Role of proteases in the regulation of cell death in caspase-inhibited leukemia cells induced by staurosporine

Doctoral thesis

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1. INTRODUCTION

Cathepsins are proteases localized into the endo-lysosomal compartment with cystein, serine and aspartate active sites. Cathepsins work in concert for long term survival of the cell and animal. There are no significant developmental defects reported for mice deficient in one of the cathepsin genes indicating their redundant functions. They have significant function in the degradation of long-lived proteins and organelles via autophagy (macro-, micro-, and chaperon-mediated autophagy). Cathepsins, released to the extra-cellular space from secretary lysosomes are the mediators of tissue remodelling. They have significant role in tumor invasion and induction of inflammation. Alternatively spliced cathepsin gene products are localized in the mitochondria and the cell nucleus regulating gene expression.

As catabolic proteases, cathepsins were proposed to function in the final stage of necrotic cell death. The role of proteases homologues to cathepsins was shown in animal models (C. elegans) to mediate necrotic neurodegeneration. Cathepsins might have role in autophagic cell death too, even though at presently it is fairly unknown how the autophagic cell death proceeds from autophagy at the molecular level.

Limited release of cathepsins to the cytosol was also shown during apoptosis. The apoptotic cell death is a precisely regulated, complex process. Its main function is the elimination of unwanted cells from the organism accompanied by an optimal, adaptive immune response. Limited proteolysis is among the most important signaling components of apoptosis. Caspases are the well-known mediators of this limited proteolysis in apoptosis. Activating signal complexes and proteolytic targets (>200) of caspases are relatively well explored. The molecular roles of cathepsins in regulation of apoptosis are considerably less known. Experiments using pharmacological inhibitors and gene silencing of cathepsins suggest that cathepsins can reinforce the activation or replace caspases in apoptotic cell death. However, the known intracellular targets of the cathepsins are one order less than of the caspases. Though, these substrates (e.g. Bid, Bcl-2) are common targets of both caspases and cathepsins, it is not established whether targeting of these proteins by

cathepsins is enough for signaling an alternative, caspase-independent apoptotic or other forms of cell death.

These two reasons lead us to develop an in vitro model to test the role of cystein cathepsins in cell death: 1) apoptotic and necrotic cell death pathways could proceed even in the absence of bulk caspase activity; 2) the role of cathepsins were barely explored either in apoptosis or necrosis.

2. AIMS OF THE STUDY

Preliminary experiments clearly showed that staurosporine- or TRAIL-induced cell death could proceed in U937 pre-monocytic leukemia (earlier histiocytic lymphoma) cell line in the presence of general caspase inhibitor (z-VAD.fmk) suppressing the bulk DEVDase caspase activity. (Staurosporine (STS) is a protein kinase inhibitor that activates the inner mitochondrial-apoptosome caspase pathway, while TRAIL is a cytokine that activates the outer death receptor-mediated caspase pathway). In this caspase-independent, alternative cell death model (U937 cells, caspase inhibition, STS induction) we examined whether alternative protease activity can replace caspases. Using cathepsin inhibitors we defined the following questions:

1. How the cathepsin inhibitors (CA-074OMe and z-FA.fmk) can modulate cell death (enhancement or decrease). Whether the effect of these inhibitors on cathepsins protease activity is correlated with their effect on cell death?

2. Which changes in cells can characterize the various cell death forms in heterogene samples for appropriate discrimination by flow cytometry?

3. METHODS

3. 1. Cell culture

U937 and HL-60 cell lines were cultured in RPMI 1640 + 10% FBS, and cells were treated in 48 well plates. Inhibitors were added before cell death induction by staurosporine (STS; 1μM, for 8 h): geldanamycin (GA; 1 μM, -12 h); z-FA.fmk (z-FA; 1 μM, -30 min); and/or z-VAD(OMe).fmk (z-VAD.fmk; 50 μM, -30 min).

3. 2. Detection of cell death associated functional changes by flow cytometry

The terms of various flow cytometric representations (profiles) used in the following paragraphs include sample preparation, the profile of flow cytometric detection, gating strategy and data analysis.

3.2.1. [PI uptake] profile of plasmamembrane damage

The necrotic cells take up the membrane impermeable fluorescent propidium iodide (PI) dye. PI binds to DNA enhancing its fluorescence intensity.

