peptides only bound HLA-A1 very weakly. Subsequently, we scrutinized PBL from HLA-A1 + cancer patients of different origins by means of ELISPOT against the Mcl-1(177-185) peptide as described.[3] Indeed, strong and frequent CTL responses were detected against this peptide in cancer patients of different origins. We were able to detect a response against the Mcl-1(177–185) in HLA-A1 + PBL in three out of five examined CLL patients (responders are defined as average number of antigen-specific cells $\pm 1/2$ standard deviation >25 per 10^5 cells), two out of six examined melanoma patients, and three out of four examined breast cancer patients (Figure 1b). Importantly, we were not able to detect a response in any of the seven HLA-A1 + healthy individuals we examined as controls (Figure 1b). The characterization of multiple Mcl-1 epitopes with different HLA class I restriction elements broadens the clinical potential of this target antigen in several important ways: Targeting different HLA molecules in a multiepitope vaccine will increase the number of patients eligible for immunotherapy and will further decrease the risk of immune escape by class I HLA-allele loss; loss of a single class I HLA allele is a significant component of MHC alterations described in cancer cells, whereas total loss of class I HLA expression is rare.[5] The attractiveness of using Mcl-1 for vaccination purposes relies on the fact that downregulation or loss of expression of this protein as a means of immune escape would impair sustained tumor growth. The availability of multiple Mcl-1 epitopes presented by different HLA class I restriction elements further reduces the risk of immune escape and thereby therapeutic failures. Finally, the immunodominance of a given peptide epitope differ among individual patients depending on several factors such as the T-cell receptor repertoire, which are almost impossible to predetermine. Thus, a variety of epitopes ensures that the respective immune-dominant epitope is actually used.

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Can application of serine protease inhibitors TPCK and TLCK provide evidence for possible involvement of serine protease Omi/HtrA2 in imatinib mesylate-induced cell death of BCR-ABL-positive human leukemia cells?

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TO THE EDITOR

Apoptosis was originally defined as a mode of cell death with specific morphology.[1] Since the discovery of caspase proteases, it has been evident that caspase activation was indispensable for apotosis to occur in various mammalian cell types. First reports in the early 1990s demonstrated that the broad-spectrum caspase inhibitors such as z-VAD-fmk could effectively block apoptosis in several animal cell death models.[2] Later on, it was rather surprisingly demonstrated that caspase inhibitors were unable to prevent cells from death; cells still died, although more slowly while exhibiting necrosis-like morphology.[3] Further research revealed that mitochondria play crucial role in both caspase-dependent and -independent commitment to cell death. Various extracellular and intracellular insults may eventually lead to the release of proteins that reside in intermembrane space of mitochondria resulting in cell death. Some of these proteins including cytochrome *c* and Smac/DIABLO act as caspase activators. The others, including AIF (apoptosis-inducing factor) and endonuclease G, contribute to caspase-independent cell death. Omi/HtrA2, a serine protease that also reside in intermembrane mitochondrial space, can act in both ways.[4]

Recently, Okada and co-workers[5] observed that serine protease inhibitors TLCK and TPCK prevented the z-VAD-fmk plus imatinib-induced necrosis-like programmed cell death (PCD) pathway. They further found that serine protease inhibitors alone failed to prevent imatinib-treated cells from death. Interestingly, serine protease inhibitors even promoted cell death in imatinib-treated cells.[5] Authors concluded that imatinib mesylate induced a caspase-independent, necrosis-like PCD mediated by the serine protease activity of Omi/HtrA2.[5] Their experiments were mainly

