A novel mechanism for imatinib mesylate–induced cell death of BCR-ABL–positive human leukemic cells: caspase-independent, necrosis-like programmed cell death mediated by serine protease activity


Caspase-independent programmed cell death can exhibit either an apoptosis-like or a necrosis-like morphology. The ABL kinase inhibitor, imatinib mesylate, has been reported to induce apoptosis of BCR-ABL–positive cells in a caspase-dependent fashion. We investigated whether caspases alone were the mediators of imatinib mesylate–induced cell death. In contrast to previous reports, we found that a broad caspase inhibitor, zVAD-fmk, failed to prevent the death of imatinib mesylate–treated BCR-ABL–positive human leukemic cells. Moreover, zVAD-fmk–preincubated, imatinib mesylate–treated cells exhibited a necrosis-like morphology characterized by cellular pyknosis, cytoplasmic vacuolization, and the absence of nuclear signs of apoptosis. These cells manifested a loss of the mitochondrial transmembrane potential, indicating the mitochondrial involvement in this caspase-independent necrosis. We excluded the participation of several mitochondrial factors possibly involved in caspase-independent cell death such as apoptosis-inducing factor, endonuclease G, and reactive oxygen species. However, we observed the mitochondrial release of the serine protease Omi/HtrA2 into the cytosol of the cells treated with imatinib mesylate or zVAD-fmk plus imatinib mesylate. Furthermore, serine protease inhibitors prevented the caspase-independent necrosis. Taken together, our results suggest that imatinib mesylate induces a caspase-independent, necrosis-like programmed cell death mediated by the serine protease activity of Omi/HtrA2.

Introduction

Imatinib mesylate (imatinib, Gleevec) was developed as a potent and specific inhibitor of ABL tyrosine kinase.1 Preclinical studies and clinical trials showed that imatinib exhibited a remarkable single-agent activity against BCR-ABL–expressing cells with acceptable toxicity in vitro and in vivo.1,2 BCR-ABL tyrosine kinase activates several signaling pathways such as the Ras/mitogen-activated protein kinase,4 signal transducer and activator of transcription 5,5,6 and phosphatidylinositol 3 kinase/Akt pathways;4,5 enhances nuclear factor κB (NF-κB) activity5; up-regulates the level of Bcl-XL;5,6 and suppresses the mitochondrial apoptotic pathway.5,7 Imatinib counteracts BCR-ABL tyrosine kinase and induces apoptosis in BCR-ABL–positive cells6-10 in a caspase-dependent fashion.11,12 Recently, however, it has been revealed that responses to imatinib are not necessarily necessary or durable in some patients with BCR-ABL–positive leukemia.13,22 and thus an increasing number of studies have searched for a novel therapy targeting the BCR-ABL–induced signaling pathways.5

Cell death is generally classified into 2 categories, apoptosis,23,24 and necrosis. Apoptosis is a well-documented active programmed cell death (PCD) in which the activation of caspases plays a central role.25 In contrast, necrosis has been conceived as a passive cell death without established regulatory mechanisms. However, it has recently been reported that necrosis-like cell death may be regulated by cellular intrinsic death programs.26-28 This active necrosis-like PCD is observed in various paradigms of cell death in conditions in which caspases are inhibited.26,27 Indeed, there is increasing evidence for caspase-independent cell death,29 and caspase inhibition occasionally turns the morphology of PCD from apoptosis into necrosis without inhibiting death itself.26,30,31 Although these models of caspase-independent necrosis might provide potential targets for a novel cancer therapy, little or no information is available on their signaling pathways.

There are 2 well-established pathways that lead to cell death, the death-receptor pathway and the mitochondrial pathway.25 In particular, the mitochondrial pathway is used extensively in response to various extracellular and intracellular insults. Mitochondria play a pivotal role in the induction of cell death by releasing several proteins localized in the intermembrane space.32 Among these proteins, cytochrome c and Smac/DIABLO function as caspase activators, while apoptosis-inducing factor (AIF)33 and endonuclease G (Endo G) can mediate caspase-independent cell death.29 Omi/HtrA2, another intermembrane protein, plays a dual role in cell death. These proteins are known to have caspase-independent activity, and this property is thought to be mediated by mitochondrial proteins contribute to cell death.