3.2.2. [Annexin V-FITC, PI] profile of phosphatidylserine distribution in the plasma membrane.

The exposure of the phosphatidylserine molecule on the outer side of the plasma membrane is one of the early events of apoptosis This can be detected by Annexin-V-FITC staining.

3. 2. 3. [$DiOC_6(3)$] representation of the mitochondrial trans-membrane potential

Proton gradient builds up trans-membrane potential on the inner membrane of the mitochondria. $DiOC_6(3)$ is a positively charged dye that accumulates inside of the mitochondria (negatively charged side), and the fluorescence relates to electric potential.

3. 2. 4. [AO red] profile for detection the volume of acidic compartments

Acridine orange (AO) is a week alcalaine metachrome dye providing green fluorescence when intercalated into ds-DNA and red fluorescence when amassed in the acidic compartments of the living cell.

3. 2. 5. [Sub-G1] profile for detection of oligonucleosomal DNA fragmentation

Nucleosomal fragmentation of DNA is a major marker of apoptosis. Fragmented DNA particles can be extracted from the ethanol fixed cells. Therefore DNA content of apoptotic cells decreases. DNA content can be detected by a DNA intercalating fluorescence dyes, (ethidium bromide or propidium iodide –PI) in the FL2 fluorescence channel.

Samples were prepared according to Gong et al. Treated cells were centrifuged and the pellets were suspended in 1 mL 70% ethanol (-20 ^{o}C), let the cells fixed on room temperature for 30 min and stored on –20 ^{o}C. Oligonucleosomal DNA fragments were extracted from ethanol-fixed cells in extraction buffer (200 mM phosphate-citrate buffer, pH 7.8) for 15 min, then stained with PI, 5 ug/mL for at least 15 min before measurement. Cells were gated in [FSC, FL2H] diagram for discriminating debris and analyzed on FL2H log scale histogram as percentage of cells in the marked, sub-G1 region.

3. 2. 6. [SSC, DNA content] profile for detection of light scatter change and DNA fragmentation

Cells were fixed, stained and gated according to the [sub-G1] representation. The gated populations were analyzed on the [SSC, FL2H] diagrams marked regions as (SSC^{norm}, DNA^{low}) for apoptotic cells, (SSC^{low}, DNA^{norm}) for necrotic cells and (SSC^{low}, DNA^{low}) for cells with a mixed, atypical phenotype.

3. 3. Cleaved PARP Western Blot analysis

Electrophoresis was performed in a 10 % polyacrylamide gel. Rabbit polyclonal antibody against PARP and chemilumines cence were applied for detection.

3. 4. Protease activity assays

Washed cells were lysed by Triton X-100 (0,2 %) in cathepsin or caspase buffer and protease activities were detected by fluorescence substrates (for caspases: z-DEVD.amc; for cathepsines: z-FR.amc or z-RR.amc).

3. 5. Agarose gel electrophoresis

The samples were lysed in SDS buffer containing proteinase K and RNase and loaded to agarose gel.

3. 6. Light microscopic studies

Cytospin preparations were fixed in methanol and stained with hematoxyllin and eosin.

3. 7. Electron microscopy

Samples were fixed in glutaraldehyde and in OsO_4 , and embedded in resin according to standard methods.

3. 8. Statistics

All experiments were repeated at least twice. Significance was determined by Student's t-probe (two tailed, paired).

4. RESULTS AND DISCUSSIONS

4. 1. Caspase-inhibited, staurosporine-induced leukemia cells become either apoptotic or necrotic. This two form of cell death can be easily distinguished in ethanol-fixed cells by a flow cytometric method.

Results: Most of the myeloid tumor cells exposed to STS for 8 h showed typical apoptotic morphology (condensation of cytoplasm and nuclei) and biochemical changes (DNA fragmentation, PARP cleavage, phospholipid assymetry-, mitochondrial potential- and acidic volume decrease). The caspase activity cleaving along DEVD sequence was fully inhibited in the presence of benzyloxycarbonyl-Val-Ala-Asp-fluorometilketone (z-VAD.fmk, 50 μM) caspase inhibitor. However, cell death was not halted, only delayed.

