Praca oryginalna *Original paper*

Ovocystatin tetramer – effect on humoral immune response in SRBC-immunized mice and cytotoxic activity*

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Summary

The effect of chicken egg white cystatin, similar to human cystatin C, was investigated in SRBC (sheep red blood cells)-immunized mice. Monomeric cystatin of chicken egg white was isolated and polymerized to tetramer form. Animals received ovocystatin tetramet (OT) 4 times at doses of 2, 0.2 and 0.02 mg/kg, intraperitoneally. SRBC immunization was performed 24 hours after the last dose, or 2 hours before the first dose of the tested compound. The number of antibody forming cells (AFC), total and 2-mercaptoethanol resistant serum agglutination titers were determined. OT has a varied effect on the humoral response of SRBC immunized mice. The most interesting effects were found when the OT was administered upon antigen. The inhibitory effect on AFC and total antibody titre was observed on day 4, and stimulation on total antibody titre and IgG titre on day 7 following OT administration with antigen. Additionally, OT showed no cytotoxic activity in relation to normal and cancer cell lines.

Keywords: cystatin, egg, ovocystatin, humoral immune response, mice

Production of proteins with potential use in the pharmaceutical industry is an important branch of the biotechnology industry in the world.

The hen egg is a natural food which is an important source of numerous nutritional and biological active substances, like food-derived peptides such as ovotransferrin, lysosyme, cystatin, which exhibit a physiological effect in humans (11, 14). These proteins have high potential for industrial, pharmaceutical applications, and once separated may serve as useful ingredients in the formulation of functional foods and nutraceuticals (24).

Chicken egg white cystatin (ovocystatin) is a protein belonging to a large super family of cysteine protease inhibitors, which are present in many tissues and body fluids of humans and animals (5). Human cystatin C amino acid sequence is highly similar to ovocystatin (40).

Cysteine proteases are involved in various biological and pathological processes. It is believed that a principal function of cystatin is protecting cells against uncontrolled activities of their own proteinases leading to unwanted both intracellular and extracellular protein degradation (23). An imbalance between proteases and protease inhibitors is one of the etiological factors of malignant tumors, chronic inflammation, neurodegenerative diseases (42). The control of these protease function by cystatins is of fundamental importance.

In the immune system cystatins interfere with cathepsin activities and antigen presenting cells. Cystatin C is a potent inhibitor of cathepsins S and L (29). Moreover, cystatin C secreted by mononuclear

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phagocytes is involved in modulation of phagocytosis and respiratory burst (2, 7, 20, 45). In addition, cystatin C serum concentration in humans is a suitable marker for glomerular filtration rate (GFR) and kidney function; however, caution is necessary in case of patients with increased activity of cystatin C due to malignancy or other pathologies (9, 17, 19). Numerous studies concerning cystatin C suggest its important role in neurodegenerative diseases and possible therapeutic implications for neuroprotection (35). Decreased levels in cerebrospinal fluid of multiple sclerosis patients suggest its potential utility as a prognostic biomarker (6, 12). In Alzheimer's disease, it may exert neuroprotective effects by inhibiting β-amyloid aggregation and regulating its cathepsin-B mediated degradation (22, 28, 36, 38). Ovocystatin has shown beneficial effects on cognitive deficits in APP/PS1 transgenic mice by modulation β-amyloid and tau proteins expression in hippocampal tissues (32, 34). It may reduce amyloid fibril formation and Aβ42 aggregation and toxicity indicating anti-amyloid potential (33).

Given its high structural and functional similarity to human cystatin C ovocystatin is used in medical model studies and is under investigation as a potential neutraceutical (30, 39, 41).

In our study the effects of the tetrameric form of ovocystatin (ovocystatin tetramer – OT) depending on the dose and timing of administration relative to the antigen stimulation, on the humoral immune response after sheep red blood cells (SRBC) stimulation were investigated. Additionally, *in vitro* cytotoxicity tests were carried out.

Material and methods

Obtaining and characterization of ovocystatin tetramer. Tetrameric form of ovocystatin was obtained by monomer thermall polymerization. Monomeric ovocystatin was isolated from egg white using affinity chromatography on S-carboxymethylated papain-Sepharose 4B, basically as described by Anastasi et al. (1) with slight modifications (21).

