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Ovocystatin affects actin cytoskeleton organization and induces proapoptotic activity*

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Ovocystatin is a chicken egg white protein, generally known for its inhibitory activity against cysteine proteases. However, biological activity of ovocystatin does not seem to be well recognized in respect to other possible cellular effects. Our attention has been focused on ovocystatin cytotoxic effects in relation to its influence on actin cytoskeleton organization and apoptosis induction. *In vitro* studies with human melanoma A375, human cervix HeLa cancer cells and normal human fibroblasts — NHDF were done. Cytotoxic activity of ovocystatin was seen in respect to apoptosis induction — manifested by cell shape changes, phosphatydylserine translocation and actin cytoskeleton reorganization. Normal human fibroblasts have shown lower sensitivity to ovocystatin as compared with human melanoma A375 and human cervix HeLa cancer cells. In conclusion, ovocystatin affects actin cytoskeleton organization and displays proapoptotic activity towards applied cell lines. This implicates its application as a potential anticancer drug. However, its adverse effects on normal cells should be taken into consideration.

Key words: ovocystatin, egg white cystatin, actin cytoskeleton, apoptosis

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INTRODUCTION

Cystatins are proteins that constitute a superfamily of structurally related, endogenous protease inhibitors. The actual classification of protease inhibitors can be found at the Merops database (merops.sanger.ac.uk). In regard to biological activities, they are best characterized as inhibitors of cysteine proteases of the papain family (C 1), such as cathepsins B, H, L and S. These inhibitors participate in intra- and extracellular control of proteolytic events, both in physiological and pathological states. This activity is crucial for processes such as: natural defense against pathogenic exogenous cysteine proteases, antigen processing, bone remodeling, immunomodulation, tumorigenesis and protection against neurodegenerative diseases (Bode *et al.,* 1988; Turk *et al.,* 2008).

In the recent decade new activities of cystatins, not assigned to inhibition of cysteine cathepsins, were also found. These activities are associated with alternative active centers of the inhibitors. For instance, it has been discovered that some cystatins can interact with metalloproteinases, and therefore their role in extracellular matrix stabilization has been proposed. These enzymes are also known to be concentrated in the leading edge of metastatic cells, where they degrade the ECM proteins to facilitate movement of the cells**.** Research in this field has determined that, in addition to TIMPs, inhibitors belonging to cystatin superfamily can take part in processing and stabilization of the matrix metalloproteinases, without affecting their activities towards natural substrates. Accordingly, isolated MMP-2 and MMP-9 undergo rapid autolysis without presence of TIMPs or cystatins. It has been shown that MMP-9 directly interacts with cystatin C, and member of the same superfamily – fetuin, with relatively high affinity (K_d=25 nM and 2.0 μM, respectively) (Vasiljeva *et al.,* 2007; Zavašnik-Bergant *et al.,* 2008). It is also suggested that legumain activity is essential in activation of matrix metalloproteinases including MMP-2. In addition, a novel, second reactive site for proteases of legumain family in the cystatin molecule was found. These enzymes are lysosomal cysteine proteases belonging to the C13 family. Furthermore, identification of the active sites towards legumains links the inhibitors to other groups of proteases such as caspases, clostripain and gingipains, which constitute different classes of cysteine endopeptidases (Smith *et al.,* 2012).

Cystatin C was also identified recently as a novel antagonist of TGF-β signaling due to interaction with TGF-β II receptor. It is known that TGF-β has growth suppressing activity in normal epithelial cells, but it can have progression promoting properties in cancer cells. It is suggested that cystatin C may abolish the growth promoting activity of TGF-β. Such an activity was demonstrated for a cystatin family member AHSG in colon carcinoma. Moreover, cystatin M and fetuin-B, members of the same family, had antitumor activity in lung and skin cancer (Ochieng & Chaudhuri, 2010).

