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Annual report:

Protein Disulfide Isomerase in ovarian cancer: a novel tumor-associated antigen and a potential target for cancer immunotherapy

Olga Tarasiuk

Tutor: Prof. Claudio Santoro

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Abstract

Ovarian cancer is one of the most dangerous and difficult diagnosed cancers. Epithelial cancer of ovary derives from malignant transformation of the epithelium of the ovarian surface, which is contiguous with the peritoneal mesothelium. The symptoms of ovarian cancer are nonspecific and often suggest the presence of upper abdominal disease. For cancer in the ovary, unlike cancers at many other sites, no anatomical barrier exists to widespread metastasis throughout the peritoneal cavity. Only 20% of the ovarian cancer is diagnosed while they are still limited to the ovaries. Despite a lot of research to develop a screening test for ovarian cancer, no effective test has been found so far. The treatment of ovarian cancer is still limited to expose patient to platinum based chemotherapy and more aggressive cytoreductive surgery.

Analyzing ascites of ovarian cancer patients it has been showed that ascites contain high titer of antibodies against Protein Disulfide Isomerase. Further experiments showed that anti-PDI antibodies purified from ascites of ovarian cancer patient are able to activate Complement Dependent Cytotoxicity and lead to the killing of ovarian cancer cells. It has been seen that patients whom were having higher titer of ant-PDI antibodies were having higher survival rate than the other patients.

Aim of the project is to analyze PDI as a possible target for immune therapy of ovarian cancer. One of the first aims of the current project is to understand how intracellular PDI is secreted to the cell surface and activate immunological response and antibody production. A second aim is to analyze if it is possible to stimulate immune system to produce antibodies against ovarian cancer cells by adding antigen to the cells.

Recent experiments have showed that extra added recombinant PDI protein is able to bind the cell surface of ovarian cancer cells. However not all analyzed ovarian cancer cell lines showed the binding of PDI. This information is important to understand the mechanism of immune response activation against cell surface PDI.

Background

Ovarian cancer

Epithelial ovarian cancer (EOC) is one of the leading mortality cancer among gynecological malignancies cancers. Ovarian cancer is the fifth most common cancer in women worldwide. About 70% of ovarian cancer cases are diagnosed at a late stage and therefore, poorly treatable (1). Each year, almost 22,000 women are diagnosed with ovarian cancer. More than 14,000 women die from it.

Ovarian cancer can be divided in three types, depends on from which cells it develop. The most spread type is epithelial tumor that starts from cells of the outer surface of the ovary. The second type is germ cell tumor that arrives from the cells that produce the eggs. Stromal ovarian cancer starts from structural tissue of the ovary that produces hormones such as estrogen and progesterone. About 85% to 90% of all cases of ovarian cancers are epithelial ovarian cancer, that is also called carcinomas (2).

The patients with ovarian cancer are mostly women in the age of 60 years. It has been seen that genetic predisposition of developing ovarian cancer is higher if a woman has a family history of ovarian or breast cancer. It can be related to the presence of an inherited mutation in one of two genes, known as *BRCA1* and *BRCA2*. One of the factors that increase risk of ovarian cancer is nulliparity. On the contrary use of oral contraceptive, pregnancy and lactation are associated with a reduced risk. It has been suggested that stimulation of the epithelium of the ovarian surface, which occurs in the nulliparous state as a result of uninterrupted ovulation, may predispose the epithelium to malignant transformation (3).

Despite a lot of research to develop a screening test for ovarian cancer, no effective test has been found so far. Nowadays the 2 tests used for ovarian cancer screening are transvaginal ultrasound (TVUS) and the CA-125 blood test. TVUS test makes possible to look at the uterus, fallopian tubes, and ovaries using sound waves. It can detect tumor formation but it can't actually tell if a mass is cancer or benign. As result of screening most of the masses found is not cancer. Other method is detecting of CA-125 protein in the blood as this protein is increasing in many women with ovarian cancer. However this test gives a lot falls-positive and false-negative results and can be used only as a help to guide treatment in women known to have ovarian cancer (2).

