A Novel Apoptotic Pathway in Quiescent Lymphocytes Identified by Inhibition of a Post-Proline Cleaving Aminodipeptidase: A Candidate Target Protease, Quiescent Cell Proline Dipeptidase

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A Novel Apoptotic Pathway in Quiescent Lymphocytes Identified by Inhibition of a Post-Proline Cleaving Aminodipeptidase: A Candidate Target Protease, Quiescent Cell Proline Dipeptidase¹

Murali Chiravuri, Tracy Schmitz, Kurt Yardley, Robert Underwood, Yogeshwar Dayal, and Brigitte T. Huber²

The vast majority of lymphocytes in vivo persist in a quiescent state. These resting lymphocytes are maintained through a cellular program that suppresses apoptosis. We show here that quiescent PBMC, but not activated PBMC or transformed lymphocytes, die in the presence of highly specific post-proline aminodipeptidase inhibitors. This form of death has the hallmarks of apoptosis, such as phosphatidylserine externalization and loss of mitochondrial transmembrane potential. However, it differs from apoptosis induced by gamma irradiation in the same cells or by Fas ligation in transformed lymphocytes in terms of caspase involvement. In addition, the aminodipeptidase inhibitor-induced cell death, but not gamma-irradiation-mediated apoptosis, can be prevented by inhibition of the proteasome complex. The target of these inhibitors is not CD26/DPPIV, but probably a novel serine protease, quiescent cell proline dipeptidase, that we have recently isolated and cloned. These studies will yield a better understanding of the requirements and the mechanisms that mediate quiescent lymphocyte homeostasis in vivo. The Journal of Immunology, 1999, 163: 3092–3099.

Apoptosis is an essential process in the development and maintenance of homeostasis in an organism (1, 2). It is also important for the normal functioning and maturation of the immune system (3–5). Although there are a variety of apoptotic triggers in lymphocytes, the state of development and activation of these cells dictates their susceptibility to a particular apoptotic stimulus (6). Proteases are attractive candidates as regulators of quiescent cell survival, because these enzymes can process or degrade multiple substrate molecules with little energy cost to resting cells. We tested this hypothesis by screening various protease inhibitors for their ability to induce programmed cell death (PCD) in cultures of freshly isolated PBMC. We found that inhibitors with remarkable specificity for post-proline-cleaving aminodipeptidases, particularly L-valinyl-L-boroproline (VbP) (7–9), cause cell death. This led to the identification of a novel serine protease, designated quiescent cell proline dipeptidase (QPP) (10).

Apoptosis triggered by inhibitors of proteolytic enzymes is well described. There is a large body of work on the apoptosis initiated by specific inhibitors of the proteasome complex. Lactacystin (11, 12) and other proteasome inhibitors have been shown to cause apoptosis in a number of cell lines (13–15). Different mechanisms seem to operate in this type of cell death induction, depending on the cell type, i.e., c-Jun N-terminal kinase up-regulation is associated with proteasome inhibition in U937 cells (14), while increased levels of p27kip1 were seen in HL60 cells (15). It is interesting to note that the susceptibility pattern of death induction by proteasome inhibitors and VbP is opposite in terms of activation state of the cells; namely, activated, cycling cells, but not quiescent cells, are susceptible to apoptosis caused by lactacystin (13–15), while the opposite pattern is seen for VbP. Even proteases that have more specialized roles, such as aminopeptidases, seem to play an essential role in maintaining cellular homeostasis. Inhibitors of puromycin-sensitive aminopeptidase, a widely expressed amino peptidase, cause apoptosis, possibly due to the toxic accumulation of uncleaved puromycin-sensitive aminopeptidase substrates (16). QPP is a 58-kDa glycoprotein that is found in lysosomes, but is also secreted in an active form. QPP cleaves dipeptides from the amino terminus of proteins that have a proline or an alanine at the penultimate position, an activity attributed to dipeptidyl peptidase IV (CD26/DPPIV) (17, 18). Although CD26/DPPIV and QPP have similar substrate specificities at neutral pH, they can be functionally and biochemically distinguished (10).