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Abbreviations: BANA, Nα-Benzoyl-DL-arginine β-naphthylamide; ECM, extracellular matrix; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle Medium; MEM, minimal essential medium; MMP-2, matrix metalloproteinase-2; MMP-9, (matrix metalloproteinase-9; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TGF-β, Transforming growth factor beta; TIMP, Tissue inhibitor of metalloproteinase

The potential role of cystatins in regulation of metalloproteinase activities, as well as modulation of TGF-β signaling pathway makes it interesting to investigate their role in the pathophysiology of apoptosis. Influence of cystatin C on apoptotic events has been recently suggested in a study on cystatin C knockout mice subjected to multistage epithelial carcinogenesis (Yu *et al.,* 2010).

Ovocystatin (egg white cystatin, chicken cystatin) is one of the best characterized members of cystatin superfamily and it has been frequently used for pathophysiological studies as the model protein, representative of this superfamily. Its application has been supported by high structural similarity to human cystatin C (66%, based on NMR analysis) as well as several common biological activities. Moreover, as opposed to cystatin C, which is difficult to isolate because of its low concentration in biological fluids — ovocystatin can be readily obtained in substantial amounts from eggs (Abrahamson *et al.,* 2003).

In this study we have investigated influence of ovocystatin on normal and cancer cells proliferation, actin cytoskeleton organization and activation of proapoptotic pathways.

MATERIALS AND METHODS

Isolation and characterization of ovocystatin. White Leghorn hen eggs (*Gallus gallus domesticus*) were purchased from a local farm for chicken cystatin isolation. The inhibitor was prepared by affinity chromatography on S-carboxymethylated papain-Sepharose 4B, according to the method worked out by Anastasi *et al.,* 1983. The process was scaled up for a 500 ml-bed. The yield of the preparation and specific activity of the ovocystatin were calculated on the basis of inhibitory activity against papain and protein concentration in egg white homogenate and the final product. The antipapain activity was assayed using BANA (Nα-Benzoyl-DL-arginine β-naphthylamide) as a substrate (Barrett, 1972; Tombaccini *et al.,* 2001). Protein concentration was determined using extinction coefficient $A_{280nm}^{0.1\%} = 0.87$ (Anastasi *et al.,* 1983). The purified protein was stored in 20% glycerol (v/v) at −80°C (stock solution).

Cell culture. Human melanoma A375 and human cervix cancer HeLa ATCC CRL-1619 cells were obtained as a gift from Laboratory of Medical Biochemistry and Molecular Biology, University of Reims, and human normal fibroblast (NHDF) were available at the Institute of Immunology and Experimental Therapy (Polish Academy of Sciences, Wroclaw, Poland). A375 cells were grown in DMEM; HeLa and NHDF cells in MEM Alpha with fetal bovine serum supplementation at 5% or 10% concentration. Cells were cultured in 25 cm^2 tissue culture flasks (Sarstedt, Germany) at 37°C in 5% CO_2/95% humidified air, and passaged twice a week, using 0.25% trypsin solution with 0.05% EDTA added.

An appropriate amount of ovocystatin was diluted with cell culture medium (without fetal bovine serum addition) to obtain the required protein concentration. Cells were seeded on 6 or 96 well plates. They were grown for 24 h with or without inhibitor addition and further analyzed.

Viability assay. Ovocystatin cytotoxicity was tested by MTT assay (Mosmann, 1973). This assay is a simple colorimetric method to measure cell proliferation and survival. Cells were grown in 96-well plates and treated with 45 μl cell culture medium and 5 μl MTT stock solution (5 mg/ml). Cells were incubated for 4 h at 37°C. Following removal of the medium, 50 μl of dimethyl sulfoxide (DMSO) was added to the cells, to dissolve formazan crystals. Plates were stirred for 10 min and 6.25 μl Sörensen buffer was added. Optical densities (OD) of dissolved formazan were read at 570 nm using a microplate spectrophotometer (μQuant).