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role as a caspase activator (acting as an inhibitor of the inhibitor of apoptosis [IAP] proteins) and as a caspase-independent death effector (acting by virtue of its serine protease activity). Overproduction of reactive oxygen species (ROS) resulting from a dysfunction in the mitochondrial respiratory chain may also lead to caspase-independent cell death.\textsuperscript{34}

In the present study, we show that a broad caspase inhibitor, zVAD-fmk (zVAD), fails to prevent the imatinib-induced cell death in BCR-ABL-positive human leukemic cells, that this zVAD + imatinib-induced cell death exhibits a necrosis-like morphology accompanied by the cytosolic release of Omi/HtrA2, and that this caspase-independent necrosis is prevented by serine protease inhibitors. Our results suggest that imatinib induces a caspase-independent, necrosis-like PCD in BCR-ABL-positive human leukemic cells, which is mediated by the serine protease activity of Omi/HtrA2.

**Materials and methods**

**Cells**

BV173 was kindly provided by Dr Matsuo (Hiroshima University, Japan) and K562 was purchased from RIKEN Gene Bank (Tsukuba, Japan). BV173 is an established human leukemic cell line derived from a patient with chronic myelogenous leukemia (CML) lymphoid blastic crisis, and K562 is derived from a patient with CML erythroid blastic crisis. Cells were with chronic myelogenous leukemia (CML) lymphoid blastic crisis, and BV173 is an established human leukemic cell line derived from a patient

**Reagents**

Imatinib was kindly provided by Novartis Pharma (Basel, Switzerland). zVAD was from Peptide Institute (Osaka, Japan). Serine protease inhibitors, N-acetyl-L-lysine-chloromethyl ketone (TLCK) and L-L-p-tosylamino-2-phenylethylchloromethane ketone (TPCK), were from Research Organics (Cleveland, OH). All these reagents were dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan) and stored at

**Cell death assessment and determination of mitochondrial transmembrane potential (ΔΨm)**

Cell death was defined by PI staining, and mitochondrial transmembrane potential (ΔΨm) was determined by DiOC6(3). For each condition, 0.5 to 1.0 × 10\(^6\) cells/mL were incubated with 40 nM DiOC6(3) and 5 μg/mL PI at 37°C for 15 minutes, and subsequently their fluorescence was measured using FL1 and FL3 channels of FACSCalibur (Beckton Dickinson, Franklin Lakes, NJ).

**Morphologic evaluation by light and electron microscopy**

After treatment, 2 to 3 × 10\(^4\) cells were spun down onto slides with cytospin, stained with Diff-Quick (International Reagents, Kobe, Japan), and subsequently examined by light microscopy for morphologic evaluation. A minimum of 400 cells was scored for each sample, and the percentage of each morphologic type was determined.

For the observation by transmission electron microscopy, 5 × 10\(^7\) treated cells were spun down into pellet, fixed in 2% glutaraldehyde in 0.1 M phosphate buffer at 4°C, washed in isotonic phosphate-buffered sucrose solution, refixed in phosphate-buffered 1% osmium tetroxide solution, dehydrated in a graded series of ethanol, and embedded in Luveak 812 (Nacalai). Sections were cut 70- to 90-nm thick with a diamond knife on a Sorvall MT-5000 ultramicrotome (Kendro, Newtown, CT), stained with uranyl acetate and lead citrate, and observed with a Hitachi H-7000 electron microscope (Hitachi, Tokyo, Japan).

**Assessment of internucleosomal DNA fragmentation and DNA content**

For internucleosomal DNA fragmentation assay, DNA was extracted using ApopLadder Ex (Takara, Shiga, Japan) according to the manufacturer's instructions. In brief, DNA extract from 3 × 10\(^7\) treated under each condition was dissolved with 50 μL TE buffer (pH 8.0; Nacalai) and electrophoretically resolved in a 2% agarose gel. After incubation of the gel in distilled water containing 0.5 μg/mL ethidium bromide for 30 minutes, fragmented DNA was visualized under ultraviolet light.