The monomer was dialysed against PBS, pH 7.4, brought up to a concentration of 0.5 mg/ml by ultrafiltration using Amicon apparatus on 3 kDa cut-off membranes. Polymerization was achieved by incubation of the solution at 90°C for 1.5 h. The proteins were characterized by gel filtration HPLC using Bio Sec-5 column (Agilent Technologies) (Fig. 1). Preparation of ovocystatin monomer and tetramer was performed as described by Gołąb (13). The purity of ovocystatin preparation determined by HPLC was higher than 95%.

The inhibitory activity of the preparations against papain was measured using Nα-Benzoyl-DL-arginine β-naphthylamide (BANA) as a substrate (3, 37). Protein concentration was determined using extinction coefficient of 0.871 at 280 nm for a 0.1% ovocystatin solution (1). Specific antipapain activity of the purified monomeric ovocystatin was in the range of 20-25 U/mg protein. There was no detectable activity in the tetrameric form of the protein.

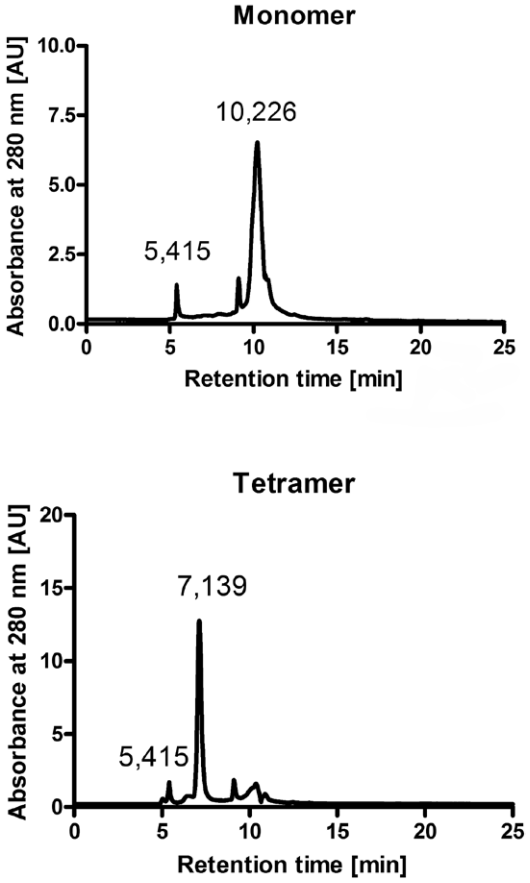

Fig. 1. HPLC elution profile of ovocystatin preparation
Explanations: Twenty microliters of the sample were loaded on the Bio SEC-5 column and eluted with phosphate buffered saline, pH 7.4. Protein in the eluent was measured by UV absorption at 280 nm. The retention time determined for monomeric and tetrameric forms of ovocystatin was 10.2 min and 7.1 min, respectively.

Cell lines and cell culture. For *in vitro* tests two normal and two cancer cell lines were used. Normal lines were represented by murine macrophage cell line J774.E, originally isolated from BALB/cN mice ascites, and D10.G4.1 (murine lymphoblasts) obtained from the collection of the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland. The neoplastic cell lines used in the study were GL-1 (canine B-cell leukemia) and CL-1 (canine T-cell lymphoma) were provided by Yasuhito Fujino and Hajime Tsujimoto from the University of Tokyo, Department of Veterinary Internal Medicine (26, 27).

All the cell lines were cultured in RPMI-1640 medium (Institute of Immunology and Experimental Therapy, Wrocław, Poland). For J774.E, D10.G4.1 and GL-1 cell lines the medium was supplemented with 10% fetal bovine serum (Sigma-Aldrich, Steinheim, Germany), 2 mM glutamine (Sigma-Aldrich, Steinheim, Germany), 100 U/ml of penicillin (Sigma-Aldrich, Steinheim, Germany) and 100 mg/ml of streptomycin (Sigma-Aldrich, Steinheim, Germany). CL-1 cell line medium was supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 U/ml of penicillin and 100 mg/ml of streptomycin.

Cytotoxicity assay. For the experiments, cells from all of the cell lines were seeded in a 96-well plate (NUNC, Denmark) in a concentration of 1×10^5 cells per well.