Confocal microscopy and actin visualization. Cells were grown on sterile glass coverslips, fixed with 4% paraformaldehyde (PFA) for 20 min, permeabilized with 0.1% Triton X-100 in PBS for 3 min. Next, coverslips were blocked for 30 min with 3% bovine serum albumin in PBS. Phalloidine-rhodamine staining was applied for polymerized, filamentous actin visualization. After incubation with phalloidine-rhodamine (Invitrogen, USA) diluted 1:100 in PBS and washing steps the coverslips were mounted in Cytomatic fluorescent mounting medium (DAKO, Denmark). An Olympus Flow View 500 confocal microscope was used for fluorescence observation. Fields containing about 25 cells were photographed every time in three independent experiments and the representative cells of every line are presented.

Apoptotic markers. Detection of phosphatidylserine on the cell surfaces was performed by FITC-annexin V staining (BD Bioscience, USA). Cystatin-treated and control cells were washed twice with cold phosphate-buffered saline (PBS) and once with annexin binding buffer (AB buffer; 10 mM Hepes-OH, pH 7.4; 140 mM NaCl; 5 mM $CaCl_2$). Cells were then incubated for 15 min with phosphatidylserine labeling solution (Annexin V-Fluos, diluted 1:10 in AB buffer). The nuclear morphology was analyzed by staining with 5 μg/ml Hoechst 33342 or propidium iodide in PBS for 10 min. Cells were then washed with AB buffer and observed directly by fluorescence microscopy.

Staurosporine treatment. Staurosporine, known for its apoptotic effect in a variety of human tumor cell lines (Bertrand *et al.,* 1994), was dissolved in DMSO (5 mg/ml) and 1 μl staurosporine was added to the cell culture medium. Effects on the cells were observed after 90 min incubation. Control observations of the effect of DMSO were made in parallel. At the tested concentration, DMSO itself did not change the cell or nuclei morphology.

RESULTS

Biological activity of cystatin seems to have a much wider spectrum than its ability to control cysteine proteinases (Yu *et al.,* 2010; Majewska *et al.,* 2012). Our main interest in these studies was focused on the ovocystatin cytotoxic effects with special attention to its influence on actin filament organization and ability of apoptosis induction.

Isolation and characterization of ovocystatin

In order to perform the study, substantial amounts of pure ovocystatin were needed. We have successfully scaled up the method, originally described by Anastasi and coworkers (1983), without affecting the yield and quality of the preparation. The protein could be prepared from 50 egg whites with the yield of 70% (calculated as a ratio of total inhibitory activity in the final preparation over the activity in the initial material), giving up to 50 mg of pure inhibitor per one preparation. The obtained inhibitor was judged to be of high purity as determined by SDS-PAGE electrophoresis. Specific antipapain activity of the preparation was 25 U/mg protein. There were no aggregates in the preparation

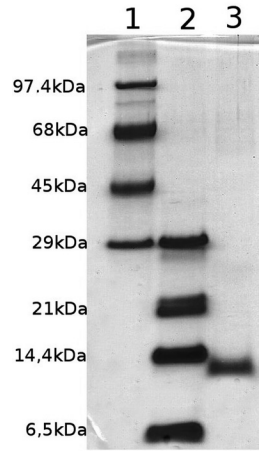


Figure 1. SDS-PAGE of purified ovocystatin preparation.
Molecular weight markers (Lane 1, 2) ovocystatin — 10 μg (Lane 3). The gel was stained with Coomassie Brilliant Blue.

(Fig. 1). The characteristics of the preparation were consistent with the original method.

Cell viability

Viability of human melanoma A375 and human cervix HeLa cancer cells grown in the presence of different ovocystatin concentrations has been shown on Figs. 2A and 2B. The results are compared with those obtained for normal human fibroblasts. (Fig. 2C). Ovocystatin concentration range was chosen based on preliminary experiments, in which the significant effect on cells was observed at 5 μM concentration. For comparison, an effect of 1 μM and 10 μM concentration is presented. Since ovocystatin aggregates at higher concentration with loss of its inhibitory activity (Gołąb *et al.,* 2011) we did not expand the concentration range in our experiments. In the case of HeLa cells a distinct response to the presence of ovocystatin in the culture media could be seen at 5 μM concentration. Increase of concentration to 10 μM did not further decrease the cell viability. The number of living cancer cells grown in the presence of ovocystatin was around 40% lower, as compared with the cells grown in the medium without ovocystatin addition. A comparable effect on A375 cells was seen at 10 μM concentration. Cytotoxic effect of ovocystatin could be clearly seen for cancer cells. However, ovocystatin seems to also affect the growth of normal human fibroblasts but not in statistically important manner.

