described previously (33, 34). The in vitro enzyme activity of ceramide synthase was also measured using microsomal preparations of the cells by monitoring the conversion of [3H]dihydrosphingosine into ceramides in the presence of stearoyl- or palmitoyl-CoAs by TLC as described previously (24).

Plasmids and Transfections—The pCMV-exSVneo/LASS1 was kindly provided by Dr. S. M. Jawzwiniski. The human LASS2, LASS5, and LASS6 were cloned from a human cDNA library by PCR and subcloned in pCMV-exSVneo. Transfection of human CML cells was performed using an Effectene transfection kit (Qiagen) as described by the manufacturer. The target siRNA (100 nM for 48 h) using Dharmaconfection reagent as described by the manufacturer. The target siRNA (100 nM for 48 h) using Dharmaconfection kit (Qiagen) as described by the manufacturer.

Results in the Development of Resistance to Apoptosis—To explore the mechanisms involved in the development of resistance to imatinib-induced apoptosis, human K562 CML cells were exposed to stepwise increasing concentrations of the drug (50–1000 nM) for several months, and the sub-clones that expressed resistance were selected. First, the degree of resistance was determined by measuring the IC50 values of imatinib at 72 h using MTT assay. As shown in Fig. 1A, K562 cells that survived upon chronic exposure to 200 or 1000 nM imatinib, respectively (Fig. 1A). Similarly, treatment with 200 and 2000 nM imatinib resulted in 2.3- to 19-fold resistance, as compared with their respective parental CML cells, respectively (Fig. 1A).

Quantitative real-time PCR for the measurement of mRNA levels of human SK1, Ab1, LASS1, LASS2, LASS4, LASS5, LASS6, and rRNA were performed using TaqMan® gene expression kit and gene-specific real-time-PCR TaqMan primer/probe mix (Applied Biosystems) with ABI 7300 Q-PCR system as described by the manufacturer.

Western Blot Analysis—The protein levels of Bcr-Abl, SK1, and β-actin were detected by Western blot analysis (33). In short, total proteins (50 μg/lane) were separated by 5–15% SDS-PAGE (Bio-Rad), blotted onto a nylon membrane, and Bcr-Abl, SK1, and β-actin proteins were detected using 1 μg/ml of mouse monoclonal anti-c-Abl Ab-3 (Calbiochem), rabbit polyclonal anti-SK1 (29), rabbit polyclonal anti-β-actin (Sigma), or mouse monoclonal anti-FLAG (Sigma) antibodies, and peroxidase-conjugated secondary anti-rabbit antibody (1:2500). The proteins were visualized using the ECL protein detection kit (Amersham Biosciences) as described by the manufacturer.

Statistical Analysis—Experiments were performed at least in two independent trials as duplicates or triplicates. Statistical significance was examined using analysis of variance or Student’s t test analysis, and p < 0.05 was considered significant.

RESULTS

Long Term Exposure to Increasing Concentrations of Imatinib Results in the Development of Resistance to Apoptosis—To explore the mechanisms involved in the development of resistance to imatinib-induced apoptosis, human K562 CML cells were exposed to stepwise increasing concentrations of the drug (50–1000 nM) for several months, and the sub-clones that expressed resistance were selected. First, the degree of resistance was determined by measuring the IC50 values of imatinib at 72 h using MTT assay. As shown in Fig. 1A, K562 cells that survived upon chronic exposure to 200 or 1000 nM imatinib, which were referred to as K562/IMA-0.2, and -1, respectively, expressed ~2.3- to 19-fold resistance, as compared with their parental sensitive counterparts. The IC50 values of imatinib were 240, 565, and 4,600 nM for K562, K562/IMA-0.2, and -1 cells, respectively (Fig. 1A). Similarly, treatment with 200 and 2000 nM imatinib resulted in 2.3- to 19-fold resistance, as compared with their respective parental CML cells, respectively (Fig. 1A).
500 nM imatinib for 48 h decreased the viability of K562 cells by 30 and 50%, respectively, whereas there was no significant change in the viability of resistant cells in response to imatinib at these concentrations as measured by trypan blue exclusion assay (Fig. 1B).