According to morphological studies (electron and light microscopy) one part of STS+z-VAD.fmk treated cells turned to apoptotic (DNA and cytoplasmic condensation), and another part became necrotic (vacuoles, degraded membranes an organelles). There was no sign of cells with mixed morphology (for instance: condensed nuclei and strongly vacuolized cytoplasm) (Figure 1.A.).

During our experiments we have observed that STS+z-VAD.fmk -induced necrotic cells showed decreased side light scatter (SSC) intensity, measured by flow cytometry. This feature of necrotic cells was preserved even after fixation of the samples in ethanol. Since fixation of samples allowed us to detect apoptosis based on DNA content analysis thus the necrotic (decreased SSC) and apoptotic (decreased DNA content) cells could be distinguished in SSC and DNA content two parameters diagram (Figure 1.B.). It was shown that in the case of STS+zVAD.fmk treatment, the percentage of necrotic cells, detected by SSC decrease method correlated well with the percentage of necrotic cells, measured in non-fixed cells with PI staining (figure 1.C.). Hydrogen peroxide (2 mM) also induced necrosis, and the necrotic cells also have decreased SSC intensity, although less considerably than in STS+z-VAD.fmk-treated samples. Furthermore, secondary necrosis ensuing STS-induced apoptosis had only slight decrease in SSC parameter (Figure 1.D.).

In conclusion, the new flow cytometry method established by our work confirmed that the mode of cell death after STS+z-VAD.fmk treatment diverges (bifucates) into two separate populations: primary necrotic and (primary) apoptotic

Discussion: Heterogene cell death forms can be well analyzed by flow cytometric methods. Flow cytometry can detect different functional changes in cells if the function can be translated to “the language of the light” by appropriate staining methods. Necrotic cells lost their plasma membrane integrity and most of the cytometric methods so far exploited almost exclusively this fact to detect necrotic cells and to discriminate them from apoptotic ones. For this reason 1) the samples might be not fixed, 2) primary necrotic cells could not be separated from secondary necrotic cells.

The cytometric method established by our work allowed not only application of a fixation procedure but also the discrimination of primary and secondary necrotic cell. The primary necrotic cells devoid of small scale DNA fragmentation for a while, while secondary necrotic cells do not. Furthermore, primary necrotic cells lost the SSC intensity, while secondary necrotic cells do not, or only moderately at later time. The light scatter (FSC or SSC) changes related to cytotoxicity of various drugs and effects have been investigated earlier. However, the correlation of DNA content with SSC changes is a new approach in cytometry.

SSC might decrease in primary necrotic cells because of decrease of the heterochromatin that can be the source of wide angle light scatter. Electron microscopy revealed that necrotic cells might lose their cytoplasma partially or fully and this can be also behind the SSC loss. Loss of SSC is a feature not only for STS+z-VAD.fmk-treated cells but H_2O_2- or nigericine ionofore-treated samples as well. Generally, not all kind of necrotic cells lose the SSC intensity: as mentioned earlier, secondary necrotic cells do not. This difference may allow better characterization of necrotic cells. This cytometric method was established by using the U937 and HL-60 cells, but other cell lines (RKO colon and Jurkat lymphoblast) were also tried and SSC loss can be detected after induction of necrosis.