Investigated compound was diluted in the culture medium at selected concentration range from 1 to 200 µg/ml (1, 2, 5, 10, 20, 50, 100 and 200 µg/ml) and added to the wells. Mitomycin C (Sigma-Aldrich, Steinheim, Germany) in a concentration of 1 µg/ml was used as a positive control for normal cell lines and doxorubicin (Sigma-Aldrich, Steinheim, Germany) in a concentration of 0.5 µg/ml was used as a positive control for cancer cell lines. The cells were incubated for 72 h. After the suitable incubation time, the MTT assay [(3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay] was carried out. 20 µl of MTT (5 mg/ml) [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide] (Sigma-Aldrich, Steinheim, Germany) was added into each well and after another 4 h of incubation 80 µl of lysis buffer was added. The lysis buffer consisted of 225 ml of dimethylformamide – DMF (Sigma-Aldrich, Steinheim, Germany), 67.5 g of sodium dodecyl sulfate – SDS (Sigma-Aldrich, Steinheim, Germany) and 275 ml of distilled water.

The optical density was measured after 24 h using a spectrophotometric microplate reader (ELx800, BioTek, USA) at 570 nm with reference wavelength of 630 nm. The control group was constituted by untreated cells. The IC50 values were obtained as the mean ± SD from three independent experiments conducted in triplicate.

Animals. The studies were conducted on Balb/c mice (8-10 weeks of age; female), each weighing 18-20 g. The mice were obtained from a Breeding Center of Laboratory Animals at the Institute of Occupational Medicine, Łódź, Poland. The animals were kept under the conventional conditions. The mice were fed a commercial granulated food and water *ad libidum*. The study protocol was approved by the Local Ethics Committee (No. 12/2009).

Study design/Experimental protocol. The studies were performed on sheep red blood cells (SRBC)-immunized mice. The SRBC suspension was prepared *ex tempore* in PBS. The mice were immunized intraperitoneally (i.p.) with 0.2 ml of 10% SRBC suspension (4×10^8 cells per mouse), previously obtained in a sterile manner Alsever's solution and it was kept at least three days at 4°C. The immunization was performed 24 hours after the last dose, or 2 hours before the first dose of the ovocystatin tetramer.

Ovocystatin tetramer as lyophilisate was dissolved in sterile phosphate buffered saline (PBS, Institute of Immunology and Experimental Therapy Polish Academy of Sciences, Wrocław, Poland). The test compound was administered intraperitoneally to mice four times at 24 hour intervals at doses of 2, 0.2 and 0.02 mg/kg body weight, in a volume of 0.4 ml. The control groups were treated with PBS intraperitoneally (0.4 ml). Each experimental and control group consisted of 7 mice.

Antibody forming cell (AFC) assay. The animals were anesthetized with isoflurane (Aerrane, Baxter) and then killed by cervical dislocation on day 4 after priming. Following euthanasia, the murine spleens were removed and placed into sterile, ice-cold Hank's saline solution (Institute of Immunology and Experimental Therapy, Wrocław, Poland). Cells were isolated passing the spleen through by a nylon mesh and then centrifugation the cells suspension (2250 g, 15 min, 4°C) on a layer of Ficoll 400 (Pharmacia, Fine Chemicals AB, Uppsala, Sweden)/Urografin 76% (diatrizoate sodium and megluminediatrizoate, Bayer Schering Pharma, Poland) at 1 : 3 ratio, at a density of 1.076. Next, the splenocytes were collected from the interphase and washed twice (375 × g, 8 min, 4°C) with sterile ice-cold Hank's saline. After the second wash, the cells were suspended in Hank's saline at 1×10^6 cells/ml. The viability of cell suspension was 92-98%, estimated with trypan blue dye-exclusion assay. The number of splenocytes producing hemolytic anti-SRBC antibodies (antibody forming-cells, AFC) was determined by a local hemolysis technique in agar gel (25) on day 4 after priming.

Determination of anti-SRBC antibodies in serum. The blood samples were taken from retro-ocular arteries of isoflurane-anesthetized mice. The sera were obtained by blood centrifugation and inactivated at 56°C for 30 min. The total and 2-mercaptoethanol (2-ME) resistant serum hemagglutinin titres were determined by active hemagglutination test carried out on microplates (16). The titre of 2-ME antibody is roughly equivalent to that of IgG in serum, so a greater titre obtained without 2-ME is due to IgM. The results were expressed as a value of $\log_2$. It was found that the serum of non-immunized mice did not contain spontaneous anti-SRBC antibodies. The number of anti-SRBC hemagglutinin titres in SRBC-immunized mice was determined on days 4 and 7 after challenging.