We show here that highly specific inhibitors of post-proline cleaving aminodipeptidases cause cell death in quiescent lymphocytes, but not activated or transformed lymphocytes, in a stereospecific manner. This cell death has apoptotic features, such as phosphatidylserine exposure and gradual loss of mitochondrial potential, and can be blocked by the broad spectrum caspase inhibitor zVAD-fmk. The molecular events associated with this form of...
PCD differ from those seen after gamma irradiation or Fas ligation, as evidenced by differential caspase activation pathway and involvement of the proteasome complex. The target of these inhibitors specific for post-proline cleaving dipeptidases is not CD26/DPP IV, but is probably QPP, because a strong correlation is seen between the inhibition of QPP activity by these inhibitors and the amount of cell death induced. Thus, blocking of QPP seems to induce this novel type of apoptosis. These data will help us understand the role played by proteases in the maintenance of homeostasis, particularly in the context of quiescent cells.

Materials and Methods

Cells

Human PBMC were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia, Uppsala, Sweden) of blood obtained from healthy donors. Briefly, a 1/1 blood/PBS mixture layered over Ficoll and centrifuged. The cells were extracted from the buffy coat, washed, and resuspended in AIM-V medium (Life Technologies, Gaithersburg, MD) supplemented with 100 IU of penicillin and 10 mg/ml streptomycin. Jurkat and H9 cells were grown in RPMI 1640, supplemented with 10% FCS, 100 IU penicillin, and 10 mg/ml streptomycin, while all assays were conducted in AIM-V medium. Primary cells were activated with 5 μg/ml PHA (Sigma, St. Louis, MO) for 48 h, followed by culture in AIM-V medium, supplemented with 100 U/ml of human rIL-2.

Reagents

The peptidase inhibitors Lys-piperidide (piperidide), VbP, and its D-enantiomer, 1-valinyl-D-boropropylide (D-VbP), were provided by R. Snow and A. Kabacelln (Boehringer Ingelheim, Ridgefield, CT). The caspase inhibitors benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk), BOC-Lys-piperidide, and BOC-Lys-piperidide (4-NO2) were purchased from Enzyme Systems Products (Dublin, CA). The caspase substrate Ac-DEVD-pna was provided by R. Talianan (BASF, Waterside, MA). Lactacystin was purchased from E. J. Corey (Harvard University, Boston, MA), and N-carbonylvalxoxyn-1-4-leucyl-l-ornvalinal (LnnV) was purchased from Enzyme Systems Products (Dublin, CA). Annexin V-FITC was purchased from Molecular Probes (Eugene, OR), and annexin V and DiOC 6 staining was performed by measuring propidium iodide (PI) uptake; cells were resuspended in isotypic PI buffer (PBS, 1% FCS, 0.01% NaN3, and 10 μg/ml PI), and PI uptake was measured using a FACScan (Becton Dickinson, Mountain View, CA).

Cell death assays

Cells were incubated with QPP inhibitors, gamma irradiated by exposure to 2500 Rad, or incubated with 1 μg/ml of the anti-Fas Ab, CH-11 (Upstate Biotech, Lake Placid, NY). Unless otherwise indicated, cell death assays were performed by measuring propidium iodide (PI) uptake; cells were resuspended in isotypic PI buffer (PBS, 1% FCS, 0.01% NaN3, and 10 μg/ml PI), and PI uptake was measured using a FACScan (Becton Dickinson, Mountain View, CA).

Annexin V and DiOC 6 staining

For annexin V-FITC staining, cells were washed twice in PBS and resuspended in binding buffer A (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, and 0.1% BSA); 10 μl of annexin V-FITC was added to 100 μl of annexin V-FITC staining buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, and 0.1% BSA); 10 μl of annexin V-FITC was added to 100 μl of annexin V-FITC staining buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, and 0.1% BSA). The nuclei were spun down, the supernatant was subjected to a 30,000 × g spin. For QPP analysis samples were subjected to a 100,000 × g centrifugation for 30 min. The protein concentration was measured using the BCA protein estimation kit (Pierce, Rockford, IL). DEVDase activity was measured using the chromogenic substrate Ac-DEVD-pna (100 μM). QPP activity was measured using the fluorescent substrate AP-AFC (2 mM in 50 mM HEPES buffer, pH 7.5) on an Fmax fluorescence plate reader (excitation, 410 nm; emission, 510 nm), while the chymotrypsin activity of the proteasome was measured using zGGL-AMC (excitation, 390 nm; emission, 460 nm; Molecular Devices, Menlo Park, CA).