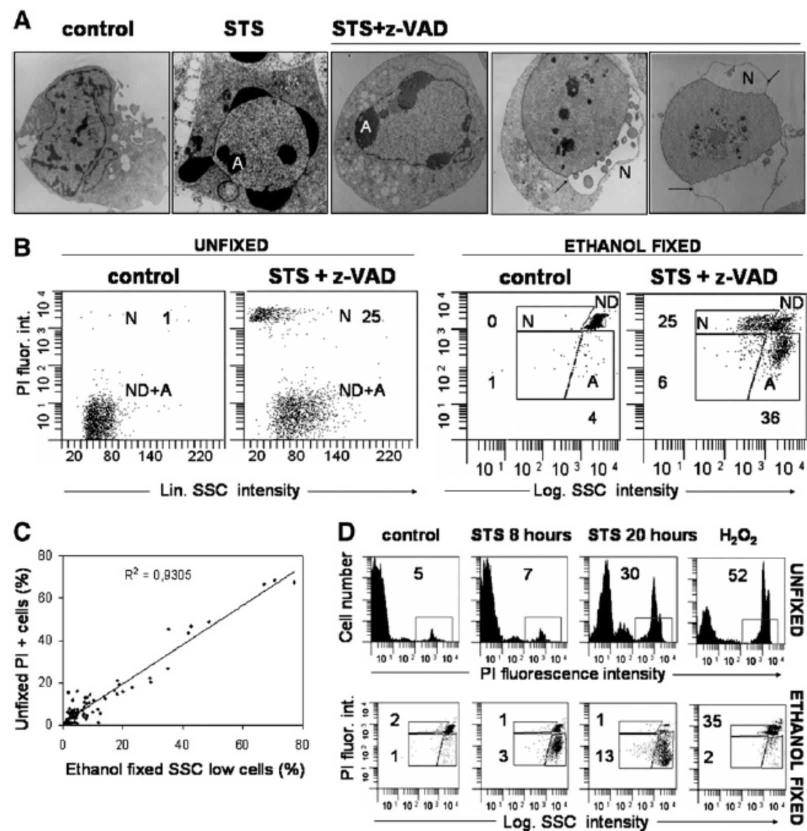

Figure 1: **Staurosporine-induced apoptotic or necrotic phenotypes related to distinct caspase-compromised cells**

(A) U937 cells were exposed to STS (1μM) in the presence or absence of z-VAD.fmk (50μM) for 8 hours. Samples were prepared for ultrastructural investigation. Black arrows indicate unstuck nucleus membrane. A: apoptotic, N: necrotic cell. (B) Cells were exposed to STS (1μM) in the presence of z-VAD.fmk (50μM) for 8 hours and prepared for flow cytometry measurement. PI stained UNFIXED samples were presented in FL2-SSC cytogram (SSC in linear scale). PI stained ETHANOL FIXED cells evaluated in [SSC, DNA content] representation (SSC in logarithmic scale). Inserted values on representative histograms show the percentage of the marked population. A: apoptotic, N: necrotic, ND: not dead cells. (C) Correlation between absolute percentage of PI + cells in unfixed samples and SSC low intensity cells in ethanol fixed samples. Points represent *various* treatments. (D) Cells were exposed to STS (1μM) for 8 or 20 hrs or H_2O_2 (2,5 mM) for 8 hrs and prepared for flow cytometry measurement. Histograms of PI stained UNFIXED samples evaluated in [PI uptake] representation. Histograms of PI stained ETHANOL FIXED cells evaluated in [SSC, DNA content] representation. Inserted values on representative histograms shows the percentage of the marked population

4. 2. In staurosporine-induced caspase-inhibited leukemic cells z-FA-fmk could block apoptosis, while geldanamycin halt necrosis.

Results: Specific, 89 kD fragment of PARP (detected by Western blot) and small scale fragmentation of DNA (detected by agarose gel-elfo) were induced by STS+z-VAD.fmk treatment even in the case of complete caspase inhibition. Both events might be the consequence of protease activation. (In the case of DNA fragmentation: cleavage of ICAD can result in the release of active CAD DNase). Supposing the role of cystein cathepsins, we investigated the effect of z-FA.fmk inhibitor on cell death.

z-FA.fmk significantly decreased the number of apoptotic cells, however significantly increased the necrotic population in STS+z-VAD.fmk treated samples detected by flow cytometry. z-FA.fmk also inhibited the processing of PARP and formation of apoptotic morphology. Concentration dependent inhibition of cathepsin activity (detected by z-FR.amc and z-RR.amc fluorescent substrates in cell lysate) and inhibition of cell death was correlated well indicating that the inhibition of apoptosis succeeded via blocking cathepsin activity.

RIP1 kinase is one of the main regulator of necrotic pathways. RIP-1 is a client protein of Hsp90 heat shock protein, therefore RIP-1 expression can be downregulated by geldanamycin (GA), the pharmacological inhibitor of Hsp90. Inhibition of Hsp90 promotes degradation of RIP1 by proteasome.

Prolong (12 h) pretreatment of cells with GA significantly decreased necrosis, while increased the number of apoptotic cells in samples exposed to STS+z-VAD.fmk detected by flow cytometry in various profiles. GA halted also the formation of the typical morphological changes of necrosis.