DNA content was assessed by flow cytometry. Cell permeabilization and staining with PI have been described elsewhere.\textsuperscript{35} Briefly, after treatment, 0.5 to 1.0 × 10\(^7\) cells were washed, fixed with 4 mL ice-cold 95% ethanol added dropwise during continuous vortexing, and incubated on ice overnight. Cells were resuspended in 1 mL 1% bovine serum albumin (BSA; Sigma)-phosphate-buffered saline (PBS) and washed and treated with 0.25% Triton X-100 (Nacalai) for 5 minutes. Cells were then washed 3 times with 1% BSA-PBS. DNA was stained with 500 μL 1% BSA-PBS, 100 μg/mL RNase A (Sigma), and 20 μg/mL PI. Nuclear emitted fluorescence was measured by FACSCalibur, and the percentage of the sub-G1 fraction was determined using ModFit LT 2.0. (Verity Software, Topsham, ME).

**Caspase activity assay**

For caspase activity assay, the procedure was performed using the Caspase Fluorometric Protease Assay Kit (MBL, Nagoya, Japan) according to the manufacturer’s instructions. Briefly, cell lysate from 1.0 × 10\(^6\) cells was incubated at 37°C for 1 hour with 50 μM DEVD-AFC, IETD-AFC, and LEHD-AFC substrate to measure caspase-3-, -8, and -9 activity, respectively. AFC fluorescence was determined (excitation, 390 nm; emission, 510 nm) with the Wallace ARVO XLS 1420 Multilabel Counter (PerkinElmer Life Sciences, Boston, MA) and expressed as fold increase on the basal level (DMSO-treated cells).

**Measurement of ROS production**

Intracellular ROS production was assessed using DCFH-DA. DCFH-DA is a peroxide-sensitive fluorescent probe that is nonpolar and diffuses into the cell. Intracellular esterases cleave the diacetate ester group and entrap the fluorescent DCF. After the indicated treatments, 5 × 10\(^4\) cells were incubated in RPMI1640 containing 50 μM DEVD-AFC, IETD-AFC, and LEHD-AFC substrate to measure caspase-3, -8, and -9 activity, respectively. AFC fluorescence was determined (excitation, 390 nm; emission, 510 nm) with the Wallace ARVO XLS 1420 Multilabel Counter (PerkinElmer Life Sciences, Boston, MA) and expressed as fold increase on the basal level (DMSO-treated cells).

**Immunofluorescent staining of apoptosis-inducing factor (AIF)**

After treatment, 2 to 4 × 10\(^6\) cells were spun down onto slides, fixed with 4% paraformaldehyde in PBS for 60 minutes, permeabilized with 0.1% sodium dodecyl sulfate (SDS; Nacalai) in PBS for 10 minutes, and blocked with 10% FBS in PBS for 20 minutes. For the simultaneous staining of AIF and AIF protein, the cells were labeled with monoclonal mouse anti-poly(adenosine diphosphate–ribose) polymerase (PARP) antibody (1:100 dilution, clone 7D3-6; Pharmingen, San Diego, CA) and polyclonal anti-AIF (Becton Dickinson, Franklin Lakes, NJ) antibodies.
rabbit anti-AIF antibody (1:250 dilution, clone H-300; Santa Cruz Biotechnology, Santa Cruz, CA), followed by secondary staining with cyanin 3 (Cy3)–conjugated goat anti–mouse immunoglobulin G (IgG, 1:100 dilution; Jackson ImmunoResearch, West Grove, PA) and AlexaFluor 488–conjugated goat anti–rabbit IgG antibody (Santa Cruz) for one hour. Blocking and incubations with primary and secondary antibodies were all performed at room temperature. The membrane was washed and bound antibodies were visualized with an enhanced chemiluminescence detection system as specified by the manufacturer (Amersham, Arlington Heights, IL). After stripping the membrane in stripping buffer (62.5 mM Tris-HCl [pH 6.7], 2% SDS, 100 mM 2-mercaptoethanol) for 30 minutes at 56°C, it was reprobed with anti–β-actin antibody (Sigma) to assess the comparability of the protein loading. Intensity of Omi/HtrA2 bands was evaluated using ATTO Lane & Spot Analyzer version 6.0 software (Atto Bioscience, Rockville, MD) and compared with the intensity of the corresponding β-actin bands on the same membrane.