Despite progressive development of medical treatment last decades, the main treatment methodology for ovarian cancer remain chemotherapy and surgery (4). Nowadays combination carboplatin and paclitaxel is a standard accepted therapy for advanced ovarian cancer. It’s obviously that new therapeutic approaches have to be developed instantly. One of the promising alternative and rational therapeutic approaches is immunotherapy. Nowadays there are more evidences supporting a protective role of the immune system against cancers and clinical success of immunotherapy using monoclonal antibodies (5).

A characteristic of cancer cells is its ability to undergo extensive proliferation through overproducing growth factors or its receptors. Tumor antigens have been explored in blood cancer patients compare to the blood of healthy people. In the cancer patients these antigens elicit an immune response towards cancer cells. Tumor cells express specific antigens on the cell surface, usually within the MHC molecules. Tumor cells cannot stimulate a T-cell response by naive T-cells partly because they lack necessary costimulatory molecules. However, dendritic cells (a type of APC, found in most parts of the body, in the circulation and on the epidermis as Langerhans cells) can provide the stimulus by attracting tumor antigens to its surface by a variety of mechanisms. The dendritic cell can then present the tumor antigens on their surface, lodged within MHC molecules, in a ready state to activate T-cells. Once the T-cells are activated, they are capable of recognizing and destroying antigen-expressing tumor cell. Antigen uptake receptors on dendritic cells provide efficient imitation of antigen-specific adaptive immunity. The recent recognition of dendritic cells as powerful APC capable of inducing primary T-cell responses in vitro and in vivo in combination with identification of tumor-specific antigens emphasizes the role of dendritic cells in antigen recognition. (6).

Carefully matching individual therapy to patient characteristics and tumor biology is essential for immunological therapy to be successful. It is necessary to search for more effective tumor predictive biomarkers. One of the biggest disadvantages of chemotherapies is that it's not tissue specific and gives low therapeutic effect. Cytotoxic agents preventing cell growth at the level of DNA precursors, damaging DNA template, disrupting the mitotic apparatus and this way affecting a lot normal dividing cells in the organism. In contrary, molecular targeted therapies directed against cancer-associated molecules are more tissue specific and less toxic. To design molecule directed drug is important to understand specific biochemical processes, molecular pathways occurring within the cancer cell, its microenvironment and attempts it to a treatment profile. It is not known if the future immunotherapy will bring crucial treatment solution but an active research to discover future possibilities is highly important (3).

Protein Disulfide Isomerase

Protein Disulfide Isomerase (PDI) is a 57-kDa chaperone protein located in the endoplasmic reticulum. PDI can function as reductase, oxidase, isomerase and maintaining cellular homeostasis by mediating oxidative protein folding, catalyzes disulfide bond breakage, formation, and rearrangement. It has been showed that PDI has also chaperone activity independent of the redox status of active site thiols. As a chaperone PDI makes wrongly folded proteins reach a correctly folded state without enzymatic disulfide shuffling.

It has been known that PDI consist of four domains a, b, b' and a' (Fig.1). Domains a and a' are considered to be active as containing two conserved cysteine residues within the CGHC motif.