We shown that co-treatment with these two agents (z-FA.fmk and GA) significantly decreased both apoptosis and necrosis detected in [sub-G1], [PI uptake] profile ($p<0,00002$). All the flow cytometric parameters of apoptosis (annexin V positivity and sub-G1 cells) and necrosis (PI uptake, SSC decrease) was inhibited by z-FA.fmk+GA co-treatment in samples induced by STS + z-VAD.fmk for 8 h. Co-

treatment successfully preserved the cytoplasmatic and nuclear structure of cells as well as the mitochondrial membrane potential and the volume of endo-lysosomal compartments. On the contrary the volume of the whole cells irreversibly decreased compared to control sample.

Despite the volume loss, the co-treatment (z-FA.fmk+GA) prevented cells from death for prolonged time. These results were supported by morphological analysis (18 hours) and flow cytometric data (42 hours).

z-FA.fmk and GA were ineffective on STS-induced apoptosis and H_2O_2 (2 mM) induced necrosis, indicating the specificity of inhibition.

Discussion: For the time being only few substrates of cystein cathepsins are known in the cytoplasm. BID and PARP are two of them. Bid is cleaved by cathepsins similarly to caspase 3, while the pattern of PARP cleavage is quite different in the two cases. There is no data about cathepsin mediated cleavage of ICAD protein and activation of CAD as a consequence. Although, the inhibition of cathepsin activity is well correlated with the inhibition of apoptosis in the case of z-FA.fmk treatment, it does not exclude that the inhibitor making this effect through sticking to a protein different from cathepsins.

GA blocks the ATP binding site of Hsp90 chaperone. This inhibition resulted in inactivation and degradation of numerous (over 100) client proteins of the Hsp90. However, only RIP1 is known sp far as taking part in the regulation of necrosis among this client proteins.

Based on our results two alternative signal pathway schemes can be proposed in our model. In one of these, the decision between necrotic and apoptotic cell death is regulated by a cystein cathepsin (or its target) with close physical interaction with Hsp90 client protein as a molecular switch (bifurcation scheme). In the other scheme, the necrotic and apoptotic pathway run parallel, independently of each other (parallel scheme). In this case the decision between apoptosis and necrosis is depend on which pathway reached more promptly to the point of no return. Getting to the point of no return may take a few hours, while the forming of cell death

just for minutes (10-30 minutes). For this reason, once the cell get to the point of no return it will die in minutes and there is not enough time for the completion of the other cell death pathway to change the fate of the cell anymore. Although these two schemes are alternatives, in both cases inhibition of both Hsp90 and cathepsin activities together are essential to halt cell death completely in caspase inhibited cells.

At this time it is not known whether this kind of cell death regulation works in other cell types when caspase activity is compromised. According to published data, cell death after STS treatment in caspase inhibited cells is dominantly either necrotic or apoptotic. The sources of this discrepancy are not definite yet. Methodological problems may be one of the sources. Myeloid cells used in our studies get to the point of no return relatively quickly, approximately 8 hours after treatment. In the case of other cell lines (for example: lymphoid cells) getting to the point of no return is slower and more inhomogeneous in time (24-36 hours). For this reason the applied experimental methods may significantly influence the proper judgment of cell death type.

4. 3 CA-074OMe prevented both apoptotic and necrotic cell death forms in staurosporin-induced caspase-compromised cells, but cathepsin B blocking was not enough for this effects.

Results: CA074-OMe cathepsin inhibitor halted both apoptotic and necrotic forms of cell death in caspase-inhibited cell induced by STS+z-VAD.fmk treatment (EC_{50}=5-15 μM). CA07-OMe also prevented mitochondrial depolarization and the volume loss of endo-lysosomal compartments.

We measured the cathepsin activity using fluorescent substrates (cathepsin B, L, H: z-FR.amc; cathepsin B: z-RR.amc) in Triton X-100 detergent lysed cells. We have found that CA-074OMe inhibited cathepsin activity at EC_{50}=0,01-0,04 μM and completely inhibited cathepsin B activity at 0,1 μM concentration. These results suggest that 1) citoplasmatic, bulk cathepsin B is not necessery for cell death induced in caspase-inhibited cells; and 2) blocking cell

death by CA-074OMe is mediated by a target molecule different from cathepsin B.