Statistical analysis. The data are presented as mean ± SD. The differences between the groups were assessed using the one-way ANOVA and *post-hoc* analysis using the Duncan test and STATISTICA 10 software (Statsoft Inc., Oklahoma, USA). Differences were considered significant at $p < 0.05$.

Results and discussion

To our knowledge there have been no studies yet describing the impact of ovocystatin on the humoral immune response in mice. In our study, the OT showed no cytotoxic activity in relation to all tested cell lines, which represent different types of immunological cells. Even after incubation with the highest concentration (200 µg/ml), no effect on cell viability was observed (IC 50 > 200 µg/ml).

Administration of OT four times prior to SRBC-immunization did not change the number of AFC. Whereas OT administered four times after priming strongly decreased the number of cells producing hemolytic anti-SRBC antibodies, but only at the highest dose – 2 mg/kg ($p = 0.0060$) (Fig. 2).

OT administered four times prior immunization decreased total antibody titre at all three investigated doses 2, 0.2 and 0.02 mg/kg (p respectively in order $p = 0.0025$, $p = 0.0074$, $p = 0.0012$) on day 4. Moreover, also after priming at all three investigated doses (p respectively in decreasing order $p = 0.0012$, $p = 0.0001$, $p = 0.0001$) a decrease in total antibody titre on day 4 was observed. Furthermore, only an increase in total antibody titre on day 7 was observed at the lowest dose – 0.02 mg/kg ($p = 0.0431$) given after priming. Effects were not dose dependent (Fig. 3).

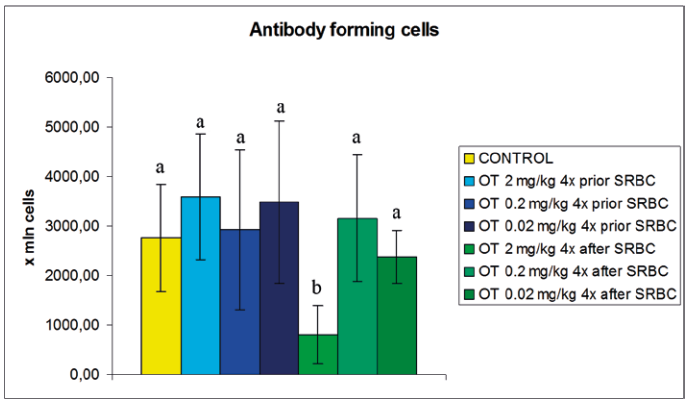

Fig. 2. The number of antibody forming cells (AFC) in SRBC-immunized mice treated with OT – ovocystatin tetramer (2, 0.2, 0.02 mg/kg) four times at 24 h intervals prior and after priming

Explanations: Values are presented by mean ± SD; n = 7 mice for each group tested by ANOVA *post-hoc* Duncan test. Data with different superscript letters show significant differences ($p < 0.05$).

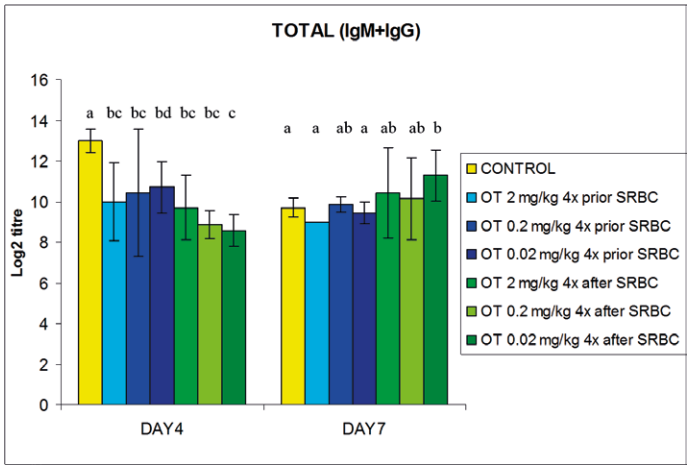

Fig. 3. The number total anti-SRBC antibody titre (IgM + IgG) in mice treated with OT – ovocystatin tetramer (2, 0.2, 0.02 mg/kg) four times at 24 h intervals prior and after priming

Explanations: Values are presented by mean ± SD; n = 7 mice for each group tested by ANOVA *post-hoc* Duncan test. Data with different superscript letters show significant differences ($p < 0.05$).