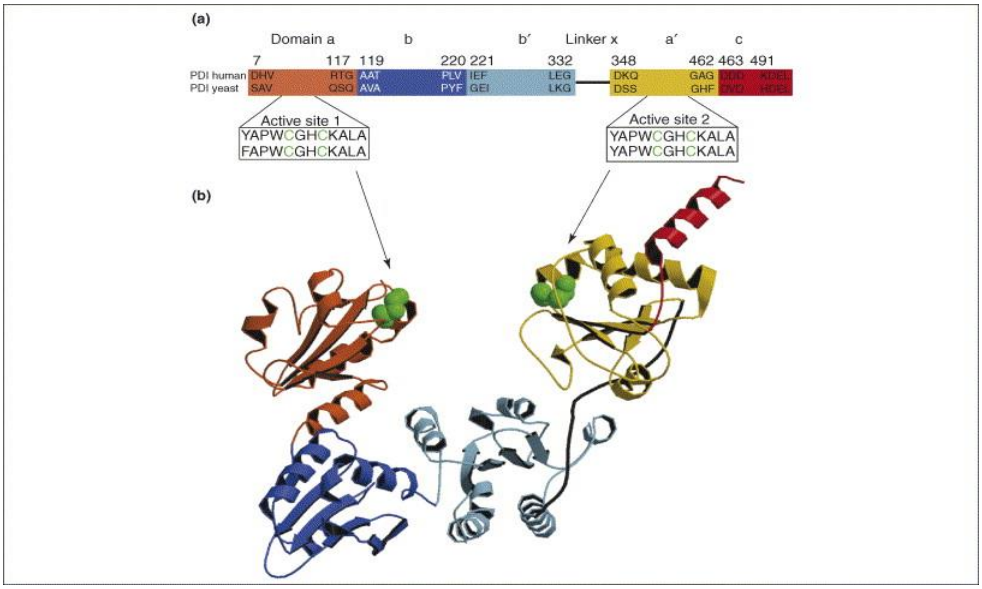


Fig.1. Domain organization of PDI. **(A)** Models of human PDI and yeast PDI. **(B)** Ribbon diagram based on the crystal structure of yeast PDI showing the active-site cysteines in green space-filling representation. Colors of the domains are the same as in (A) (7).

Due to this sequence PDI is able to change between oxidized (disulfide) and reduced (dithiol) states. The active site domains are linked by the b and b' domains. These two domains are considered to be not active as it not having cysteine residues but are playing a role in recruitment of substrates. Between b' and a' domains is located a small interdomain region known as the x-linker. Domain b' has been identified as a chaperone domain. It has been seen that amino acid residues of the b' domain was able to interact with unfolded RNase A, an often used enzyme to assay the chaperone activity of PDI. The b' domain contains a large multivalent hydrophobic surface allowing for a structurally promiscuous binding site (8). Extensive research has assessed the roles of b' domain to be the primary

peptide- or protein-binding domain (9). It has been shown that expression of PDI is increasing in stress conditions, as ER stress is leading to unfolded protein response.

Different research groups showed that PDI is involved in the development of other diseases. It has been shown that internalization of some pathogens is modulated by PDI. It has been hypothesized that PDI plays a role in the reduction of the disulfide bonds present on the parasite, which may help with it internalization. Resent research has described the role of PDI in HIV infections. Both PDI and thioredoxin1(Trx1) have been shown to reduce disulfides on the viralgly coprotein gp120 causing the internalization of HIV-1. PDI on the surface of platelets plays a crucial role in the formation of thrombus in collagen-coated platelets. In monocytic cells, cell surface PDI is required for antithymocyte globulin decryption of tissue factor. Recent studies have shown that free thiols and isomerization are associated with the process of coagulation. Protein aggregation has been shown to occur in a variety of neurodegenerative diseases, such as cerebral ischemia and amyotrophic lateral sclerosis, Alzheimer disease and Parkinson's disease (8). In neurodegenerative diseases nitric oxide (NO)-induced S-nitrosylation of PDI and inhibits its enzymatic activity, leading to the accumulation of polyubiquitinated proteins, and activates the unfolded protein response, which lead to ER stress and apoptosis in neuronal cells (10).

Latest research associated increased PDI expression in a variety of human cancers, including ovarian, prostate and lung cancers as well as lymphoma, glioma, acute myeloid leukemia and melanoma (1).

The endoplasmic reticulum (ER) is the principal intracellular organelle responsible for protein folding, translocation and post-translation modification. Disturbance in the ER environment by biochemical, physiological and pathologic stimuli causes nutrient deprivation, altered glycosylation, calcium depletion, oxidative stress, DNA damage and energy disturbance/ fluctuation, resulting in ER stress with subsequent accumulation of unfolded or misfolded proteins in the ER. It activates unfolded protein response (UPR). Through the UPR process cells seek to maintain appropriate folding processes in the ER and the expression of chaperones is increases (11). Compared to normal tissues, protein disulfide isomerase (PDI) is overexpressed in ovarian tumors (4). It has been shown that inhibition of PDI activity leads to apoptosis in cancer cells (1).

Due to it PDI is being considered to be a promising druggable target. Beside cancer cells grow, inhibition of PDI is leading to inhibition HIV-1 entry into cells. Bacitracin is one of the PDI inhibitors intensively investigated last period. Unfortunately bacitracine is having high level of nephrotoxicity and low membrane permeability that is limiting it use. Therefore, the development of safer and more effective small-molecule PDI inhibitors remains an attractive approach for cancer treatment (1).

Introduction

A current project is the persecution of an ongoing project in our laboratory. It is important to make a small introduction of this work and represent some result for a better understanding of further aims of a current project.