Discussion: CA-074 inhibits cathepsin B much more effectively than cathepsin L or H *in vitro*, *ex-cell*. As CA-074 has ionic character, it penetrate cell membranes quite bad. Its methylated form as a pro-drug can get into cells more easily, where aspecific estherases cleave the methyl group and form the start-up drug. However, methylation can affect the inhibitory properties of the compound negatively, according to published data. Compared to z-FA.fmk cystein protease inhibitor, which only inhibited apoptosis, CA-074OMe inhibited both forms of cell death in caspase-compromised cells. Furthermore, we showed that the concentration of CA-074OMe needed to inhibit the bulk cathepsin activity was significantly smaller than concentration needed to inhibit cell death. These results let us to conclude that CA-074OMe has an unidentified target molecule, independent of cathepsin B.

In the literature, CA-074OMe was used in cell death studies usually at the range of 10-100 μM. The inhibition of cathepsin B activity was tested only at these concentrations. Our new results call for carefully interpretation of experimental data obtained with CA074-OMe.

The cathepsin B-independent target of CA074-OMe was positioned in the signal pathway upstream of mitochondrial and lysosomal damage, since CA07-OMe inhibited both mitochondrial depolarization and the volume loss of endo-lysosomal compartments.

5 CONCLUSIONS

Our data indicates that either apoptotic or necrotic cell death can occur in STS-treated myeloid cells with delayed kinetics in the presence of caspase inhibitor by 8 hours.

Applying inhibitors of cystein proteases (CA-074OMe és z-FA.fmk) we have shown that necrotic cell death could not be inhibited by these agents at the concentration range where cathepsin activity was totally blocked. This suggested that cathepsins are not direct mediators of necrotic cell death in our experimental system. It is possible that some type of cathepsin play role in the regulation of decision point, where it is decided if the cell dies by necrosis or apoptosis (bifurcation signal hypothesis), but they do not have effector functions. Moreover we cannot exclude the possibilty that the apoptotic and necrotic pathway run parallel and with different speed in each cells. The faster signal can get more quickly to the decesion point and results in one of the special forms of cell death (paralell signal hypothesis). In the last case, cathepsins are not necessary for regulating necrosis. This necrotic process is probably not related to autophagic cell death, in which lysosomal cathepsins may play important role. Necrosis was inhibited by necrostatin-1 (not published), which is an inhibitor of death receptor and RIP-1 mediated necroptotic cell death (necroptosis: „active cell death with necrotic morphology that is mediated by death receptors)

Role of cathepsins in caspase-inhibited apoptosis is also questionable. Although, both cathepsin inhibitors halted apoptosis, we can not exclude the possibility of alternative hypothesis. In the case of CA-074OMe significant difference in the concentration was detected between inhibition of cathepsin activity and apoptotic cell death. However we can not exclude the possibilty that a cathepsin, independent of cathepsin B, mediates this process. In the experiments performed with z-FA.fmk treatment, the inhibition of cathepsin activity was well correlated with the inhibition of apoptosis. However it is also possible that the inhibition of an alternative target molecule rather than cathepsins, results in the speeding up the necrotic signal pathway rather than the inhibition of

apoptotic pathway. Based on published data, caspase-2 is less sensitive to z-VAD.fmk than other caspases. Moreover it was described that caspse-2 activity is inhibited by z-FA.dmk in higher concentrations. It needs further study to clarify whether caspase-2 may be a mediator of caspase-inhibited cell death and is the target molecule of z-FA.fmk in our experimental model.

In conclusion: Although we was not able to establish unambiguously whether cathepsins are the mediators of the cell death in our model system, but we have shown that cell death forms can be switched to each other with pharmacological methods. Apoptotic cell death often silences immune reaction and suppresses inflammation. Quite oppositely, necrotic cell death is accompanied by inflammation. Inflammation may increases cell proliferation locally by the infiltration and activation of macrophages. However, necrosis can promote maturation and activation of dendritic cells, which stimulates the adaptive immunity.

A hypothetical question is whether a specific “cell death cocktail” can be mixed in tumors, which helps to develop optimal immune response against tumors using all advantages of natural and adaptive immune system. It requires and deserves further examinations. In other fields, secondary tissue damage is decreased by turning necrosis to apoptosis to avoid total loss of beta cells and manifestation of diabetes in experimental animal models of pancreatitis.

The flow cytometric technique established by our work, in which SSC parameter indicates necrosis, might be suitable for better characterization of pharmacological agents inducing mixed cell death forms. From this point of view it is to consider that cell death markers (SSC decrease and DNA degradation) corrode postmortem. For this reason our method can be applied only for quantitative analysis by detecting relatively synchronized cell death processes.

