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Ex vivo measurement of calpain activation in human peripheral blood lymphocytes by detection of immunoreactive products of calpastatin degradation

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Abstract: Limited proteolysis of multiple intracellular proteins by endogenous Ca-dependent cysteine proteases - calpains - is an important regulatory mechanism for cell proliferation, apoptosis etc. Its importance for cellular functions is stressed by existence of endogenous calpain inhibitors - calpastatins. The calpain-calpastatin system within living cells is in a fragile balance, which depends on both partners. The interdependence of calpain - a protease - and calpastatin - an endogenous inhibitor and at the same time a substrate for this enzyme makes any assessment of actual activity of this enzyme in the cells very difficult. In this work we made an attempt to estimate and compare the activity of calpain in human peripheral blood lymphocytes by assessing the levels of limited proteolysis of calpastatin in these cells by western blot, while at the same time the levels of calpain protein inside these cells was measured by flow cytometry. Our results indicate that it is possible to compare (semi-quantitatively) the activities of calpain in peripheral blood $CD4^+$ and $CD19^+$ lymphocytes from various donors that way. Preliminary results showed that calpain activity is increased in the $CD4^+$ T cells isolated from peripheral blood of rheumatoid arthritis patients as compared to control lymphocytes. Extremely high intrinsic activity of calpain was detected in chronic lymphocytic leukemia ($CD19^+$) cells. All this confirms the detection of immunoreactive products of calpastatin as a good maker of endogenous calpain activity.

Key words: Calpain - Calpastatin - Cysteine proteases - Ex vivo - Lymphocytes

Introduction

Calpains belong to a family of cytoplasmic cysteine proteases activated by calcium ions [1-4]. They can be divided into two groups depending on their tissue distribution: the first group is ubiquitously present in all tissues, and the second one is tissue-specific [5]. Among the ubiquitous calpains are the μ-calpain (calpain 1), characteristic for blood cells, and m-calpain (calpain 2), present for example in the striated muscle [6]. These two isoforms are activated by different concentrations of calcium ions - m-calpain requires milimolar Ca^{2+} concentrations for reaching a full activity (0.1 - 1 mM Ca^{2+}), but μ-calpain only micromolar (1 - 100 μM) Ca^{2+} [4,7].

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Calpains have a very broad spectrum of action; so far at least a hundred cellular substrates were described. Among them there are transcription factors (for example c-Jun, c-Fos), transmembrane receptors, enzymes (kinases, phosphatases, caspases), cell cycle proteins (including p53 and cyclin D), cytoskeleton proteins and many others [7,8]. This list of calpains' substrates suggests their involvement in basics cellular functions, including proliferation, apoptosis and the general regulation of genes' expression [2,3,7,8]. In these aspects the calpains are unique, because they not only can cause total degradation of substrates, but - what is more important - they are able to perform limited proteolysis, changing the substrates' activity [7].

Being so actively involved in vital cellular processes, the calpains need a very tight control of their proteolytic activity. It is maintained by two major mechanisms: first, in every cell there is a specific, endogenous inhibitor of calpain - calpastatin; and second, cal-

pain itself is limiting its activity by autolysis [3,9]. Calpastatin is one of the calpain substrates [10,11]; its limited proteolysis by active calpain is considered necessary for its inhibitory activity and thus it is being postulated as an element of balance of the calpain-calpastatin system, preventing uncontrolled activity of the enzyme, and allowing its physiological function [12]. Seventy kilodalton form of calpastatin is the most often detected, native form of this enzyme in human tissues, including the blood cells [13].

We have shown earlier the μ-calpain overexpression in the B-cell chronic lymphocytic leukemia (B-CLL) cells at both mRNA and protein levels and demonstrated its high total activity correlated with low susceptibility to apoptosis [14]. Also, we had demonstrated there that the μ-calpain activity is greatly decreased in the normal T and B lymphocytes obtained from healthy elderly individuals [14]. In that study we used the casein zymography technique, which is detecting the total available calpain activity in the cell lysate (proportional to enzyme concentrations in cells) [15], but tells nothing about the actual activity of the enzyme in the living cells. In fact, every currently available method of assessment of calpain activity (regardless of it being detected by zymography or by cleavage of fluorogenic substrates) allows for estimation of the total available activity of the enzyme. In our opinion, actual calpain activity in the living cells prior to their isolation and lysis should be of even more physiological (and possibly pathological) relevancy, including that related to the functions of immune cells. Thus, in the current paper, we explore the possibility of endogenous (actual) calpain activity measurement in human peripheral blood T lymphocytes, through the estimation of the proportion of native form of calpastatin and its immunoreactive calpain-dependent cleavage products detected by western blot and its relation to calpain protein content in the T cells freshly isolated from peripheral blood measured by flow cytometry. We show that the method is viable by presenting the differences in the abovementioned proportions in the $CD4^+$ T cells isolated from healthy volunteers and patients with rheumatoid arthritis (RA), and in the B-CLL cells.