Main goal of the previous work was to identify noble antigen specifically expressed by tumor cells in ascitic fluids of ovarian cancer patients. Ascitic fluids were analyzed in order to understand which proteins, secreted by ovarian cancer cells are able to trigger immune response and antibody production.

Preliminary study was analyzing if ascitic fluid from ovarian cancer patient is having antibodies against ovarian cancer cells. To understand it ovarian cancer cells were incubated by ascites from ovarian cancer patients. Afterwards bounded antibodies from ascites fluid have been visualized. As result it has been seen that antibodies from ascites of ovarian cancer patient are able to bind the cell surface of ovarian cancer cells (Fig. 2A). On the contrary ascites from patients with other diseases showed no staining (Fig. 2B). Further has been performed Cell surface ELISA experiments where ovarian cancer cell were incubated by ascites from ovarian cancer, other cancer or non-cancerous ascites. This experiment showed that ovarian cancer ascites giving significantly higher binding of antibodies than other cancer ascites or non-cancerous ascites (Fig. 2D). Further performing of CDC assay showed that antibodies from ovarian cancer ascites are able to activate Complement Dependent Cytotoxicity and lead to ovarian cancer cells killing. This result was significantly higher than result of antibodies from non-cancerous ascites (Fig. 2E).

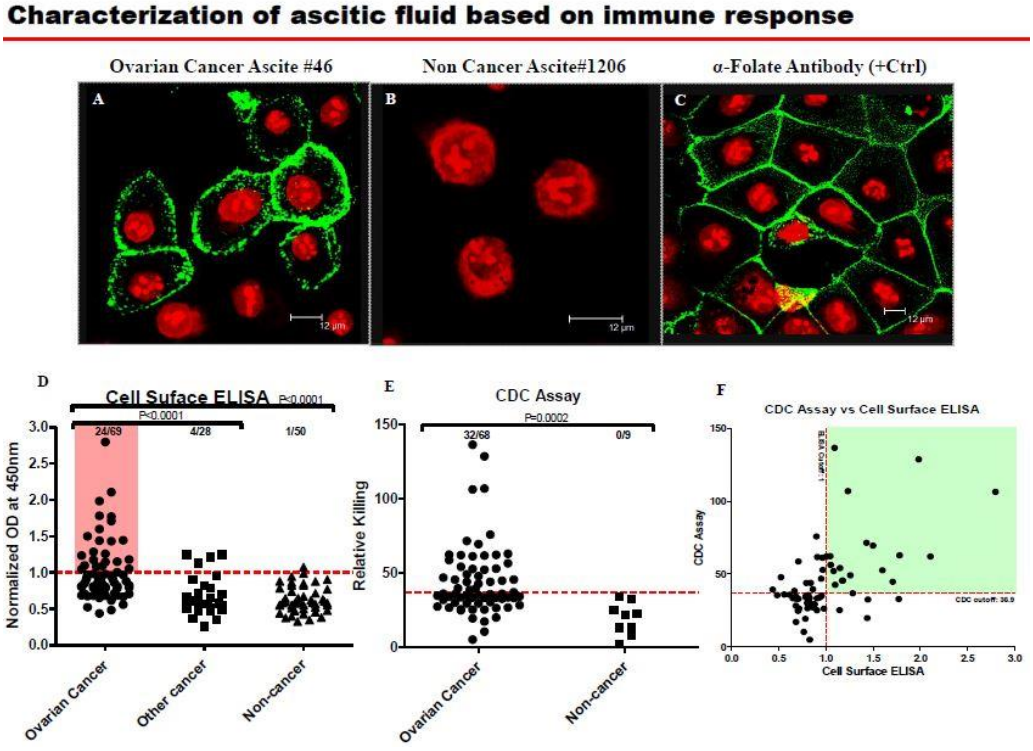
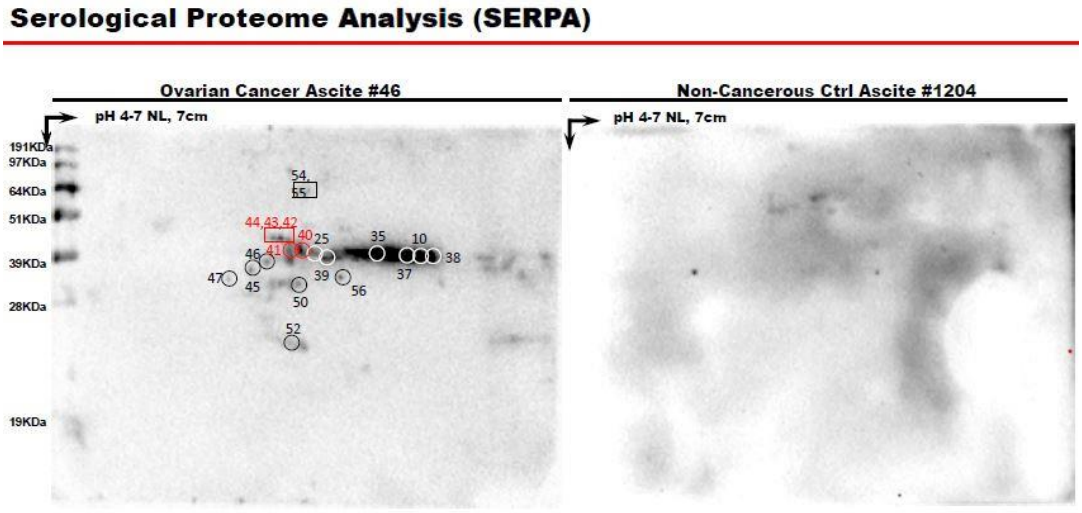