Whole cell lysate was analyzed by the same Western blotting procedure using anti-ABL antibody (Pharmingen) or antiphosphotyrosine antibody (Santa Cruz).

**Statistical analysis**

Values are expressed as means ± SEM. To evaluate the difference in means between 2 groups, the Mann-Whitney U test was used. StatView-J version 4.5 software (SAS Institute, Cary, NC) was used for all statistical analyses and significance was defined as a P value less than .05.

**Results**

**Inhibition of caspases fails to prevent the imatinib-induced cell death**

To confirm caspase dependency in the imatinib-induced cell death of BCR-ABL–positive human leukemic cells, BV173 cells and K562 cells were preincubated with DMSO or zVAD and subsequently treated with or without imatinib. After treatment with imatinib alone, the percentage of PI-positive cells (dead cells)
increased in a time-dependent fashion both in BV173 cells (Figure 1A; 70.5 ± 3.3% after 72 hours) and in K562 cells (Figure 1B; 44.7 ± 1.8% after 72 hours). Unexpectedly, however, the dead cells also increased after treatment with zVAD + imatinib both in BV173 cells (Figure 1A; 51.3 ± 2.7% after 72 hours) and in K562 cells (Figure 1B; 24.2 ± 0.7% after 72 hours), although the appearance of dead cells was slightly delayed compared with that in imatinib-treated cells. Importantly, neither DMSO nor zVAD was cytotoxic and, as shown later, the concentration of zVAD was sufficiently high to inhibit the caspase activities throughout these experiments.

**Imatinib induces classical apoptosis, while zVAD + imatinib induces atypical cell death**

The results shown in Figure 1 suggested the existence of caspase-independent cell death in zVAD + imatinib–treated cells. To determine whether this mode of cell death is apoptosis or not, we performed a morphologic evaluation by light microscopy in the early phase of cell death. In imatinib-treated BV173 cells, apoptotic cells characterized by cell shrinkage, nuclear condensation, and nuclear fragmentation (Figure 2A) began to appear after 3 hours and increased up to 8.2 ± 0.5% after 12 hours (Table 1). In zVAD + imatinib–treated BV173 cells, such apoptotic cells were barely detectable. Instead, dying cells exhibited an atypical morphology with cellular pyknosis and a lack of nuclear fragmentation. As expected, imatinib-treated cells exhibited internucleosomal DNA fragmentation (Figure 2B) and DNA content (Figure 2C), consistent with their apoptotic morphology (4.8%, 5.3%, and 8.2% at 3, 6, and 12 hours, respectively; Table 1). In contrast, zVAD + imatinib–treated cells exhibited neither internucleosomal DNA fragmentation nor any decrease in DNA content even after 12 hours (Figure 2D), when the percentage of atypical cells reached 6.6% (Table 1). These signs of atypical cell death induced by zVAD + imatinib treatment were consistent with the necrotic mode of cell death.
The activation of caspase-9/caspase-3 is involved in imatinib-induced apoptosis, while caspase activities are completely inhibited in zVAD + imatinib–induced necrosis

As previously described,⁶⁻¹² imatinib induces the mitochondrial apoptotic pathway, which causes the release of cytochrome c into the cytosol, followed by the activation of caspase-9 and caspase-3. To examine whether this is also the case in our system and to confirm that caspases are completely inhibited after zVAD preincubation, we measured the caspase activities using fluorogenic substrates. As expected, in imatinib-treated cells, caspase-3 and caspase-9 were significantly activated after treatment for 3 hours (Figure 5), confirming the activation of the mitochondrial apoptotic pathway by imatinib. Importantly, in zVAD + imatinib–treated cells, the activities of caspase-3, -8, and -9 were all constantly maintained at or below the control level throughout the experiments (Figure 5).