Poly-ADP ribose polymerase (PARP) immunoblots

Cells (1–2 × 10^6) were suspended in reducing lysis buffer (62.5 mM Tris (pH 6.8), 6 M urea, 10% glycerol, 2% SDS, 0.003% bromophenol blue, and 5% 2-ME). The lysates were sonicated to break up the DNA and detach PARP from the DNA. The sonicated lysates were run on SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membranes and probed with the C2–10 anti-PARP Ab, purchased from Dr. Guy Poirier (Montreal, Canada).

Electron microscopy

PBMC (40 × 10^6) were fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide, and dehydrated with a graded ethanol series. The cells were embedded in Epon, and sections were stained with uranyl acetate and lead citrate. Samples were analyzed with an electron microscope (Phillips, Mahway, NJ).

Enzyme assays

Cells (1–2 × 10^6) were resuspended in lysis buffer (20 mM HEPES, 1.5 mM MgCl2, 2 mM EDTA, 10 mM KCl, 0.1% Nonidet P-40, 5 μg/ml antipain, and 5 μg/ml leupeptin) for 30 min at 4°C. The nuclei were spun out at 2,000 rpm on a microcentrifuge for 10 min. The postnuclear supernatant was subjected to a 30,000 × g spin. For QPP analysis samples were subjected to a 100,000 × g centrifugation for 30 min. The protein concentration was measured using the BCA protein estimation kit (Pierce, Rockford, IL). DEVDase activity was measured using the chromogenic substrate Ac-DEVD-pna (100 μM). QPP activity was measured using the fluorescent substrate AP-AFC (2 mM in 50 mM HEPES buffer, pH 7.5) on an Fmax fluorescence plate reader (excitation, 410 nm; emission, 510 nm), while the chymotrypsin activity of the proteasome was measured using zGGL-AMC (excitation, 390 nm; emission, 460 nm; Molecular Devices, Menlo Park, CA).

Cell sorting

The anti-CD26 Abs TA1 and 134-2C2 were obtained from E. Reinherz (Dana-Farber Cancer Institute, Boston, MA). T cells were purified by SRBC rosetting, stained with the anti-CD26 mAb TA1, and sorted into CD26^+ and CD26^- populations using a FACStar™ dual laser cytometer (Becton Dickinson). Cells expressing the highest level of CD26 (top 5%) were designated CD26^+; cells expressing the lowest level of CD26 (bottom 10%) were designated CD26^- . The purity of these cell populations was >90% as determined by staining with the anti-CD26 mAb 134-2C2, which is directed against a different epitope on CD26 than that recognized by TA1.
Results

Inhibitors of a post-proline cleaving dipeptidase induce cell death in PBMC

When we screened PBMC for sensitivity to protease inhibitors, we found that cultures containing a peptidyl boronic acid inhibitor of post-proline-cleaving aminodipeptidases, namely, VbP (boroproline is the boronic acid analogue of proline; see Fig. 1) (7) had a markedly increased number of dead cells compared with cultures containing medium alone. VbP is a highly specific inhibitor of the relatively rare post-proline-cleaving aminodipeptidases (7–9).

A detailed analysis showed that concentrations as low as 10 μM VbP in the culture medium caused ≥30% death in PBMC, as measured by vital dye exclusion (Fig. 2A). Using this method, dead cells were apparent as early as 4 h after the addition of VbP, with maximal death occurring within 24 h (data not shown). The inactive stereoisomer, d-VbP (19), did not cause cell death in resting lymphocytes, indicating that a stereospecific interaction such as that between a substrate and enzyme active site is required for this cell death to occur (Table I).