6. LIST OF PUBLICATIONS

6. 1. Publications related to the dissertation:

1. Mihalik R, **Imre G**, Petak I, Szende B, Kopper L. Cathepsin B-independent abrogation of cell death by CA-074-OMe upstream of lysosomal breakdown. Cell Death Differ 2004;11(12):1357-60. **IF: 8,2**

2. **Imre G**, Dunai Z, Petak I, Mihalik R. Cystein cathepsin and Hsp90 activities determine the balance between apoptotic and necrotic cell death pathways in caspase-compromised U937 cells. Biochim Biophys Acta 2007; 1773(10):1546-57. **IF: 6, 900**

6. 2. Publications not related to the dissertation:

3. Nagy K, Petak I, **Imre G**, Barna G, Gezane-Csorba M, Sebestyen A, Houghton JA, Mihalik R, Kopper L. Proteasome inhibitors abolish cell death downstream of caspase activation during anti-microtubule drug-induced apoptosis in leukemia cells. Anticancer Res 2005;25(5):3321-6. **IF: 1,6**
4. Felfoldi B, **Imre G**, Igyarto B, Ivan J, Mihalik R, Lacko E, Olah I, Magyar A. In ovo vitelline duct ligation results in transient changes of bursal microenvironments. Immunology 2005;116(2):267-75. **IF: 3,5**
5. Mihalik R, **Imre G**. Kaszpázok, apoptózis, sejtelhalás: (jel)útvesztőben. Orvosképzés 2006, LXXXI. évfolyam, 3. szám: 151-157

6. 3. Lectures, posters:

6. **Imre Gergely**, Dunai Zsuzsanna**:** TPCK és TLCK szerinproteáz inhibitorok nem az Omi/Htra2 proteáz inaktiválásán keresztül gátolják a kaszpázfüggetlen elhalást leukémia sejtekben. Semmelweis Egyetem Doktori Iskola PhD

Tudományos Napok, Budapest 2006. április 13-14. absztrakt szám: E-III/7

7. Z. Dunai, **G. Imre** and R. Mihalik: Staurosporine-induced caspase independent cell death is abrogated by the Omi/Htra2 inhibitor UCF101 by preserving mitochondrial trans-membrane potential. 30th FEBS Congress and 9th IUBMB Conference, Budapest, Hungary 2-7 July 2005, The FEBS Journal, Volume 272 Supplement 1, poster abstract: N5-019P
8. **Imre Gergely**, Dr. Mihalik Rudolf: Apoptózis-nekrózis reosztát kaszpáz gátolt leukémia sejtekben. Semmelweis Egyetem Doktori Iskola PhD Tudományos Napok, Budapest 2005. április 14-15. absztrakt szám: E-V/1
9. **G. Imre**, Z. Dunai and R. Mihlaik: Abrogation of caspase-independent cell death (CICD) by TPCK or TLCK (unlike to UCF101) is not mediated by Omi/Htra2. 13th Euroconference on Apoptosis, Budapest, Hungary October 1-4, 2005, poster abstract: P-91
10. **Gergely Imre**, Rudolf Mihalik. To kill two birds with one stone: simultaneous detection of apoptosis and necrosis in ethanol fixed cells by flow cytomery.(2004) XXII Internationa l Congress of the International Society for Analytical Cytology (ISAC), LeCorum, Montpellier, France 2004. május 22-27. Cytometry, abstract issue, part A, 59A, number 1, abstract number: 95719
11. **Imre Gergely**, Dr. Mihalik Rudolf (2004) Programozott sejtelhalás szabályozása kaszpázgátolt leukémia sejtekben: a katepszinek és a Hsp90 szerepe, PhD. Tudományos Napok, Semmelweis Egyetem, Budapest, 2004. április 8-9., Absztrakt gyűjtemény, 62-63.o.
12. **Imre Gergely**, Dr. Mihalik Rudolf (2004) Két legyet egy csapásra: az apoptózis és a nekrózis egyidejű detektálása etanol fixált sejteken áramlási citometriával. Modern sejtanalitikai módszerek, IV. Magyar Sejtanalitikai Konferencia, Semmelweis Egyetem Budapest, 2004. május 6-8. absztrakt katalógus,150.

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