In addition, the cell cycle profiles of sensitive and resistant cells were examined by flow cytometry. The data revealed that, although exposure to 500 nM imatinib for 48 h caused apoptosis in ~70% of the population in sensitive K562 cells, there was no detectable apoptosis in resistant K562/IMA-0.2 cells (Fig. 2, A and B, respectively). There were no significant changes in the cell cycle profiles of K562/IMA-0.2 cells as compared with their parental controls (Fig. 2, A and B). These data demonstrate that continuous exposure results in the selection of cells that express resistance to imatinib-induced cell death in human K562 cells.

These results were also confirmed by measuring various parameters of apoptosis such as loss of mitochondrial membrane potential, or the activation of procaspase-3. Treatment with 200–500 nM imatinib for 48 h caused a significant loss of mitochondrial membrane potential, as measured by increased accumulation of cytoplasmic monomeric form of JC-1 (31), in sensitive K562, but not in resistant K562/IMA-0.2 cells (Fig. 2C). In addition, treatment of sensitive K562 cells with 200 or 500 nM imatinib for 48 h resulted in a significant activation of caspase-3 (~10- or 13-fold, respectively), whereas treatment of K562/IMA-0.2 or -1 cells at these concentrations did not have any significant effects on the activation of caspase-3 (Fig. 2D).

Thus, these data confirmed that K562/IMA-0.2 and -1 cells exert significant resistance to imatinib-induced caspase activation and loss of mitochondrial membrane potential.

Ceramide Levels Increase in Imatinib-sensitive, but Not in Resistant Cells, in Response to Imatinib Treatment—To examine whether ceramide synthesis/metabolism is involved in mechanisms of resistance to imatinib, the levels of endogenous ceramide in K562 and K562/IMA-0.2 cells, treated in the absence or presence of 500 nM imatinib (48 h), were measured by HPLC/MS. Interestingly, the data showed that treatment with imatinib resulted in a significant increase in the generation of endogenous ceramides, particularly in C18-ceramide (~30-fold), and to a lesser extent, C14- and C16- ceramides (~2-to 8-fold) in sensitive K562 cells when compared with untreated controls (Fig. 3, A and B, and supplemental Table S1). On the other hand, in resistant K562/IMA-0.2 cells, ceramide generation was unaffected by the addition of imatinib.

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FIGURE 1. Effects of imatinib on the growth of K562, K562/IMA-0.2 and -1 cells in situ. A, the IC_{50} concentration of imatinib was determined by MTT assay for each cell line as described. The MTT assays were performed using triplicate samples in at least two independent experiments. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and p < 0.01 was considered significant. B, the effect of imatinib on cell viability was determined using trypan blue dye exclusion assay. Cells, grown in 6-well plates (5 × 10^4 cells/well), were treated in the absence or presence of imatinib for 48 h. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs.

FIGURE 2. Analysis of cell cycle profiles, mitochondrial membrane potential, and caspase-3 activity in response to imatinib in parental versus resistant K562 cells. The effects of imatinib (500 nM for 48 h) on cell cycle profiles of K562 (A) and K562/IMA-0.2 (B) cells were determined and compared with that of untreated cells using flow cytometry. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. C, mitochondrial membrane potential was measured in K562 and K562/IMA-0.2 cells using the JC-1 kit by flow cytometry. D, caspase-3 activity in K562, K562/IMA-0.2, and -1 cells was measured by fluorometry as described under “Experimental Procedures.” The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs.
other hand, treatment of resistant K562/IMA-0.2 cells with imatinib (500 nM for 48 h) did not cause significant changes in the levels of ceramide, except C24:1-ceramide (Fig. 3, A and B, and supplemental Fig. S1). These data showed also that C18-ceramide in response to imatinib remained at very low levels in resistant cells, whereas its levels were elevated ~30-fold compared with untreated controls in sensitive K562 cells (Fig. 3, A and B, and Table S1), suggesting that it might play a role in imatinib-induced apoptosis. The absolute levels of C18-ceramide were 0.047 and 1.45 in K562 and 0.043 and 0.074 pmol/nmol P1 in K562/IMA-0.2 cells, in the absence or presence of imatinib, respectively (Fig. S1).

Moreover, effects of imatinib on the generation of endogenous ceramide was further investigated by measuring the levels of C18-ceramide after treatment of sensitive K562 cells with 500 nM imatinib for various time points (0, 12, 24, and 48 h). These data showed that C18-ceramide levels increased in a time-dependent manner in response to treatment with imatinib by ~4-, 10-, and 30-fold at 12, 24, and 48 h, respectively, when compared with untreated controls (Fig. 3C). Interestingly, the levels of SM also increased in both sensitive and resistant cells after treatment with imatinib at 500 nM for 48 h (supplemental Fig. S2), suggesting that increased ceramide levels in response to imatinib are likely to be due to de novo synthesis and less likely to hydrolysis of sphingomyelin.