Cell death has hallmarks of apoptosis

To characterize this cell death in more detail, we analyzed the morphology of VbP-treated resting PBMC. Before cell lysis, apoptotic cells undergo externalization of phosphatidylserine molecules, normally found exclusively in the inner leaflet of the cytoplasmic membrane (20, 21). To test for the loss of phosphatidylserine asymmetry in VbP-treated PBMC, we costained these cells with the phosphatidylserine binding reagent annexin V-FITC and the vital dye PI. Apoptotic cells stain annexin V⁺/PI⁻, while necrotic cells nonspecifically take up both annexin V

![Graph showing % Death vs VbP (μM)](http://www.jimmunol.org/)

![Graph showing Dot plots of annexin V-FITC/PI costaining](http://www.jimmunol.org/)

![Graph showing Histograms of DiOC₆ staining](http://www.jimmunol.org/)

![Electron micrographs of untreated, VbP-treated (100 μM for 12 h), and necrosed (10% ethanol for 90 min) lymphocytes](http://www.jimmunol.org/)
and PI (21). As shown in Fig. 2B, 5.5 h of treatment with VbP resulted in >12% of PBMC staining annexin V+/PI−, compared with 2.4% for the untreated controls, and within 8 h, 29.6% of VbP-treated PBMC and 2.8% of untreated PBMC stained annexin V+/PI−. This shows that VbP-treated PBMC acquire an apoptotic annexin V+/PI− phenotype in a time-dependent manner.

Another early feature of apoptotic cells is mitochondrial damage and the subsequent loss of mitochondrial transmembrane potential, \( \Psi_{m} \) (22). Mitochondrial damage is thought to commit the cell to death due to the release of caspase activators, the loss of electron transport, a change in cellular redox potential, or a combination of the three (23). To determine the effects of VbP on the mitochondrial potential of resting PBMC, we analyzed the mitochondrial function of these cells using the cationic dye DiOC₆. Cells that have undergone mitochondrial damage and lost mitochondrial transmembrane potential stain DiOC₆low. Within 3 h of VbP treatment, 13.1% of VbP-treated PBMC stained DiOC₆low, and this number increased to 32% after 8 h, showing a time-dependent loss of mitochondrial potential (Fig. 2C). No significant loss of DiOC₆ staining was seen in untreated control cells. The percentage of PBMC showing mitochondrial damage after 8 h of VbP treatment (32%) correlated with the number of cells exposing phosphatidylserine on the surface at this time point (29.6%). In agreement with the findings of others (22), the kinetics of the earlier time points showed a loss of mitochondrial function before the loss of membrane phosphatidylserine asymmetry.

Ultrastructural analysis of cell death

Electron microscopy was used to analyze the ultrastructural features of VbP-treated PBMC. These cells, treated with VbP (100 μM) for 10 h, showed a retention of cytoplasmic and nuclear membrane integrity and a preservation of cytoplasmic ultrastructure (Fig. 2D). In these cells the overall structure of organelles such as mitochondria was retained, although the mitochondria in VbP-treated lymphocytes appeared more dense than those in the untreated controls. This altered mitochondrial phenotype may be associated with the observed loss of mitochondrial potential. On the other hand, necrotic cells exhibited a nonspecific ablation of cytoplasmic and intracellular membranes, organelles, and nuclei (Fig. 2D). These data further suggest that VbP-treated PBMC undergo PCD, rather than nonspecific necrotic cell death.

Only quiescent lymphocytes in \( G_0 \) are sensitive to death induction by dipeptidase inhibitors