Materials and methods

Blood donors and sample preparation. Venous blood from five healthy volunteers, nineteen RA patients, and five B-CLL patients was collected into the tubes with EDTA.

Peripheral blood mononuclear cells (PBMC) were isolated by Histopaque™ (Sigma, USA) flotation. Isolated PBMC were washed and suspended in PBS for further studies. For the Western blot determination of calpastatin contents, $CD4^+$ T cells were isolated from peripheral blood of healthy volunteers and RA patients, and $CD19^+$ B cells were isolated from peripheral blood of B-CLL patients.

Intracellular calpain staining. Isolated PBMC (5x105 cells per sample) were incubated with monoclonal anti-CD4 or anti-CD19 antibody conjugated with RPE-Cy5 (DAKO Cytomation) for $CD4^+$ or $CD19^+$ cells identification respectively. Surface-stained cells were fixed with 2% paraformaldehyde for 15 minutes at room temperature. Saponin buffer (0.25% saponin in PBS, Sigma) was used to wash and permeabilize fixed cells. Fixed-permeabilized cells were further resuspended in saponin buffer before adding 20 μl of PE-anti-μ-calpain monoclonal antibody (BD Bioscience-Pharmingen, USA, clone B27D8). After 30 minutes' incubation at +4°C, cells were washed once with saponin buffer and then with PBS and analyzed by flow cytometry. Appropriate fluorochrome-conjugated irrelevant mouse immunoglobulins of corresponding isotypes (DAKO Cytomation and BD Bioscience-Pharmingen, USA) were used as controls. Fluorocytometry analysis was performed with the FACScan system (Becton Dickinson, USA). All fluorocytometric data were analyzed with the WinMDI 2.9 (J. Trotter, The Scripps Institute, La Jolla, CA) software.

Purification of $CD4^+$ and $CD19^+$ cells. $CD4^+$ T lymphocytes were purified from PBMC by immunomagnetic negative selection method, using the $CD4^+$ Negative Selection kit (Invitrogen, Dynal Biotech, USA) according to manufacturer instructions. After separation, the purity of $CD4^+$ T cells was checked by flow cytometry. At least 90% pure $CD4^+$ T cells were used for further molecular studies.

Purification of the $CD19^+$ B-CLL cells was not necessary, because all of the samples from B-CLL patients included into study had exhibited the proportion of $CD19^+CD5^+$ leukemia cells above 90%, as confirmed by flow cytometry.

Detection of native and degraded calpastatin by Western blot. Lysates from $CD4^+$ T cells and $CD19^+$ B-CLL cells were prepared using the isolation kit (Complete Lysis-M, EDTA-free, Roche) and the protease inhibitor cocktail (leucopeptin, aprotinin, iodoacetamid, PMSF - all in concentration of 10 μg/ml); the amounts of total proteins were measured by Bradford method. Purified domain I of human calpastatin (Sigma, USA) was used in lieu of a standard degraded fragments of the protein. Lanes were loaded with the lysates from 1×10^6 cells each, corresponding to the 20 μg protein per lane. Protein resolution was performed by electrophoretic separation in polyacrylamide gel under reducing conditions (SDS-PAGE) according to Laemmli [16] in a Mini Protean II (Bio Rad) apparatus at room temperature, using constant voltage of 190 V for 90 minutes. Electrotransfer of separated proteins from the gel to nitrocellulose membrane (Schleicher & Schuell BioScience, Germany), was performed by semi-dry transfer (TRANS-BLOT SD, BioRad, USA).