Therefore, these data also suggest that increased ceramide generation and/or accumulation might be involved in mediating imatinib-induced apoptosis and that defects in C18-ceramide generation and/or metabolism might play a role in increased resistance to imatinib-induced apoptosis in these cells.

Role of LASS1, Which Is Specifically Involved in the Generation of C18-ceramide, in Imatinib-induced Cell Death—To test possible roles of endogenous C18- and C16-ceramides, whose levels were altered in the resistant cells as compared with sensitive cells in response to imatinib treatment (Fig. 3), the expression of LASS1, LASS5, and LASS6 were partially inhibited using siRNAs (Fig. 4 A), and their effects on imatinib-induced caspase activation were examined using non-targeting scrambled siRNA treatment as controls (Fig. 4B). The specificity and effectiveness of these siRNAs (100 nM for 48 h) for the inhibition of transcription were confirmed using real-time PCR (Fig. 4A), as described under “Experimental Procedures.” Importantly, inhibition of hLASS1 expression, and not hLASS5 or hLASS6, by siRNA prevented the activation of caspase-3 in response to imatinib (100 nM for 48 h) in K562 cells (Fig. 4B), suggesting a role for LASS1/C18-ceramide in imatinib-induced apoptosis. Moreover, partial inhibition of hLASS2 or hLASS4 transcription did not have any significant effects on imatinib-induced caspase activation (data not shown). Because the

FIGURE 3. Analysis of ceramide levels in parental versus resistant cells. The concentrations of C14-, C18-, C18:1-, and C20-ceramides (A) and C16-, C24-, and C24:1-ceramides (B) in K562 and K562/IMA-0.2 cells in the absence and presence of imatinib were measured by HPLC/MS. C22-ceramide was below the detection limits. The levels of ceramide were normalized to P1 concentrations. C, percent changes of the levels of C14- and C18-ceramide levels were calculated for K562 cells that were exposed to 500 nM imatinib for 12-, 24-, and 48 h. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs.
mRNA levels of hLASS3 were under detectable levels in K562 cells, siRNA against hLASS3 was not used in these studies.

The role of human LASS1/C\textsubscript{18}-ceramide in imatinib-induced apoptosis was further explored. The expression of LASS1 was partially inhibited using siRNA, and its effects on cell viability in response to imatinib (500 nM for 48 h) were examined by trypan blue dye exclusion assay. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and $p < 0.05$ was considered significant.

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FIGURE 4. Examining the role of LASS1 in the regulation of C\textsubscript{18}-ceramide generation and cell viability using siRNA in K562 cells. A, the effects of siRNAs against hLASS1, hLASS5, and hLASS6 on their mRNA levels (left, middle, and right panels, respectively, were examined using real-time PCR, as described under "Experimental Procedures"). B, the effects of siRNAs against hLASS1, hLASS5, and hLASS6 on imatinib-induced caspase-3 activation was measured in K562 cells, as described under "Experimental Procedures." Non-targeting scrambled siRNA (scr) was used as a control. C, levels of C\textsubscript{18}-ceramide in K562 cells transfected with non-targeting (control) and LASS1 siRNA were examined by HPLC/MS. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. D, cell viability of control and LASS1 siRNA transfected K562 cells were determined by trypan blue dye exclusion assay. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and $p < 0.05$ was considered significant.

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FIGURE 5. The role of overexpression of hLASS1 and hLASS6 in imatinib-induced apoptosis in K562/IMA-1 cells. A, overexpression of human LASS1 and LASS6 mRNAs in K562/IMA-1 cells were confirmed using RT-PCR and compared with that of vector-transfected controls. B, fold changes in caspase-3 activity were determined in control, hLASS1-transfected, and hLASS6-transfected K562/IMA-1 cells, exposed to 3μM imatinib for 48h using the caspase-3 colorimetric assay. C, fold changes in endogenous ceramide levels using HPLC/MS (Fig. 6A). D, alterations of the balance between ceramide and S1P via overexpression of SK1—First, to examine whether mechanisms by which K562/IMA-1 cells express resistance to imatinib-induced cell death involve the down-regulation of hLASS1 expression, the mRNA levels of hLASS1 in these cells as compared with their parental sensitive controls were examined by real-time PCR. Unexpectedly, the data in Fig. 6A showed that there was no decrease in the mRNA levels of LASS1 in resistant K562/IMA-1 cells, and in fact, they overexpress hLASS1 2.5-fold compared with their parental cells. These data suggested that there might not be any defect in the generation of C18-ceramide via LASS1 in response to imatinib in resistant cells.