Resting PBMC are resistant to Fas-mediated death (24–26), but are susceptible to gamma-irradiation-mediated death (27), while activated PBMC and transformed lymphocytes are susceptible to both forms of apoptosis. Given that the activation state of a lymphocyte is important in determining its susceptibility to apoptotic triggers, we compared the effects of three apoptotic triggers, VbP, Fas ligation, and gamma irradiation, on lymphocytes in different activation states. Resting PBMC, activated PBMC (5 μg/ml PHA), and transformed (Jurkat) lymphocytes were treated with VbP (10 μM), 2500 Rad, or 1 μg/ml anti-Fas mAb CH-11. As shown in Table I, the addition of 10 μM VbP caused significant cell death in resting lymphocytes (34.4%), but not activated PBMC (7.8%) or transformed (Jurkat) T cells (2.7%). This was the opposite of the pattern observed for Fas/FasL-mediated death. All three cell types, however, showed significant cell death following gamma irradiation. The difference in VbP-mediated susceptibility for cell death between resting and transformed cells was not due to differential cell permeability. This was tested by the ability of VbP, added to intact cells, to block the activity of intracellular QPP in all the cell types (data not shown). VbP did not cause cell death in resting lymphocytes (Table I), precluding a general nonspecific toxicity. Quiescent T and B cells were equally sensitive to death induction by VbP regardless of whether they were primary cells or long term memory cells, as long as they were in the \( G_0 \) stage of the cell cycle (data not shown).

**Caspase involvement**

The caspase family consists of postaspartate-cleaving cysteine proteases that are downstream effectors of most, if not all, known apoptotic pathways (28). To establish the involvement of caspases in VbP-mediated death induction of PBMC, we used peptide-fluoromethyl-ketone (fmk) inhibitors. These inhibitors are cell permeable, relatively nontoxic, and specific for postaspartate-cleaving caspases (6). bD-fmk and zVAD-fmk are broad spectrum caspase inhibitors (29, 30), but bD-fmk has been reported to be more specific for caspase-3-like proteases than zVAD-fmk (6). Thus, these two caspase inhibitors were used to determine whether the downstream effector molecules involved in VbP-mediated PCD were different from those involved in gamma irradiation or Fas-mediated apoptosis.

As shown in Fig. 3A, the addition of zVAD-fmk blocked VbP-mediated PCD in resting PBMC by >50%, while the control reagent, zFA-fmk, had no effect. The addition of the caspase inhibitor, bd-fmk, did not block this type of cell death. On the other hand, cell death induction in quiescent lymphocytes by a different apoptotic stimulus, gamma irradiation, was prevented by both zVAD-fmk and bd-fmk (Fig. 3A). We also observed that bd-fmk and zVAD-fmk blocked Fas/FasL-mediated cell death in the Jurkat T cell tumor line (Fig. 3A).

**Caspase substrates**

Caspase-3-like caspases are known to be activated following DNA damage (27, 31, 32) or Fas/FasL interaction (33, 34). To further analyze whether different caspases were activated following the addition of QPP inhibitors compared with those induced after gamma irradiation or Fas ligation, we tested for caspase-3-specific
DEVDase-cleaving activity and the cleavage of the caspase substrate PARP. We were able to detect the cleavage of the chromogenic caspase-3 substrate Ac-DEVD-pna and the model substrate PARP in gamma-irradiated resting lymphocytes as well as in Fas-cross-linked H9 T cells (Fig. 3, B and C). Interestingly, neither DEVDase activity nor PARP cleavage was seen in resting lymphocytes treated with VbP (Fig. 3, B and C). To rule out that the QPP inhibitors were directly acting on caspase-3-like caspases, we analyzed PARP cleavage in the presence or the absence of 100 μM VbP in the Fas-mediated death pathway. PARP was cleaved in anti-Fas mAb treated H9 cells in the presence or the absence of VbP (Fig. 3 D), demonstrating that VbP does not block PARP cleavage.

Proteasome involvement

Recent reports have indicated the involvement of the proteasome in the execution of some apoptotic pathways (35). To determine whether the proteasome complex plays a role in the VbP-induced cell death pathway in PBMC, we cultured VbP-treated cells in the presence or the absence of lactacystin. Lactacystin, a metabolite of streptomyces, is a highly specific inhibitor of the proteasome that binds irreversibly to the active site threonine of the β subunit (11). As shown in Fig. 4A, PCD induced by VbP in resting lymphocytes was blocked by lactacystin in a dose-dependent manner. A 20-μM concentration of lactacystin blocked up to 50% of the VbP-induced cell death. Identical results were obtained with another proteasome inhibitor, LLnV (15) (data not shown). On the other hand, lactacystin did not block cell death in response to gamma irradiation in
resting lymphocytes; in fact, the addition of proteasome inhibitor seemed to potentiate this form of PCD (Fig. 4A). As described above, VbP causes a loss of mitochondrial membrane potential that can be detected by DiOC₆ staining. The loss of mitochondrial potential after 5.5 h of 100 µM VbP treatment was almost completely inhibited by the addition of lactacystin (20 µM; Fig. 4B). These data suggest that the proteasome complex plays a role in the upstream pathway of VbP-induced cell death.