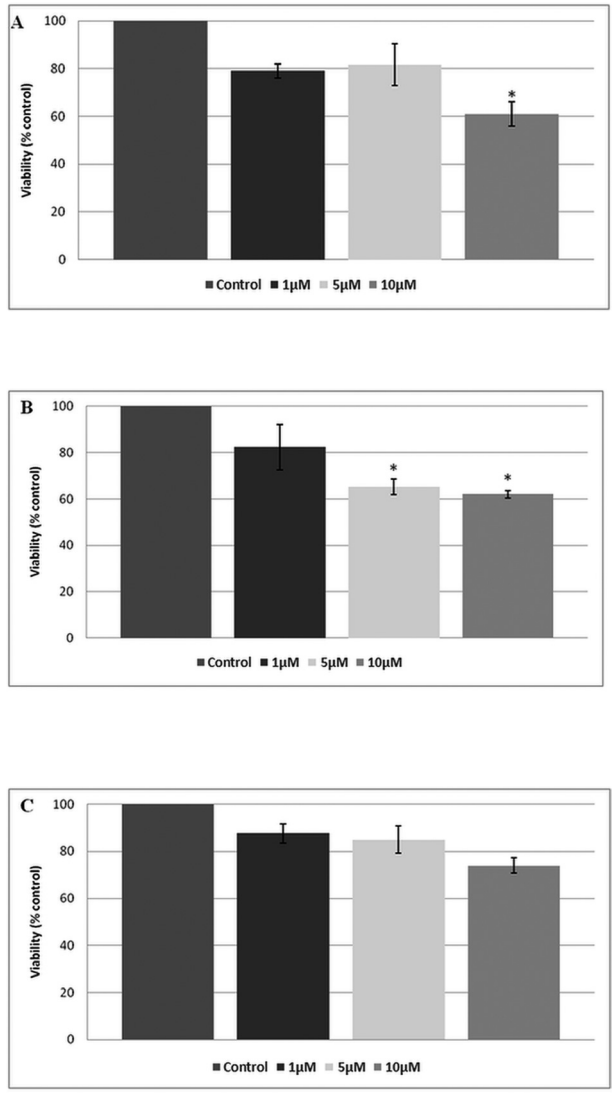


Figure 2. The effect of ovocystatin on cell viability.
Panel A: A375 cells, **panel B**: HeLa cells and **panel C**: NHDF cells. Cells were grown in a medium with or without ovocystatin supplementation at 1 μM, 5 μM and 10 μM final concentration for 24 h. Cell viability was measured with MTT test. Statistical significance of the differences was confirmed using the Student's *t*-test, $p<0.05$;* indicates values significantly different for cells treated with ovocystatin in comparison to non-treated control cells.

Cystatin affects actin cytoskeleton organization

Actin cytoskeleton is responsible for cell shape, migration ability and many other basic life processes such as signal transduction from cell environment to the nucleus, and reverse from the nucleus through cytoplasmic environment to ECM. This structure also undergoes substantial rearrangement in the cells directed to apoptotic pathway. Confocal fluorescence microscopy and phalloidine-rhodamine staining are the best tools for polymerized actin visualization.