Alteration of the Balance between Ceramide and S1P via Overexpression of SK1—Next, endogenous ceramide synthesis activity in K562 and K562/IMA-1 cells was measured after pulsing the cells with exogenous 17C-dihydrophosphoglycerine, and monitoring the generation of 17C-dihydro-C18- or 17C-dihydro-C16-ceramides (Fig. 6B) in the presence or absence of imatinib. These data showed that there were no alterations in the activity of ceramide synthase for the generation of dh-C18 and dh-C16-ceramides in the absence or presence of imatinib in resistant K562/IMA-1 cells when compared with sensitive K562 cells (Fig. 6B). Thus, these data suggest that alterations in the levels of endogenous ceramide in resistant cells in response to imatinib are not due to decreased levels of hLASS1 mRNA or enzyme activity.

compared with controls. More impressively, inhibition of hLASS1 expression by siRNA almost completely prevented the induction of C18-ceramide generation in response to 500 nm imatinib (48h) in these cells, demonstrating a role of imatinib in the regulation of C18-ceramide generation via LASS1 activity. The levels of C18-ceramide were 0.047, 1.45, and 0.025 and 0.062 pmol/nmol P in cells transfected with control and LASS1 siRNAs in the absence or presence of imatinib, respectively. Importantly, hLASS1 mRNA partially but significantly prevented (~50%) cell death in response to 500 nm imatinib for 48h (Fig. 4D). Specifically, treatment with 500 nm imatinib resulted in ~45% apoptosis in response to control siRNA, whereas imatinib caused ~23% apoptosis in hLASS1 siRNAs transfected cells, respectively (Fig. 4D). These data, therefore, demonstrate that hLASS1 via the generation of C18-ceramide is necessary, but insufficient, in imatinib-induced cell death in K562 cells.

In addition, overexpression of hLASS1, and not hLASS6, in resistant K562/IMA-1 cells further enhanced caspase-3 activation in response to 3μM imatinib (for 48h) by ~2.5-fold, when compared with controls, which were transfected with vector DNA (Fig. 5B). In these experiments, overexpression of LASS1 and LASS6 was confirmed by RT-PCR (Fig. 5A) and by measurement of endogenous ceramide levels using HPLC/MS (Fig. 5C), and the data showed that LASS1 overexpression resulted in ~4-fold increase in steady-state levels of C18-ceramide when compared with vector controls (Fig. 5C). Moreover, treatment of hLASS1-overexpressing cells with imatinib induced the generation of C18-ceramide ~16-fold in resistant K562/IMA-1 cells, whereas its levels in vector-transfected resistant cells were increased ~5-fold in response to the drug (Fig. 5C). On the other hand, overexpression of hLASS6 did not have a significant effect on the levels of C18-ceramide in these cells, although it caused ~8.5- and 4-fold induction in the generation of C16-ceramide, in the absence or presence of imatinib, respectively, as compared with controls (Fig. 5D).

Taken together, these data show that hLASS1, via the generation of C18-ceramide, plays an important role in imatinib-induced apoptosis via the activation of caspase-3/7. Also, these results further suggest that defects in the generation, or accumulation/metabolism of C18-ceramide, might result in increased resistance to imatinib-induced cell death in K562 cells.
but might be due to attenuation of ceramide accumulation and/or metabolism.

Then, after observing an increase in LASS1 expression and activity, but a decrease in ceramide levels in resistant cells, we considered the possibility that sphingoid base substrates would be channeled into the SK1 metabolic pathway. Therefore, we then measured the levels of S1P in these cells by HPLC/MS, and the data showed that S1P levels were significantly higher in resistant K562/IMA-1 cells when compared with sensitive K562 cells (Fig. 6C). These data also showed that, whereas imatinib slightly reduced the levels of S1P in sensitive cells, it further increased the levels of S1P~25% in resistant cells (Fig. 6C). Next, the levels of SK1 expression were analyzed in these cells by Western blotting. Indeed, the data showed that K562/IMA-1 cells overexpress SK1~2.5-fold when compared with controls (Fig. 6D, lanes 2 and 1, respectively). Thus, these data suggested that overexpression of SK1 results in increased generation of S1P, altering the balance between C18-ceramide and S1P, which might be involved in drug resistance.