Loss of mitochondrial transmembrane potential (ΔΨm) is observed in the early phase of necrosis

Because the loss of ΔΨm represents mitochondrial dysfunction and involvement in cell death,⁷⁻⁴¹ we examined the change in ΔΨm during necrosis by means of the ΔΨm-sensitive fluorochrome DiOC₆(3). After treatment with imatinib or zVAD + imatinib, cells were incubated with DiOC₆(3) and PI, followed by cytofluorometric analysis. Viable cells exhibited a DiOC₆(3)high and PI⁻ phenotype, dying cells with a loss of ΔΨm were found in DiOC₆(3)low and PI⁻ fraction, and dead cells were DiOC₆(3)low and PI⁺. As shown in Figure 6, imatinib-treated or zVAD + imatinib–treated BV173 cells (A) and K562 cells (B) exhibited an increase of the DiOC₆(3)low and PI⁻ fraction before the increase of DiOC₆(3)low and PI⁺ population. Thus, during zVAD + imatinib–triggered necrosis as well as imatinib-triggered apoptosis, the ΔΨm dissipation preceded the disruption of plasma membrane integrity.

Neither the overproduction of ROS nor the nuclear translocation of AIF is required for the execution of necrosis, while Omi/HtrA2 is released into the cytosol during necrosis

The results shown in Figure 6 suggest the involvement of mitochondria in necrosis. There are some mediators of caspase-independent cell death in association with mitochondrial dysfunction: overproduction of ROS, nuclear translocation of AIF or Endo G, and release of serine protease Omi/HtrA2 into the cytosol. Because Endo G causes internucleosomal DNA fragmentation,⁴² we ruled out the possibility that Endo G was the mediator in our system. Thus we examined the involvement of ROS, AIF, and Omi/HtrA2 in caspase-independent cell death in parallel with that in apoptosis. First, we measured intracellular ROS production after treatment with imatinib or zVAD + imatinib for 3, 6, and 12 hours. Intracellular ROS production did not change after 3 and 6 hours and slightly decreased after 12 hours compared with that in control...
imatinib–treated cells was demonstrated only in the cytoplasm (or mitochondria), not in the nuclei, after 12 hours, while staurosporine–treated cells (as a positive control) showed the nuclear AIF (Figure 7B), as has already been described.44,45 Also, AIF was not localized to the fragmented nuclei of apoptotic cells. Moreover, subcellular fractionation followed by Western blotting showed that the cytosolic release of AIF was absent both in imatinib–treated cells and in zVAD + imatinib–treated cells (Figure 7C). Finally, we examined the release of Omi/HtrA2 into the cytosol. We found that Omi/HtrA2 was released into the cytosol of imatinib–treated apoptotic cells after 3 hours and zVAD + imatinib–treated necrotic cells after 12 hours (Figure 7C). Mitochondrial contamination was ruled out by the absence of cytochrome oxidase in the cytosol. Using densitometry and correction against β-actin expression, the amount of cytosolic Omi/HtrA2 in imatinib– or zVAD + imatinib–treated cells after 12 hours was found to be increased 2-fold (imatinib) or 1.7-fold (zVAD + imatinib) compared with that in control cells (Figure 7C). Quantitative analysis indicated that the amount of cytosolic Omi/HtrA2 was concordant with the percentage of dying cells in morphologic evaluation (Table 1). In addition, we showed that the direct kinase inhibitory activity of imatinib is equivalent in cells treated with imatinib alone or with imatinib plus additional agents by direct measurement of BCR-ABL phosphotyrosine content and ruled out the possibility that the additional agents directly altered imatinib cellular uptake or activity (Figure 7D).