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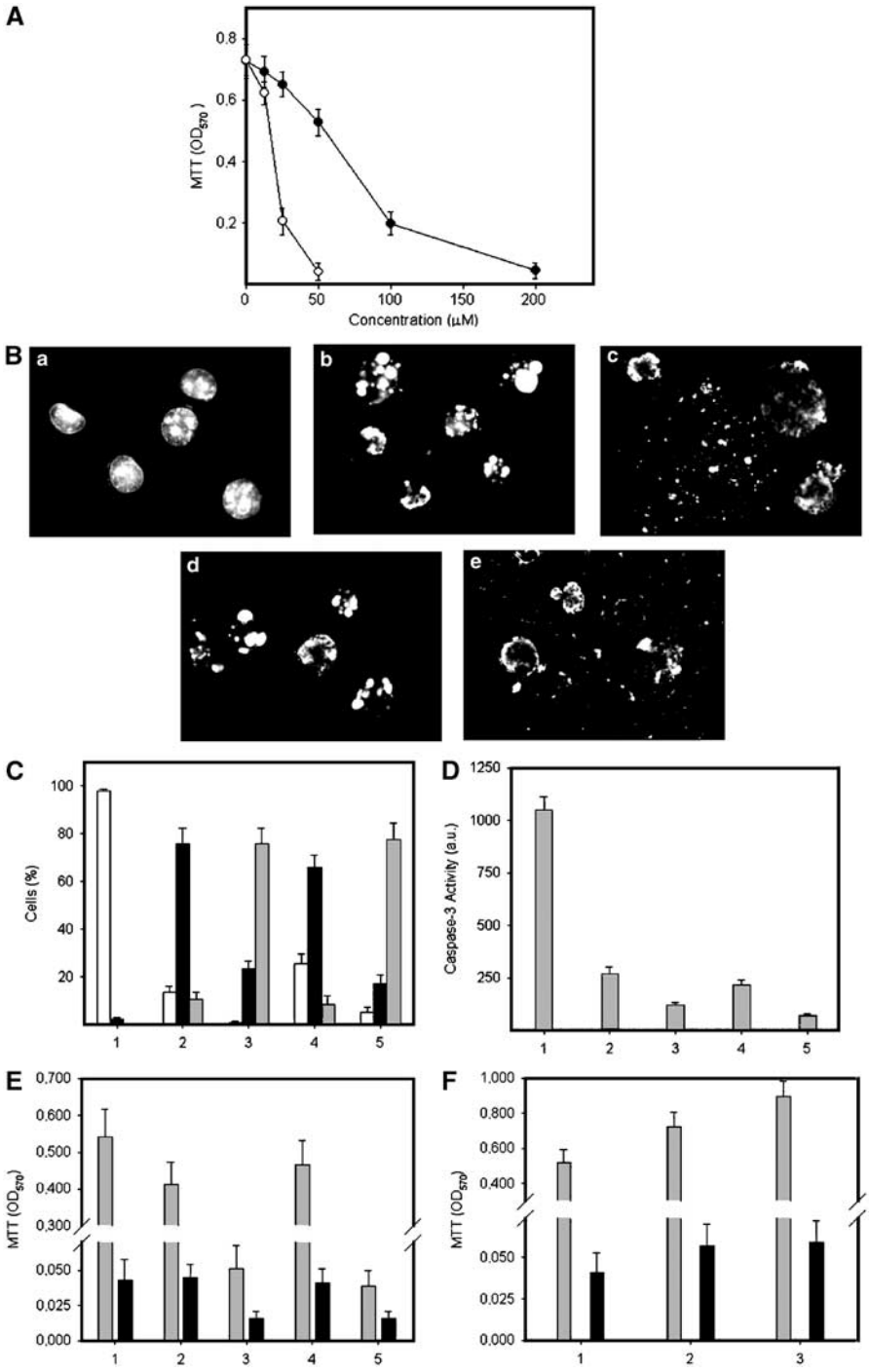

Figure 1 Effects of serine protease inhibitors on cell proliferation (A), nuclear morphology (B), cell death induction (C), caspase-3 activity (D), cell survival (E), and effect of z-VAD-fmk on cell survival (F). (A) Cells were incubated in the presence of TPCK (open circles) or TLCK (filled circles) for 72 h and then MTT assay was performed. (B) Cells were incubated in the presence of TLCK or TPCK (Sigma, St Louis, MO, U.S.A.) for 72 h, cells were stained with Hoechst 33341, and then the nuclear morphology was examined using fluorescence microscopy; control (a) 25 μM TPCK (b), 50 μM TPCK (c), 100 μM TLCK (d), and 200 μM TLCK (e). (The images were acquired using BX50 microscope (Olympus). The photography was performed using Olympus DP50 digital camera and the Viewfinder Lite version 1.0 program (Pixera, Los Gatos, CA, USA)). (C) Cells were incubated in the presence of TPCK or TLCK for 72 h and the numbers of nuclei with normal (plain columns), apoptotic (black columns) or necrotic (gray columns) morphology were counted using fluorescence microscopy; 1 – control, 2 – 25 μM TPCK, 3 – 50 μM TPCK, 4 – 100 μM TLCK, and 5 – 200 μM TLCK. (D) Activity of human recombinant caspase -3 (Sigma) was measured in the presence of TPCK or TLCK using fluorogenic caspase-3 substrate Ac-DEVD-AMC (Bachem, Bubendorf, Switzerland); 1 – Control, 2 – 25 μM TPCK, 3 – 50 μM TPCK, 4 – 100 μM TLCK, and 5 – 200 μM TLCK. (E) Cells were incubated for 72 h in medium containing a combination of serine protease inhibitors TPCK or TLCK with imatinib (Novartis Pharma, Basel, Switzerland); 1 – Imatinib, 2 – Imatinib+25 μM TPCK, 3 – Imatinib+50 μM TPCK, 4 – Imatinib+100 μM TLCK, and 5 – Imatinib+200 μM TLCK. Cells were then washed and transferred into fresh medium without inhibitors and incubation proceeded for the next 72 h prior to MTT assay. Gray columns: 0.5 μM imatinib; black columns: 1 μM imatinib. (F) Cells were incubated for 72 h in medium containing imatinib in a combination with z-VAD-fmk (Bachem); 1 – Imatinib, 2 – Imatinib+40 μM z-VAD-fmk, 3 – Imatinib+80 μM z-VAD-fmk. Cells were then washed and transferred into fresh medium without inhibitors and incubation proceeded for the next 72 h prior to MTT assay. Gray columns: 0.5 μM imatinib; black columns: 1 μM imatinib.