To demonstrate a possible role for SK1/S1P in the regulation of resistance to apoptosis in response to imatinib, expression of SK1 was partially inhibited ~50% by siRNA in K562/IMA-1 cells (Fig. 7A, lanes 2 and 1), which also caused ~50% reduction in the levels of S1P in the absence or presence of imatinib (Fig. 7B) when compared with controls. Then, its effects on the induction of apoptosis in K562/IMA-1 cells were examined by trypan blue assays. As shown in Fig. 7C, SK1 siRNA increased sensitivity and resulted in a significant induction of apoptosis (~40%) in these resistant cells in response to 1 μM imatinib, suggesting a protective role for SK1 in imatinib-induced apoptosis.

On the other hand, overexpression of SK1, confirmed by RT-PCR (Fig. 8A), in sensitive K562 cells significantly prevented cell death (Fig. 8B), and also blocked the activation of caspase-3 (Fig. 8C) in response to imatinib, when compared with vector-transfected controls. These data were in agreement with the measurement of endogenous C18-ceramide and S1P levels by HPLC/MS, which showed that overexpression of SK1 resulted in a significant increase in S1P levels, raising its levels above C18-ceramide, whereas the levels of C18-ceramide was significantly higher than S1P levels in vector transfected control cells in response to imatinib (Fig. 8D).

Thus, these data reveal a functional role for SK1 in the regulation of resistance to imatinib-induced apoptosis via altering the balance between pro-apoptotic ceramide and anti-apoptotic S1P levels in these cells.

**SK1 Mediates the Up-regulation of Bcr-Abl Expression**—To investigate possible downstream mechanisms involved in imatinib resistance, the DNA sequence of the ATP-binding site of Bcr-Abl in K562 and K562/IMA-1 cells was examined by direct sequencing. There were no mutations detected in Bcr-Abl in these cells (data not shown). Then, possible alterations in the MDR1 gene expression were analyzed using real-time PCR in these cells. There was no up-regulation of MDR1 in K562/IMA-1 compared with K562 cells (data not shown). Then, expression levels of Bcr-Abl were examined using Western blotting in these cells. The data showed that protein levels of Bcr-Abl were increased ~2-fold in the resistant cells compared with sensitive cells (Fig. 9A, lanes 2 and 1, respectively).

Moreover, to determine whether SK1/S1P plays a functional role in the up-regulation of Bcr-Abl, effects of SK1/S1P on the expression of Bcr-Abl in K562 cells were determined. The forced expression of SK1-FLAG (Fig. 9B, lanes 1 and 2), which was detected by Western blotting using anti-FLAG antibody, resulted in ~2-fold increase in the protein (Fig. 9B, lanes 3 and 4), but not in the mRNA (Fig. 9C) levels of Bcr-Abl when com-
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Figure 7. The role of partial inhibition of SK1 expression using siRNA in imatinib-induced apoptosis in K562/IMA-1 cells. A, expression levels of SK1 after exposure to control, scrambled non-targeting siRNA (lanes 3 and 5) or SK1 (lanes 2 and 4) siRNAs in K562/IMA-1 cells were examined by RT-PCR. The mRNA levels of SK1 (lanes 2 and 3) and β-actin (lanes 4 and 5) were measured as described under "Experimental Procedures." Lanes 1 and 6 contain molecular weight markers. B, percent changes in the levels of S1P in K562/IMA-1 cells transfected with control (non-targeting) or SK1 siRNAs in the absence or presence of imatinib (3 μM for 48 h) were measured by HPLC/MS as described. C, apoptotic cell death of control (non-targeting, scrambled) and SK1 siRNA transfected K562/IMA-1 cells in response to 1 μM imatinib for 48 h was determined by trypan blue exclusion assay. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical analysis was done using Student’s t test; p < 0.01 was considered significant.