To study the kinetics of the proteasome involvement in VbP-induced apoptosis, lactacystin was added at discreet time points following the addition of QPP inhibitors. As shown in Fig. 4C, the addition of 20 µM lactacystin at the same time as VbP inhibited 52% of VbP-induced cell death. However, when lactacystin was added 2 or 4 h after VbP, lactacystin blocked 38 and 23% of VbP-mediated PCD, respectively. Thus, delaying the addition of the proteasome inhibitor by 2 or 4 h resulted in less efficient prevention of PCD induced by VbP. Identical results were obtained using the QPP-specific inhibitor lys-piperidide (see Fig. 1) in place of VbP to induce PCD in PBMC (Fig. 4C).

QPP, not CD26/DPPIV is the likely target of the dipeptidase inhibitors

There are relatively few proteases that can cleave peptide bonds containing proline (36), and VbP is an extremely specific inhibitor that selectively targets post-proline-cleaving enzymes (8). CD26/DPPIV is the best known post-proline-cleaving aminopeptidase, and VbP is a potent inhibitor of CD26/DPPIV (7, 17). Thus, we performed experiments to determine whether CD26 is required for VbP-induced death; namely, we isolated CD26₁ and CD26₂ T cell subpopulations from PBMC and assayed them for susceptibility to VbP-induced death. The data show an equal sensitivity to VbP-induced PCD between the two subpopulations at varying concentrations of VbP (Fig. 5A). This indicates that VbP-induced death is not mediated through the inhibition of CD26, but through a novel target(s). This is substantiated by the observation that T cells as well as B cells (data not shown) are sensitive to VbP-induced apoptosis. To further confirm these results, we tested the fluorooefolin Ala-Y(CF=O)-Pro-NHO-Bz(4-NO₂) L125, which is a strong inhibitor of CD26 (9). As shown in Fig. 5B, L125 does not cause cell death in PBMC; thus, inhibition of CD26 does not lead to PCD in quiescent lymphocytes. It should be noted that L125 is not an effective inhibitor of QPP activity (Kᵢ > 1,000 nM) (10).

This result led us to look for an alternate post-proline-cleaving activity as a candidate target for VbP. We found an intracellular activity that had the same substrate specificity as CD26/DPPIV at neutral pH. We purified and cloned this activity, which we have termed QPP (10). To investigate any correlation between QPP inhibition and cell death in resting PBMC, we directly measured QPP enzymatic activity in control and VbP-treated cells that were later analyzed for cell death. PBMC were treated for 90 min with increasing concentrations of VbP and then thoroughly washed. Aliquots of these samples were harvested and measured for soluble QPP activity, while the rest of the cells were analyzed for cell death. As Fig. 5C shows, the inhibition of QPP activity shows a striking correlation with the amount of apoptosis measured in the resting PBMC. Furthermore, to demonstrate the specificity of this interaction, the chymotrypsin activity of an unrelated protease, the proteasome complex, was also measured. As shown in Fig. 5C, VbP did not cause any significant decrease in proteasome activity. The fact that the capacities of the various concentrations of VbP to induce cell death correlate with their inhibitory potentials of QPP, but not those of other proteases, makes QPP a strong candidate for the PCD-inducing molecular target of VbP.