Control cells (i.e. grown in a medium without ovocystatin) are characterized by polygonal shape and presence of filamentous actin (red color) throughout the whole cell body with visible condensation under cell membrane and in the cell protrusions (Fig. 3). At 5 μM ovocystatin concentration — visible actin cytoskeleton rearrangement occurs in cancer cells. HeLa cells were rounded and exhibited filamentous actin condensation with stress fibers still seen (yellow arrows). Melanoma A375 cells became elongated and resemble 'spindle shape like structure' with distinct actin condensation at the edges. At 10 μM concentration, the destruction of actin organization and remarkable cell shape change could be seen in HeLa cells. Structural rearrangement occurs in A375 cells, forming 'star shape like structure' with actin concentrated in cellular extensions. Only slight changes were seen in normal NHDF cells. Distinct stress fibers were still seen in the leading edge of cells grown in the presence of 10 μM ovocystatin. To summarize- actin cytoskeleton reorganization induced by ovocystatin was much more pronounced in the cancer cells (A375, HeLa) when compared with normal human fibroblasts (NHDF).

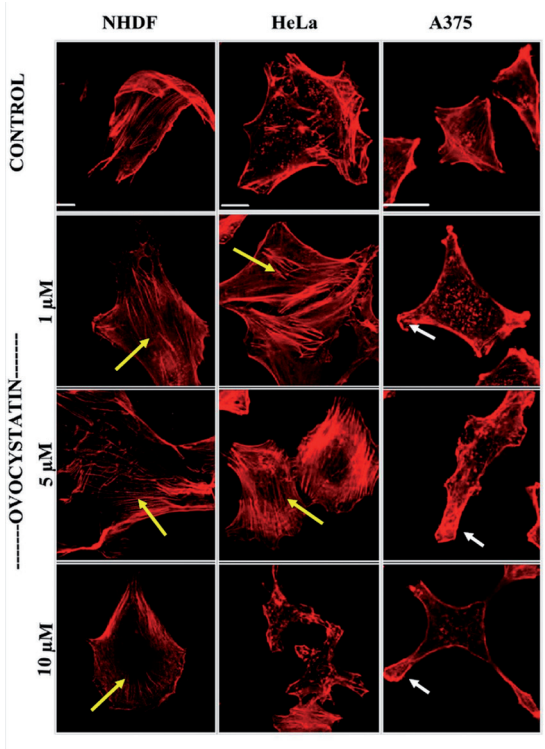
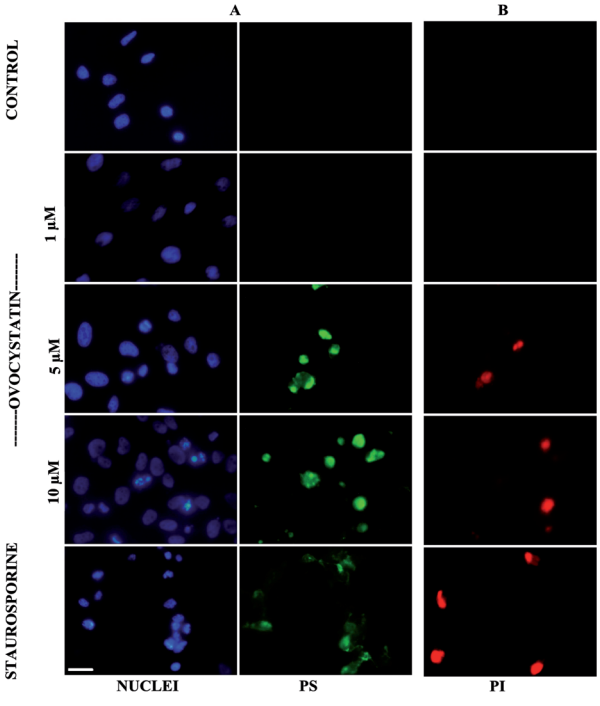
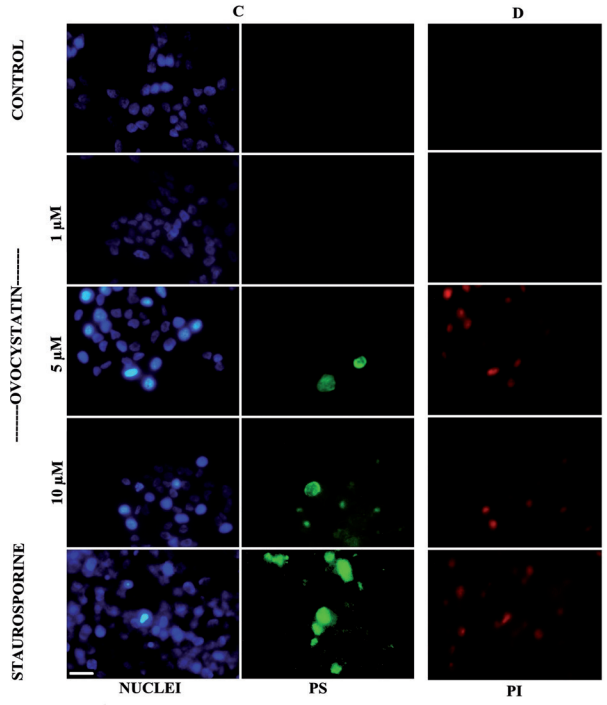