In this study, roles and mechanisms of action of ceramide metabolism in the regulation of imatinib-induced apoptosis and resistance in human K562 cells were examined. The data presented here provide novel and mechanistic information about the role of the ceramide/S1P pathway in the regulation of imatinib-mediated apoptosis, which involves the loss of mitochondrial membrane potential, and the activation of caspase-3 in K562 cells. The results showed that, although treatment with imatinib mediates the generation of C18-ceramide via LASS1, which appears to play an important role in imatinib-induced apoptosis in sensitive K562 cells, overexpression of SK1 in resistant cells prevents cell death in response to imatinib by altering the balance between C18-ceramide and S1P. Importantly, this study revealed that SK1/S1P plays a role in the regulation of Bcr-Abl expression at the post-transcriptional level, implicating that SK1/S1P-induced resistance might mechanistically be linked to the up-regulation of Bcr-Abl in these cells.

Bioactive sphingolipid ceramide involves in mediating anti-proliferative responses via various distinct mechanisms in human cancer cells (11). It has been well documented that treatment with some chemotherapeutic agents results in increased generation and/or accumulation of endogenous ceramide either via the activation of the de novo pathway, or by increased activity of sphingomyelinases (11). However, any role for imatinib in inducing the generation of ceramide in human CML cells has not been examined previously. In this report, the data showed that treatment with imatinib significantly increased the generation of ceramide, particularly C18-ceramide, in a time-dependent manner, via the action of LASS1 in K562 cells. Importantly, further evaluation of possible roles for LASS1-generated C18-ceramide by molecular approaches suggested its involvement in imatinib-induced cell death in K562 cells. However, although hLASS1 siRNA completely prevented the generation of C18-ceramide in response to imatinib in K562-sensitive cells, it only partially (~50%) prevented imatinib-induced apoptosis (see Fig. 4), indicating that there are other mechanisms and downstream targets that are involved in this process. Nevertheless, overexpression of LASS1 in resistant K562/IMA-0.2 or -1 cells, which induced the generation of C18-ceramide, increased the sensitivity to imatinib, suggesting that up-regulation of ceramide generation might help improve response to imatinib.

Our previous data revealed an important role for C18-ceramide via LASS1 activity in enhancing chemotherapy-induced cell death by a mechanism involving the activation of mitochondrial apoptotic cascade, including the activation of caspase-9/3 in squamous cell carcinomas of the head and neck both in vitro (32) and in vivo (35). These data are also in agreement with the present study showing the role of C18-ceramide in the regulation of caspase-3 activation and loss of mitochondrial membrane potential in K562 cells. However, whether imatinib treatment specifically induces the generation of C18-ceramide via LASS1 in various other CML cell lines is not known.
and needs to be determined. This may be important to examine because in a previous study it was reported that, although defects in hLASS1/C18-ceramide pathway play a role in the pathogenesis and/or progression of squamous cell carcinomas of the head and neck, the reduced levels of C16-, C18-, and C24-ceramides, products of various LASS proteins, appeared to play important roles in the pathogenesis of non-squamous head and neck cancers (32). This suggests that LASS-dependent generation of ceramide synthesis might be regulated by distinct pathways in a cell line or cell type-dependent manner. Interestingly, although absolute levels of C16- and C24-ceramides reached higher amounts than C18-ceramide in response to imatinib, LASS1-mediated C18-ceramide, and not LASS6-mediated C16-ceramide, appeared to play a role in imatinib-induced apoptosis in K562 cells. However, roles of endogenous C16- or C24-ceramides in chemotherapy-mediated apoptosis have been shown in various models previously (reviewed in Ref. 11), suggesting cell-line/type-dependent functions of these ceramides in apoptosis. These data also suggest that sub-cellular localization, membrane context, or membrane microenvironment, and/or specific downstream targets of these ceramides may determine their distinct biological functions in various cell types. Indeed, recent data demonstrated a role for LASS5-generated C16-ceramide in PMA-induced regulation of p38 phosphorylation (36). Mechanisms of distinct biological roles of endogenous ceramides with different fatty acid chain lengths generated by LASS proteins (37), are still unknown, and need to be determined.