Trypsinlike serine protease is required for the execution of necrosis

Others have already reported that extramitochondrially overexpressed mature HtrA2 induces caspase-independent cell death and that this death-inducing activity is dependent on the trypsinlike serine protease activity of HtrA2.46 To further elucidate the role of serine protease in the caspase-independent necrosis, we used 2 serine protease inhibitors, TLCK and TPCK. TLCK is a trypsinlike serine protease inhibitor, while TPCK is a chymotrypsin-like serine protease inhibitor. These agents prevented the zVAD + imatinib–induced necrosis in a dose-dependent fashion (data not shown), with maximal prevention at the indicated concentration of TLCK or TPCK (Figure 8A-B). The cytoprotective effect of TLCK and TPCK was evident both in the early phase (Figure 8A) and in the late phase (Figure 8B-C) of cell death. After treatment for 12 hours, none of the TLCK (or TPCK) + zVAD + imatinib–treated cells exhibited necrotic morphology (Figure 8A). A significant difference in the percentage of dead cells between zVAD + imatinib–treated and TLCK (or TPCK) + zVAD + imatinib–treated cells was seen after treatment for 48 (not shown) and 72 hours, and the preventive effect was greater using TLCK than TPCK both in BV173 cells and in K562 cells (Figure 8B). In addition, these serine protease inhibitors also prevented the loss of cytosolic Omi/HtrA2, indicating that these 2 pathways are independent of each other.

Discussion

This is the first report showing that imatinib induces caspase-independent necrosis-like cell death in BCR-ABL–positive cells. It has
been reported that imatinib induces caspase-dependent apoptosis in BCR-ABL–positive cells, but the present data demonstrate that a broad caspase inhibitor, zVAD, failed to prevent the imatinib-induced cell death and that dying and dead cells exhibited necrotic morphology. Previous reports that showed that zVAD prevented imatinib-induced cell death did not extensively analyze the mode or the time course of cell death. The quantification of cell death often relies on the assessment of nuclear fragmentation, internucleosomal DNA fragmentation, and phosphatidylserine exposure on the plasma membrane. This methodology might favor overestimating necrosis or caspase-independent cell death for 2 different reasons. First, necrotic cells do not exhibit nuclear or DNA fragmentation and do not necessarily turn Annexin V–positive in the early phase of cell death. Second, inhibition of caspases by zVAD often delays the manifestation of cell death. In the absence of thorough morphologic evaluation by light or electron microscopy early necrotic cells may appear “normal.” Second, inhibition of caspases by zVAD often delays the manifestation of cell death. In the absence of kinetic follow-up studies, this creates the impression that zVAD “inhibits” cell death (when, in reality, it delays cell death). Our data are consistent with a number of reports suggesting that zVAD fails to prevent cell death or even increases the sensitivity of cells to necrosis. In Jurkat cells, zVAD fails to prevent cell death induced by Bax or by oligomerization of Fas–associated death domain. In human neutrophils and L929 cells, zVAD fails to prevent tumor necrosis factor α–induced apoptosis and increases necrosis. In mouse thymocytes, zVAD prevents apoptosis induced by the mitochondrial permeability transition inducers protoporphyrin IX or the carbonyl cyanide m-chlorophenylhydrazone, while thymocytes themselves undergo necrosis. In these reports, necrosis was confirmed by electron microscopic analysis, and the following features were observed: cytoplasmic vacuolation, distension of the mitochondria, and dilatation of the nuclear envelope internal space. However, to our knowledge, there have been no reports on atypical cell death characterized by electron-dense nucleus and cytoplasm and chromatin clustering to speckles as seen in our zVAD–treated cells.

The second novel point in the present study is that the serine protease Omi/HtrA2 is likely to be involved in caspase-independent necrosis–like cell death. The modes of caspase-independent cell death are classified into apoptosis–like PCD and necrosis–like PCD, and their mechanisms are often mediated by mitochondria–associated factors such as ROS, AIF, Endo G, and...
Our study demonstrated that imatinib-induced, caspase-independent necrosis was associated with the release of Omi/HtrA2 from mitochondria, yet was not coupled to the overproduction of ROS, nuclear translocation of AIF, or the Endo G–mediated internucleosomal DNA fragmentation. Omi/HtrA2 is a serine protease that is localized in the mitochondrial intermembrane space. During apoptosis, Omi/HtrA2 is released into the cytosol and inhibits IAPs, thereby deinhbiting the activation of the caspase cascade. In addition, the serine protease activity of Omi/HtrA2 induces caspase-independent cell death with atypical morphologic changes: cell rounding and shrinkage not accompanied by membrane blebbing, apoptotic body formation, or nuclear morphologic changes. These morphologic features seemed similar to those of the caspase-independent necrosis in our study. Additionally, the release of Omi/HtrA2 was not inhibited by zVAD. These findings raised the possibility that Omi/HtrA2 might play a crucial role in zVAD + imatinib–induced necrosis.