based on measurement of changes in mitochondrial transmembrane potential ($\Delta\psi_m$), permeability of plasma membrane, and on morphological observations following application of high concentrations of TLCK or TPCK in combination with a broad-spectrum caspase inhibitor z-VAD-fmk in imatinib-treated cells.[5]

We do not think that Okada and co-workers presented the only possible interpretation of their results. First, regardless to the cell line used, their experimental arrangement permits two different interpretations. Owing to the fact that both serine and caspase inhibitors were used in combination, it is not possible to distinguish which inhibitor was rescuing what treatment. One interpretation could be that serine protease inhibitors prevented imatinib plus z-VAD-fmk-treated cells from death, as concluded by Okada and co-workers.[5] However, it is also necessary to admit a possibility that z-VAD-fmk prevented imatinib plus TLCK- or TPCK-treated cells from death. Indeed, it was found that serine protease inhibitors alone even promoted cell death in imatinib-treated cells.[5] In other words, caspases might play crucial role in this process as well. Equal attention should be paid to both interpretations; however, they implicate that different proteases might play a crucial role. Therefore, we believe that the experimental setting cannot provide unequivocal support for the conclusion made by the authors.[5]

Second, we performed similar experiments with the K562 cells. Our results were in some aspects different than those published by Okada *et al*.[5] Both inhibitors TLCK and TPCK inhibited cell proliferation and induced cell death in K562 cells at concentrations even lower than that used by Okada and co-workers (Figure 1A–C). The mode of cell death appeared to be apoptotic and at highest concentrations of TLCK and TPCK even necrotic like. Our observation is in agreement with the findings of other authors who demonstrated that protease inhibitors including serine protease inhibitors are powerful inductors of apoptosis in many cell lines.[6] Further, Okada and co-workers[5] used relatively high concentrations of TLCK and TPCK, which therefore might have exhibited nonselective inhibitory effects. This assumption was confirmed by our *in vitro* experiments. Both inhibitors effectively blocked caspase activity at relevant concentrations (Figure 1D). Our finding is consistent with the fact that chloromethyl ketone moiety in their molecule can irreversibly alkylate the cysteine residue of cysteine proteases.[7] The observation that the serine protease inhibitors TLCK and TPCK could exert a strong inhibitory effect on caspases at applied concentrations (Figure 1D) further weakened the interpretation made by Okada and co-workers. Inhibitory effects of TLCK and TPCK on caspase activity could explain the change of the mode of cell death from apoptotic to necrotic, which is induced by these inhibitors at highest concentrations used (Figure 1B and C). The finding that TLCK and mainly TPCK even promoted cell death in imatinib-treated cells (Okada *et al*[5], Mlejnek, unpublished results) is consistent with our observation that serine protease inhibitors are toxic *per se* (Figure 1A–C). Despite the toxicity of TLCK and TPCK, they maintain relatively high value of $\Delta\psi_m$, especially in the case of TLCK when applied either alone or to the imatinib-treated cells (Mlejnek, unpublished results) and/or to imatinib plus z-VAD-fmk-treated cells (Okada *et al*[5]). This effect may seem to be protective, however, we found that TLCK (or TPCK) failed to prevent cells from death. Indeed, the proliferation potential (Figure 1E) as well as ATP production (not shown) in TLCK (or TPCK) plus z-VAD-fmk plus imatinib-treated cells were not revived upon transfer of cells to a standard medium without inhibitors.

Third, Okada and co-workers used only one concentration of z-VAD-fmk (40 μM). We found that higher concentration of z-VAD-fmk (80 μM) provided stronger protection, especially when lower concentration of imatinib was applied (Figure 1F). It is necessary to note that even 80 μM z-VAD-fmk failed to prevent the cells from death; however, a larger portion of the cells recovered upon transfer of cells to standard medium without inhibitors. Although 80 μM z-VAD-fmk could exert some nonspecific inhibitory effects on other proteases, this concentration was routinely used in many mammalian cell death models to prove involvement of caspase proteases in apoptosis and must be taken into consideration. Our data are in agreement with the observation published by Jacquel and co-workers,[8] who demonstrated that imatinib-induced apoptosis in K562 cells was caspase dependent or at least that caspases are involved in both the initiation as well as in execution phase of cell death.

Taken together, our data suggested that application of serine protease inhibitors TPCK and TLCK at high concentrations does not provide sufficient experimental support to the idea that serine protease Omi/HtrA2 plays the key role in z-VAD-fmk plus imatinib-induced necrosis-like PCD pathway. Moreover, the discussed experimental setup leads to ambiguous interpretation of the results and should be modified in order to avoid the mentioned uncertainty.

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