The membranes containing separated proteins were blocked with 3% fat-free milk in TRIS-buffered saline (TBS) in order to avoid the non-specific antibody binding. Then, the membranes were incubated overnight at +4°C with monoclonal mouse primary antibody against human calpastatin in concentration 1 mg/ml, titer 1:1000 (Abcam, USA) or with the anti-β-actin antibody in concentration 0.5 mg/ml, titer 1:2000 (Abcam, USA) for assessment of gel loading uniformity. After thorough washing of unbound primary antibodies with multiple changes of TBS, the membrane was incubated with the peroxidase (HRP)-conjugated secondary antibody - polyclonal rabbit anti-mouse Ig (Abcam, USA), at concentration 2 mg/ml and titer 1:2000. Protein-antibody binding was detected by chemiluminescence using the SuperSignal™ kit (Pierce, USA). Detection and recording of specific bands was performed by exposing the membranes to photoradiographic film Medical X-Ray film Foton XS-1 (Fotochemische Werke GmbH). The developed and fixed films were digitally recorded using the GDS-8000 System and dedicated acquisition software Labworks Image Acquisition and Analysis Software Version 4.0 (UVP Bioimaging System, UK). Densitometric analysis was performed using the Scion Image Beta (version 4.0.2) program.

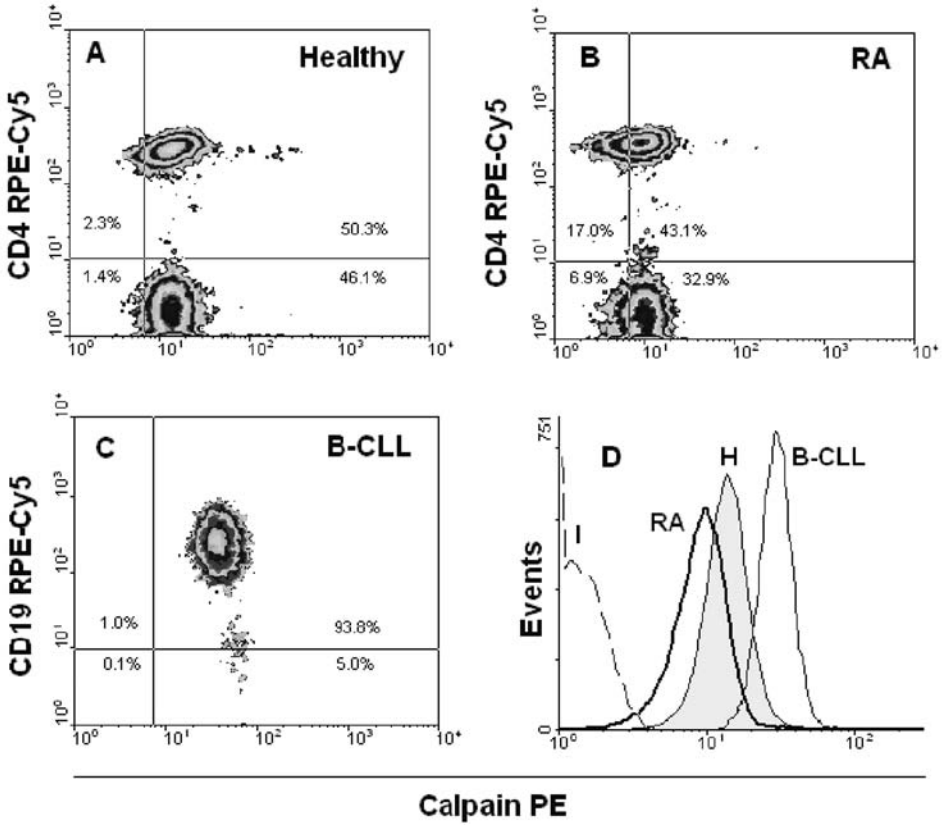

Fig. 1. Flow cytometric detection and comparison of calpain protein contents in peripheral blood lymphocytes. Representative density plots are shown for: $CD4^+$ T cells from a healthy volunteer (Fig. 1A), patient with rheumatoid arthritis (Fig. 1B), and for $CD19^+$ cells of a B-CLL patient (Fig. 1C). Due to low contents of calpain in $CD4^+$ T cells both in healthy volunteers and RA patient - the standard analysis measuring the percentage of positive cells (by putting a quadrant) could not be applied. Therefore, the histogram (Fig. 1D) was used, to show the differences in relative amounts of intracellular calpain in gated $CD4^+$ cells. There, markings at the respective histograms correspond to these used in the density plots; H means the healthy control, an I the isotype control. The histogram shows that all $CD4^+$ T cells from both healthy and RA individual contain intracellular calpain but the amount of calpain is different from that expressed by B-CLL cells.