TLCK and TPCK are used as serine protease inhibitors as previously described. TLCK is a trypsinlike serine protease inhibitor, while TPCK is a chymotrypsin-like serine protease inhibitor. Omi/HtrA2 possesses trypsinlike serine protease activity, thereby inducing atypical cell death. In our study, the caspase-independent necrosis was prevented by these serine protease inhibitors. Moreover, the trypsinlike serine protease inhibitor TLCK showed a greater effect than the chymotrypsin-like TPCK, corroborating that the caspase-independent necrosis was mediated by the trypsinlike serine protease activity of Omi/HtrA2. We considered this mode of cell death as a programmed one in the sense that it was mediated by an inherent cellular protein. In addition, TLCK (or TPCK) + zVAD + imatinib–treated cells exhibited the same degree of growth arrest as zVAD + imatinib–treated cells (not shown), indicating that the serine protease activity was not rate-limiting for the zVAD + imatinib–induced cell cycle block. Both TLCK and TPCK prevented the loss of ΔΨm, which is similar to the phenomenon that caspases can delay the ΔΨm dissipation and, consequently, cell death. These data indicate that caspases and serine proteases may contribute to the ΔΨm loss and thereby accelerate the cell death. There may be a positive feedback mechanism in the signaling pathway downstream of the mitochondria. On the other hand, there are 2 problems concerning these agents, TLCK and TPCK. First, the trypsinlike serine protease inhibitor TLCK is not necessarily specific for Omi/HtrA2. Therefore, we have not ruled out the possibility that other serine proteases than Omi/HtrA2 might be involved in the caspase-independent necrosis. Second, these 2 agents are alkylating agents as well as serine protease inhibitors. Indeed, in the absence of zVAD, these agents promoted cell death in BV173 cells and K562 cells (Figure 8C). In contrast, in the presence of zVAD, these agents prevented cell death in a dose-dependent fashion. Hence, we considered that, under the inhibition of caspase activities, these agents function as inhibitors of cell death. Accordingly, in order to investigate exactly the role of Omi/HtrA2, it would be more useful to use a specific inhibitor or a dominant-negative form for Omi/HtrA2. Although we tried ucf-101, which has recently been reported to be the specific inhibitor for Omi/HtrA2, this was cytotoxic to our cell lines at the concentration that it inhibited the serine protease activity of Omi/HtrA2. As there is not any specific inhibitor for Omi/HtrA2, others have used general serine protease inhibitors, 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) and TPCK, and concluded the involvement of Omi/HtrA2 in a p53-dependent apoptosis pathway.

Our result that caspase-independent cell death of BCR-ABL–positive cells exhibits necrotic morphology may be worthy of further investigation. Recently, necrosis has been attracting attention among immunologists and oncologists in terms of therapeutic approaches to cancers. Necrotic cancer cells are ingested by antigen-presenting cells (APCs) and thereby activated APCs in turn induce the immune responses against cancer in vitro and in vivo. Moreover, APCs stimulated with necrotic cells display better antitumor activities than those stimulated with apoptotic cells. These findings suggest that induction of necrosis in cancer cells could constitute a novel maneuver for rendering cancer cells immunogenic. Future investigation will unravel the feasibility of such an “immunotherapy.”

In conclusion, our data indicate that imatinib induces caspase-independent, necrosis-like programmed cell death mediated by serine protease activity, most likely by Omi/HtrA2, in BCR-ABL–positive human leukemic cells.

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References


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