Discussion

The target of the boronic acid protease inhibitors used in this study is almost certainly a post-proline-cleaving aminopeptidase. The prolineboronic acid dipeptides are extremely specific for post-proline-cleaving serine proteases (7–9). Coutts et al. reported that 100-µM concentrations of VbP show no inhibition of the serine proteases chymotrypsin, trypsin, leukocyte elastase, thrombin, plasmin, and trypase among others (8). In our hands, we could detect no inhibition of the chymotrypsin activity of the proteasome in cells treated with VbP. Furthermore, the specificity of VbP is
such that it is 1000-fold less efficient at blocking post-proline-cleaving endopeptidases, such as prolylendopeptidase, than post-proline-cleaving aminopeptidases (8). This again points to a post-proline-cleaving aminopeptidase as the target of VbP.

The stereoisomer D-VbP did not cause cell death, further precluding a nonspecific toxicity of VbP and showing that a stereospecific interaction, such as that between an enzyme-active site and substrate, is required for cell death to occur. D-VbP is 1000 times less effective in binding to the active site than L-VbP (19) and shows vastly diminished inhibitory capacity for blocking post-proline-cleaving aminopeptidases in vitro (8, 19).

Proteases that cleave peptide bonds containing proline are rare, and the best described post-proline-cleaving aminopeptidase is CD26/DPPIV. The boronic acid inhibitors have been extensively used to study CD26/DPPIV (7, 37–39). However, the fact that both CD26− and CD26+ T cells were susceptible to VbP-induced cell death indicates that blocking CD26/DPPIV does not cause the apoptosis in resting lymphocytes. Furthermore, these results show that both naive T cells (CD26−) as well as resting memory T cells (CD26+) are susceptible to VbP-mediated cell death. To confirm that CD26/DPPIV is not the target of death induction by VbP, we used the fluoroorolefin Ala-Y(CF3)-Pro-NHO-Bz(4-NO2) L125, an inhibitor that blocks CD26/DPPIV (Kᵢ, 188 nM) (9, 40), but is ineffective in blocking QPP (10). This agent showed no death-inducing effect on resting PBMC (Fig. 5B). On the other hand, a strong correlation was seen between the inhibition of QPP activity and the amount of apoptosis induced in PBMC by VbP, making the novel protease QPP a likely candidate for the target of the inhibitors.

Compared with activated lymphocytes, quiescent lymphocytes are relatively resistant to a number of apoptotic triggers (13, 25, 41–44) (Table I). Thus, the selective susceptibility of quiescent lymphocytes to VbP is even more unusual. The mechanism by which resting lymphocytes are more susceptible to QPP inhibitors than activated cells has not been defined. One possibility is that the activated or transformed lymphocytes, which express a large number of gene products, may have an additional system or cellular pathway(s) that renders QPP activity redundant. Likewise, these cells may down-regulate or inhibit the pathway(s) and/or caspase(s) that are activated in response to QPP inhibitors in resting lymphocytes.

Proteolytic cleavage can result in altered specificity of a protein. Amino-terminal dipeptide cleavage has recently been shown to inactivate certain chemokines, such as stromal-derived factor-1. Amino terminus (36), rendering them ideal substrates for proteases and substrate(s) of QPP will allow us to understand the unique requirements for the activation of caspases or of other molecules involved in this death pathway. If this were the case, then inhibition of the proteasome would prevent activation of the caspases, thus preventing QPP inhibitor-mediated apoptosis. Clearly, the apoptotic cascade induced by VbP differs from the cascade activated in resting lymphocytes by gamma irradiation, as PCD induced by this mode is not blocked by proteasome inhibitors.

It has become increasingly apparent that the so-called quiescent state in lymphocytes is actually dynamic, requiring the expression of specific gene products. Constant external signaling seems to be necessary for the survival of resting lymphocytes, the absence of which activates a latent apoptotic pathway (52). Published data indicate that the transcription factor LKLF is required for the maintenance of quiescence in resting T cells, while its presence seems to be dispensable in activated T cells (53). Elucidation of the substrate(s) of QPP will allow us to understand the unique requirement of this activity in quiescent cells and yield a more detailed analysis of amino dipeptidase-inhibitor-induced PCD. Identification of the caspase(s) involved in this cell death induction will help us understand the components of the apoptotic pathway in quiescent lymphocytes and ultimately yield a better understanding of homeostasis in the quiescent lymphocyte pool in vivo.

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