Ethical issues. All the individuals involved in the study were informed about the purpose of the experiment and gave their consent. The project was accepted by the Local Independent Committee for Ethics in Scientific Research at the Medical University of Gdansk.

Results

Calpain protein amount can be compared by flow cytometry in $CD4^+$ and $CD19^+$ peripheral blood lymphocytes

Flow cytometry allows to detect and compare the intracellular quantity of calpain protein in human peripheral blood lymphocytes. Figure 1 presents representative density plots of the distribution analysis of T cells of healthy volunteer (Fig. 1A) and rheumatoid arthritis (RA) patient (Fig. 1B) as well as of the $CD19^+$ leukemia cells from a B-CLL patient (Fig. 1C), stained for intracellular calpain with combination of surface staining for CD4 or CD19 respectively. The latter served as a positive control of the intracellular calpain staining, based on our earlier observation of very high μ-calpain activity in these cells [14]. The relative amount of calpain protein in the $CD4^+$ T cells from RA patient was lower than in $CD4^+$ T cells from healthy volunteers which, in turn, was a few times lower than that detected in the B-CLL leukemic cells (Fig. 1D).

Calpastatin and/or its immunoreactive cleavage fragments can be detected by western blot in human $CD4^+$ and $CD19^+$ lymphocytes and used for estimation of calpain activity

Western blot analysis of the lysates from immunomagnetically purified $CD4^+$ or $CD19^+$ cells allowed for detection of both the native calpastatin (about 70 kDa) and its immunoreactive fragments (apparent m.w. about 40 kDa) (Fig. 2). Observed quantities and therefore proportions of the native and cleaved forms of calpastatin were different for different cell types or sources. Thus, in the resting $CD4^+$ T cells isolated from healthy people exclusively the native, 70 kDa calpastatin was detected (Fig. 2, lane 1). while in the $CD4^+$ T cells isolated from RA patients usually both forms of calpastatin - native (about 70 kDa) and degraded (about 35-40 kDa) were found (Fig. 2, lane 2). Interestingly, in 3/19 RA patients' cell samples only the immunoreactive calpastatin fragments, and in another 3/19 only the native calpastatin form could be detected. As expected, high level of calpain activity reported earlier in the B-CLL cells resulted in practically total elimination (digestion) of native calpastatin, leaving only its degraded immunoreactive fragments detectable (Fig. 2, lane 3). In the latter case, in order to show that the immunoreactive calpastatin fragments are really the result of calpain-dependent cleavage, we

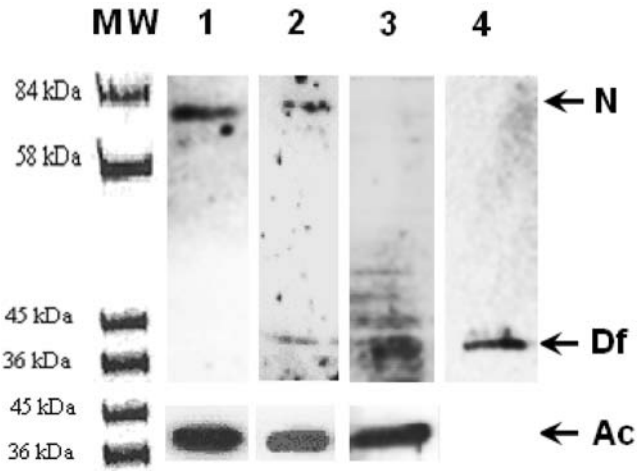

Fig. 2. Estimation of relative calpain activity by western-blot detection of immunoreactive fragments of calpastatin in the cell lysates. Representative results of detection of immunoreactive products of calpastatin cleavage by calpain, as described in Materials and methods. MW - molecular weight marker. Lanes contain the following lysates (at equal protein content): lane 1 - $CD4^+$ T cells from a healthy volunteer (no endogenous calpain activity), lane 2 - $CD4^+$ T cells from a RA patient (low endogenous calpain activity), lane 3 - $CD19^+$ B cells from a B-CLL patient (high endogenous calpain activity) , lane 4 - purified domain I of calpastatin serving as standard, immunoreactive, degraded calpastatin fragment. The native calpastatin (N, present in lanes 1 and 2) has m.w. about 70 kDa, while the immunoreactive degraded fragments of calpastatin (Df, present in lanes 2, 3 and 4) are about 35-40 kDa and have a similar molecular weight to the standard. Ac - the results of western blot detection of actin in the same samples serving as loading uniformity control.

have incubated the B-CLL cells with 2 μM membrane-permeable calpain inhibitor (Calpain Inhibitor IV, Z-LLY-FMK; Calbiochem, UK), for 24 hours at 37°C prior to lysis. Western blot analysis of the B-CLL cells treated that way had shown very faint traces of native calpastatin (not shown).