Importantly, results presented here demonstrated that continuous exposure of K562 cells to stepwise increasing concentrations of imatinib results in the selection of sub-clones expressing resistance to imatinib-induced apoptosis. A similar approach has been used in various studies to derive imatinib resistance starting with Ph chromosome-positive cell lines previously, including AR230, LAMA84, and K562 (38). The data presented here showed that the perturbations of the balance between ceramide and S1P, with opposing functions as pro-apoptotic and anti-apoptotic sphingolipids, respectively, by overexpression of SK1, and not by altered levels of ceramide synthesis, play an important role in the regulation of resistance to imatinib. These results are consistent with previous data, which showed a role for SK1 via alteration of the ceramide/S1P balance in the regulation of drug-induced apoptosis, and chemotherapy sensor both in culture and in animal models of prostate adenocarcinoma (39). In parallel with these data, increas-

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FIGURE 8. The role of overexpression of SK1 in the inhibition of apoptosis in K562 cells. A, overexpression of SK1 in K562 cells after transfections with pcDNA3 (vector) or pcDNA3/SK1 plasmids were examined by RT-PCR. B, fold changes of cell death in SK1-transfected K562 cells, exposed to 1000 nM imatinib for 48 h, were determined by trypan blue dye exclusion assay. C, caspase-3 activity was determined in control and SK1-transfected K562 cells, exposed to 1000 nM imatinib for 48 h, using the caspase-3 colorimetric assay. D, the levels of S1P and C18-ceramide in control and SK1-transfected K562 cells, in the presence (1000 nM for 48 h) or absence of imatinib, were examined by HPLC/MS. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical analysis was done using two-way analysis of variance, and p < 0.05 was considered significant.
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**A**

Bcr-Abl

K562 IM1

**B**

SK1-FLAG

**C**

Normalized mRNA Levels (Fold Change)

K562 Vector

K562-SK1

**D**

Actin

**FIGURE 9. Role of SK1 in the regulation of Bcr-Abl expression.** A, protein levels of Bcr-Abl in K562 and K562/IMA1 cells were measured by Western blotting (lanes 1 and 2), in which β-actin levels were used as controls (lanes 3 and 4), respectively. B, protein levels of Bcr-Abl in SK1-transfected cells compared with vector-transfected controls were examined by Western blotting (lanes 4 and 3, respectively). β-Actin levels were used as controls (lanes 2 and 1, respectively). C, expression levels of Bcr-Abl in SK1-FLAG and vector-transfected cells were examined by real-time PCR. β-Actin levels were used as controls. The error bars represent the standard deviations. D, effects of partial inhibition of SK1 using siRNA on SK1 (lanes 2 and 1) and Bcr-Abl (lanes 4 and 3) compared with scrambled siRNA transfected controls, respectively, were examined by Western blotting. β-Actin levels were used for loading controls (lanes 5 and 6) in these samples.

The expression of SK1 reduced the sensitivity of A-375 melanoma cells to Fas- and ceramide-mediated apoptosis that could be reversed by inhibition of SK1 expression (28). Other previous studies have shown that SK1 expression is up-regulated in colon carcinogenesis (40) and in the tumors of the patients with lung cancer (41). More importantly, it has been demonstrated previously that modulation of S1P levels increases apoptosis, or abolishes starvation-induced autophagy, which leads to increased cell death (42). Thus, perturbations in the metabolism of ceramide via overexpression of SK1 may play important roles in the development of resistance to imatinib.

It should also be noted that increased activity/expression of one or more ceramidases, required for the hydrolysis of ceramide to generate sphingosine (43–47), which is then converted to S1P by SK1, might also be involved in the development of resistance to imatinib. However, there were no significant differences in the activity of acid, neutral, and alkaline ceramidases (43–47) between K562 and K562-IMA-1 cells, as measured in vitro using total cell lysates (data not shown), as described previously (43–47). Nevertheless, although these data may suggest that alterations of ceramidase (CDase) enzyme activity might not be involved in the development of resistance to imatinib, inhibition of a specific CDase, which might preferentially utilize LASS1-generated C18-ceramide as a substrate, can still provide a novel tool to improve the efficacy of imatinib and reverse resistance to this drug. These possibilities, however, need to be further explored.

Various diverse mechanisms have been reported for their involvement in the resistance to imatinib. The most common mechanism was the overexpression/amplification of Bcr-Abl, and it has been well documented that the degree of Bcr-Abl expression appears to be directly proportional to the levels of imatinib resistance (37, 48). Another mechanism for the development of resistance was due to selection of cells with mutated Bcr-Abl in the imatinib-binding domain in various CML cell lines (49). In addition, there have been Bcr-Abl-independent mechanisms reported for driving resistance against imatinib in various CML cells. For example, overexpression of MDR1/P-gp, a classic drug efflux protein (50), or up-regulation of anti-apoptotic Bcl-2 in Lyn-kinase-dependent mechanism (51) have been shown to be involved in resistance to imatinib.