Fig.2. Characterization of ascitic fluid based on immune response. **(A)** Immunofluorescent staining of cell surface of ovarian cancer cells previously incubated by ovarian cancer ascites of patient #46. **(B)** Immunofluorescent staining of cell surface of ovarian cancer cells previously incubated by non-cancer ascites of patient #1206. **(C)** α-Folate staining of ovarian cancer cell surface. **(D)** Cell Surface Elisa. **(E)** Complement Dependent Cytotoxicity Assay. **(F)** Merged results of Cell Surface Elisa and Complement Dependent Cytotoxicity Assay experiments.

In the further steps have been performed Serological Proteome Analysis (SERPA). This approach is combining classical 2-DE SDS-PAGE with Western blotting. Patient's ascites were screened for present antibodies against cancer specific antigens. Using MALDI–TOF MS has been identified

nature and abundance of the total proteins in the analyzed sample. Fresh OVCAR3 total cell lysate pellet has been run on 2D acrylamide gel. Afterwards gel was incubated by ascites of ovarian cancer patient. One of the identified proteins that showed abundant antibody binding result was PDIA1 (Fig. 3).



Spot	Hugo Name	Accession number	Name	MW (Da)	pI	Matched peptides/25	Coverage	Mascot score
40	PDIA1	P07237	Protein disulfide-isomerase	57480	4.76	8	16%	66
41	PDIA1	P07237	Protein disulfide-isomerase	57480	4.76	9	18%	82
42	PDIA1	P07237	Protein disulfide-isomerase	57480	4.76	9	20%	84
43	PDIA1	P07237	Protein disulfide-isomerase	57480	4.76	7	16%	61
44	PDIA1	P07237	Protein disulfide-isomerase	57480	4.76	7	14%	67

Fig.3. Serological Proteome Analysis

To investigate more about PDI has been performed ELISA experiments. ELISA plates have been covered by recombinant PDI and incubated by affinity purified antibodies from ascites of ovarian cancer patient, other cancer ascites and non-cancerous ascites. Antibodies from ovarian cancer ascites showed significantly higher result compare to other cancer ascites and non-cancerous ascites (Fig.4). Analyzing overall survival of ovarian cancer patients it has been concluded that patients whom were having higher response to PDI in ELISA experiment were having higher survival rate than the other patients (Fig. 4).