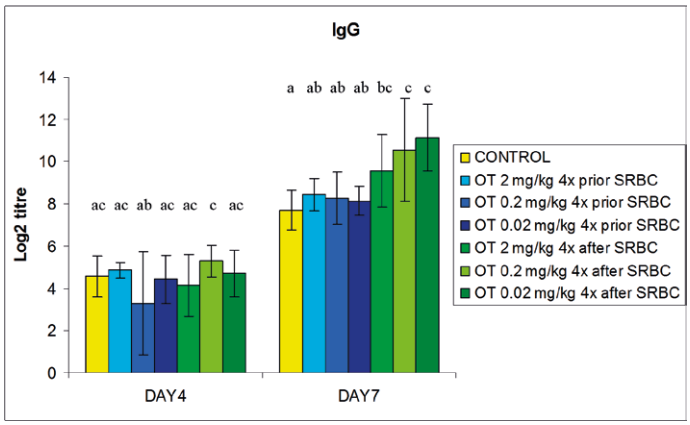

Fig. 4. The number of IgG anti-SRBC antibody titre in mice treated with OT – ovocystatin tetramer (2, 0.2, 0.02 mg/kg) four times at 24 h intervals prior and after priming

Explanations: Values are presented by mean ± SD; n = 7 mice for each group tested by ANOVA *post-hoc* Duncan test. Data with different superscript letters show significant differences ($p < 0.05$).

Administration of OT four times prior to and after SRBC-immunization did not change the number of IgG class antibody titre for determinations made on day 4. However, an increase in IgG class on day 7 for OT administration after priming for all three tested doses 2, 0.2 and 0.02 mg/kg (p respectively in order $p = 0.0375$, $p = 0.0017$, $p = 0.0002$) was noted (Fig. 4).

Our studies demonstrated that ovocystatin tetramer administered parenterally (i.p.) exerted a slightly modulating effect on the number and the activity of the immune cells involved in the humoral response. Inhibitory influence (decrease in AFC and in total antibody titre on day 4) on humoral response is consistent to some extent with the data confirming protective, inhibiting, modulating role in development of chronic inflammatory disorders (4). To date, there is no experimental evidence specifically addressing the effect of ovocystatin on total antibody titres including IgM or IgG levels or activity – its influence on humoral responses remains uncharacterized. Only a limited number of studies have examined the impact of cystatin on cells of the immune system. Cystatin, exhibits a variety of immunomodulatory functions, either by modulating the activity of cysteine proteases or through mechanisms independent of their protease inhibitory activity. These include modulation of cytokine expression, interactions with immune cell receptors, and influence on antigen presentation and immune cell function, as outlined below. For example, cystatin C contributes to MHC class II presentation complex and maturation of dendritic cells (10, 18, 29). Additionally cystatin C, cystatin B and ovocystatin have been implicated in nitricoxide production (NO) by interferon-γ (IFN-γ)-activated murine peritoneal macrophages (MPM), not through inhibition of cysteine protease, but due to increases in the synthesis of inducible NO synthetase (43). Further findings of Verdot et al. (44) showed that ovocystatin stimulates NO production through cytokine induction, specifically by enhancing the release of TNF-α and IL-10 by IFN-γ-activated MPM). Moreover, using chicken cystatin known property to induce the synthesis of NO by IFN-γ-activated MPMs, ovocystatin was used to treat *Leishmaniasis* using the mouse Balb/c model (*Leishmania donovani* infected mice). The treatment resulted in complete recovery and conferred resistance to reinfection (8). In contrast, other studies showed that ovocystatin does not stimulate microglial cells to produce inflammatory mediators – NO and IL-1β (31), indicating that its immunomodulatory effects may be cell-type specific.

The effect of examined OT on the humoral response to SRBC depends on the dose applied, and above all on the point of administration relative to immunization with SRBC. OT exhibited a varied impact on humoral immune response in mice immunized with SRBC. The most interesting results, the biphasic effect, were observed when the OT was administered after immunization. The strongest inhibition effect was found on

day 4, after administration of the test compounds upon antigen in all tested doses. Stimulation was observed on day 7 after administration of the test compounds upon antigen. The results of our research allow us to speculate that the most interesting potential dosages are 2 and 0.2 mg/kg of OT. Our *in vitro* research on normal and cancer immunological cell lines has provided important evidence that OT has no cytotoxic activity into relation to all tested lines. Separation and production of bioactive peptides from chicken egg white proteins are emerging areas with unlimited potential for new applications.

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