Figure 3. Actin cytoskeleton organization in cells treated with ovocystatin.
Analysis was performed using fluorescent confocal microscopy after staining of filamentous actin with rhodamine-phalloidin. Yellow arrows indicate actin stress fibers. White arrows indicate actin condensation in cellular extensions. Scale bar is 20 μm for all cells.





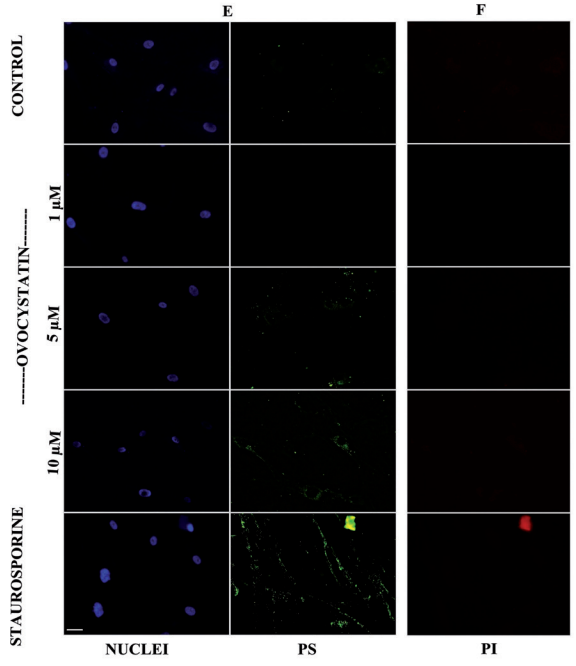


Figure 4. Induction of apoptosis by ovocystatin in HeLa and A375 and NHDF cells.
Detection of externalized phosphatidylserine (PS) by annexin V-fluorescein (**panel A** for HeLa cells, **C** for A375 cells and **panel E** for NHDF cells, right columns). Nuclei were stained with Hoechst 33342 (**panel A, C** and **E**, left columns). Dead cells were identified with propidium iodide (PI) (**panel B** for HeLa cells, **D** for A375 and **F** for NHDF cells). Results were visualized by fluorescence microscopy and compared with those observed in the presence of the known apoptosis inducer — staurosporine (1 μM). Scale bar is 30 μm for all cells.