Mechanistically, our data showed that Bcr-Abl is up-regulated at the protein level in imatinib-resistant K562/IMA-1 cells, when compared with parental-sensitive cells, and that overexpression of SK1/S1P might play an important role in the up-regulation of Bcr-Abl protein in K562 cells. However, although Bcr-Abl expression is increased ~8-fold in the resistant K562/IMA-1 cells compared with sensitive cells, overexpression of SK1 resulted in the up-regulation of Bcr-Abl to ~2-fold in sensitive cells, suggesting that SK1/S1P is necessary, but insufficient for the overexpression of Bcr-Abl. This was also consistent in reciprocal experiments, in which partial inhibition of SK1 expression resulted in a slight decrease in Bcr-Abl protein levels in the resistant K562/IMA-1 cells that resulted in increased apoptosis in response to imatinib. Nevertheless, these data, then, suggest that SK1/S1P is involved in the regulation of Bcr-Abl expression mainly at the post-transcriptional level in these resistant cells. However, there are clearly other mechanisms for its up-regulation. Mechanisms by which SK1-generated S1P, endogenously or exogenously via S1PR paracrine, regulates Bcr-Abl expression are still unknown and need to be determined.

To examine the potential role of S1P/S1PR signaling in resistance to imatinib, first, the mRNA levels of S1PRs (S1P1, S1P2, S1P3, S1P4, and S1P5) were measured in the resistant compared with sensitive K562 cells using conventional RT-PCR. There was a slight increase in the S1P2 and S1P3 mRNAs in the resistant cells, whereas there was no difference in the levels of S1P1 mRNA between sensitive and resistant cells (supplemental Fig. S3A). The levels of S1P5 mRNA were below detectable levels in these cells (data not shown). In addition, the data showed that mRNA levels of S1P4 were markedly elevated (~4-fold) in the resistant K562/IMA-1 cells when compared with sensitive K562 cells (supplemental Fig. S3A). Then, to determine possible roles of S1P4 in the development of resistance to imatinib-induced apoptosis in K562/IMA-1 cells,
effects of siRNA against S1P4 on cell viability in response to increasing concentrations of imatinib were examined using MTT assays. Interestingly, the data showed that, although siRNA treatment resulted in a significant reduction in the expression of S1P4 (supplemental Fig. S3B, right panel), it did not have any significant effect on the IC_{50} value of the drug when compared with controls, which were treated without non-targeting scrambled siRNA (Fig. S3B, middle panel). Thus, these data suggest that S1P4 might not play a major role in the development of resistance to imatinib-induced apoptosis in these cells. However, pre-treatment of cells with pertussis toxin, which is a known inhibitor of G-protein-coupled receptors, to S1P4, which couple to G_{i} G_{q} and/or G_{12/13} subfamilies of G-proteins (52–54), resulted in increased sensitivity (~2-fold) to imatinib in K562/IMA-1 cells (Fig. S3B, left panel). This reduced the IC_{50} value of the drug to ~2 μM (from ~4.2 μM), suggesting that other S1PRs may play a role in this process. However, because pertussis toxin is not a specific S1PR inhibitor, possible roles of S1PRs, if any, in the development of resistance to imatinib remain unknown and need to be determined in more comprehensive studies.

At any rate, the data presented here have important implications for designing novel therapies for the treatment of CML. For example, in addition to the inhibition of Bcr-Abl, targeting ceramide metabolism by increasing its synthesis, and/or modulating its metabolism to S1P might provide improved strategies for the treatment of CML. In addition, specific antagonists for S1PRs (55, 56), might also improve the efficacy of imatinib, and help prevent resistance, after establishment of their roles, if any, in SK1/S1P-mediated resistance to this drug.

In conclusion, these results show that LASS1-dependent generation of endogenous C_{18}-ceramide plays important roles in imatinib-induced apoptosis in sensitive K562 cells. More importantly, the data presented here also imply that alterations of the ceramide/S1P balance by overexpression of SK1 is involved in the regulation of imatinib resistance in K562 cells.

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Alterations of Ceramide/Sphingosine 1-Phosphate Rheostat Involved in the Regulation of Resistance to Imatinib-induced Apoptosis in K562 Human Chronic Myeloid Leukemia Cells

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