Densitometric analysis of the western blot data allowed to calculate the rate of degraded to native forms of calpastatin as a relative coefficient of calpain activity in $CD4^+$ T cells isolated from RA patients. We have found that this coefficient was 0.53 ± 0.49 (mean ± SD) for the cells of healthy individuals and 1.19 ± 1.08 for $CD4^+$ lymphocytes of RA patients. The difference was not significant (p=0.134, N=5, Welch's modification of unpaired T test) due to relatively high variability of the results.

Discussion

In the current study we were able to detect the presence of calpain protein in peripheral blood lymphocytes by flow cytometry, in combination with a surface staining of lymphocytes. However, the presence of enzyme does not give any information about its activity which, in the case of calpains, has a broad significance for the functioning of many cellular systems. Previous methods of measurements (casein zymography and cleavage of fluorogenic substrates) allowed to detect the total available calpain activity after separation or inhibition of calpastatin. However, the calpain-calpastatin system works in living cells and thus a method to detect its actual, momentous activity is desirable as a tool for its study. We believe we found such a method, which allows to detect the endogenous activity of calpain in circulating peripheral blood lymphocytes and probably might be applied to other cell types.

The method we propose is similar to one utilized for example to assess the endogenous activity of caspase-3 by detecting and quantifying the degraded fragments of its natural cellular substrate, the PARP [17,18,19].

We based our method on the fact that calpastatin is a natural, ubiquitous intracellular substrate and inhibitor of calpain. By Western-blot technique we were able to show both the native and degraded forms of calpastatin in freshly isolated $CD4^+$ T cells from peripheral blood. $CD4^+$ T cells from RA patients were characterized by presence of both forms of calpastatin, with prevalence of degraded form, and in $CD4^+$ T cells isolated from healthy volunteers the majority (if not all) of calpastatin was in native form. This result suggested a relatively high activity of calpain in the resting $CD4^+$ lymphocytes of RA patients and practically lack of such activity in the cells of healthy individuals. Paradoxically, apparent calpain levels in the $CD4^+$ cells of RA patients was decreased compared to that seen in the lymphocytes of healthy individuals, while the analysis of calpastatin degradation products suggested higher activity of the enzyme in the patients' cells. However, calpain autocleavage ('self-destruction') upon activation had been earlier reported [12]. Taken together, in our opinion low calpain contents plus the presence of degraded calpastatin in the $CD4^+$ cells of RA patients strongly suggests the increased activity of the enzyme in these cells.

Relatively high variability of the results of calculation of the proportion of degraded to native calpastatin as a measurement of actual calpain activity in the tested $CD4^+$ cells of both healthy individuals and RA patients may indicate additional factors (like, for instance, the individual's age and or disease activity) playing a role in the final result. These factors require further study on bigger groups of both healthy and RA individuals.

We used an already known model of high calpain activity - the B-CLL cells, for which the extremely high total available calpain activity was earlier measured by zymography - as a positive verification of our method [14]. In B-CLL lysates only the degraded form

of calpastatin was detected, with no trace of native calpastatin, in accord with the notion of very high activity of the enzyme.

Obtained results are in agreement with the postulated model of calpain-calpastatin balance. Thus, cells that were containing active calpain at the moment of harvest, did also contain calpastatin, in either both forms: native and degraded or at least just its degraded immunoreactive fragments. We observed changes in proportion of native to fragmented calpastatin indicating variable endogenous calpain activity in the lymphocytes isolated from patients and healthy volunteers. It is very likely that in the situation of very high calpain activity (as in B-CLL cells and possibly also a few cases of RA) calpain is responsible for such an extensive degradation of calpastatin, that only little immunoreactive fragments can be found inside the cells.

Summarizing, we describe here a new approach for detection of actual, endogenous calpain activity by measuring the amounts of immunoreactive fragments of calpastatin and relating it to the calpain contents in the cells. We also demonstrate its potential usefulness for the determination of actual calpain activity in various cellular systems, by showing the differences between the contents of immunoreactive fragments of calpastatin in the lymphocytes of healthy, RA and B-CLL individuals.

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