Apoptotic markers

The decrease in cell viability, accompanied by the changes in their shape and actin filament architecture reorganization, are known symptoms of exposure to cytotoxic compounds in the cell environment. Those may induce apoptosis or even necrosis of the cells, depending on their concentration and time of exposure (Bertrand *et al.,* 1994). To reveal the physiological "status" of cells cultured in the presence of ovocystatin — some apoptotic markers have been investigated. Surface phosphatidylserine, an early apoptotic marker, was visualized with fluorescently labeled annexin V (green fluorescence — Fig. 4). The cells were counterstained using propidium iodide (PI), which enables detection of morphological changes in the nucleus undergoing late apoptosis/necrosis (red fluorescence). In addition, the cells were stained with Hoechst 33342, which enables nuclei morphology and chromatin condensation visualization. Staurosporine, a known inducer of apoptosis, was used in the control experiment. For both cancer cell lines studied (A375 and HeLa), apoptotic cells were clearly seen, beginning with 5 μM concentration of ovocystatin in the culture media. Phosphatidylserine (PS) exposure and nuclei disintegration, as well as chromatin condensation were observed. The effect of 10 μM ovocystatin on apoptosis induction was comparable with that of 1 μM staurosporine. On the other hand, phosphatidylserine exposure in NHDF cells could be observed at higher concentration of ovocystatin.

DISCUSSION

In these studies, cytotoxicity of ovocystatin and its proapoptotic effects were presented. Human melanoma A375, human cervix HeLa cancer cells and human dermal fibroblasts NHDF were treated *in vitro* with three different ovocystatin concentrations. Ovocystatin induces cytotoxic effects in the applied cellular models. Cells growing in the presence of ovocystatin had decreased viability, accompanied by shape changes and signs of apoptosis induction. Apoptotic effects were seen in respect to phosphatydylserine externalization (recognized as an early apoptotic marker), distinct actin cytoskeleton rearrangement and nuclei disintegration.

The molecular mechanism by which ovocystatin can induce apoptosis remains unclear. One way of action could be inhibition of papain-like proteases such as cathepsin B, whose role in cancer progression and metastasis has been well established. However, the role of cathepsin B in the process is rather restricted to extracellular matrix degradation by excreted isoform of the enzyme or involvement in angiogenesis, which obviously cannot be the case under cell culture conditions. From the inhibition of intracellular cathepsin B one could expect an opposite effect, as the enzyme itself takes part in apoptosis induction (Chwieralski *et al.,* 2006). It seems that anti-legumain activity of ovocystatin cannot explain the findings too, as the enzyme is involved in activation of proapoptotic cathepsins. (Liu *et al.,* 2008).

It is tempting to speculate that the proapoptotic action of ovocystatin is mediated *via* interaction with TGF beta II receptor. It has been recently demonstrated that human orthologue of the inhibitor — cystatin C — antagonizes TGF beta signaling by binding to TGF-β type II receptor and abrogating the interaction of TGF-β. This cytokine is known to act as a growth promoting factor for cancer cells (Tian *et al.,* 2009; Arteaga 2006). Thus, ovocystatin could possibly abrogate proliferation of cancer cells in our studies by directing them to the apoptotic pathway. On the other hand TGF-β prevents proliferation of normal cells by inducing apoptosis (Magister & Kos, 2013). This might explain the influence of ovocystatin on viability of NHDF cells in our experiments. Ovocystatin could be considered as a potential anticancer drug; however its activity towards normal cells should be taken into consideration.

The goal of our studies had character of "preliminary recognition" if ovocystatin affects actin organization in normal and cancer cells. We have to remember, however, that these effects are very much dependent on the kind of surface the cells are growing on and many different actin binding proteins (ABP), affecting actin polymerization, and filaments dissociation, and organization (Radwańska *et al.,* 2008). Based on our preliminary experiments, ovocystatin may induce the changes seen on cellular level through other, not yet recognized interactions.

Recent reports (Gole *et al.,* 2012; Wallin *et al.,* 2013; Liang *et al.,* 2011; Wegiel *et al.,* 2009) suggest that we also have to give much more attention to the role of cystatins not only in respect with their ability to cysteine protease inhibition but also to cancer cell migration and invasive abilities. As it is shown in our experiments — ovocystatin may also affect dynamic actin polymerization and reorganization — the key events in cancer cell invasion and migration ability. Studies on recognition of inhibitory effect of ovocystatin on cancer cell migration capability in correlation with actin cytoskeleton organization should be continued with different cancer cell models and application of their normal counterparts.

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