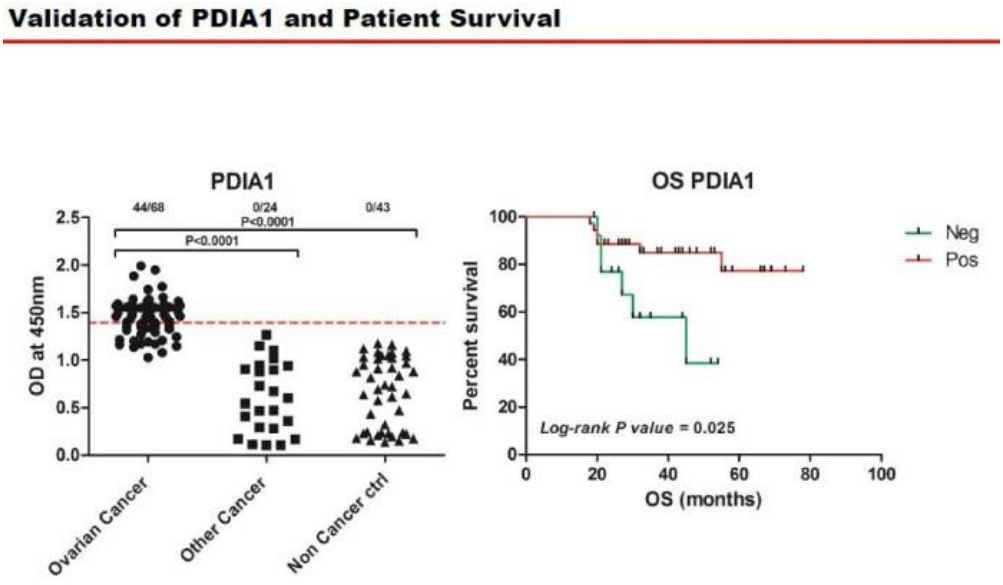


Fig. 4. ELISA experiment of antibody binding to PDIA1 and Overall patient Survival analysis.

Further experiments have showed that anti-PDI antibodies from ovarian cancer ascites are able to activate Complement Dependent Cytotoxicity. In this approach has been tested commercial anti-PDI antibodies (SIGMA) and affinity purified antibodies from ascites. Anti-PDI antibodies, blocked by added PDI protein has been tested as negative control (Fig. 5).

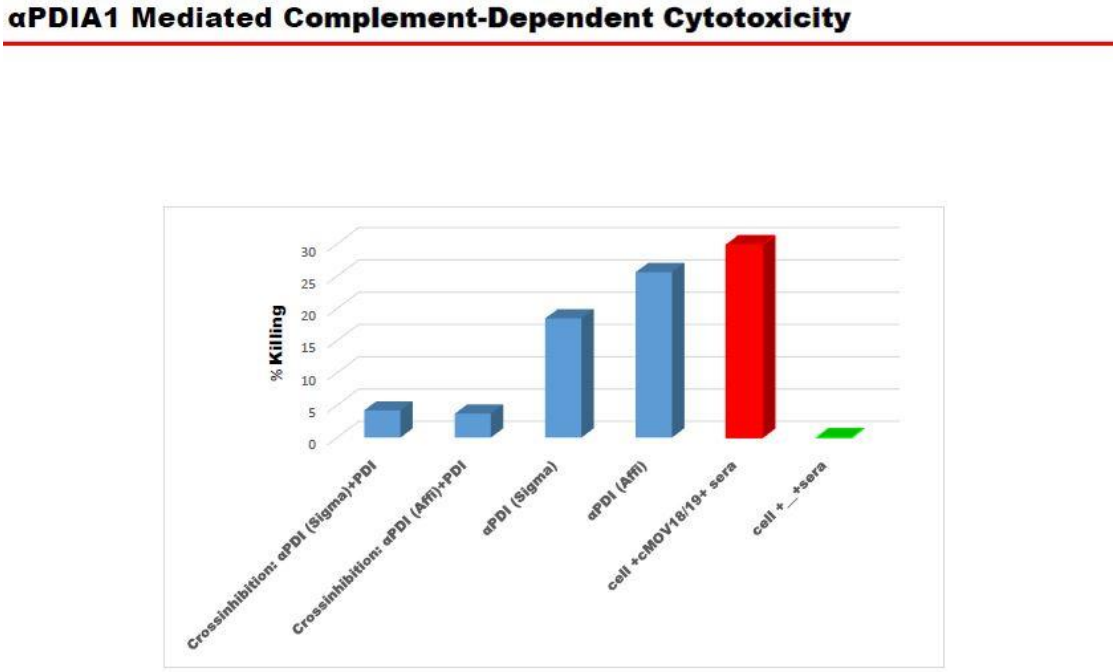


Fig.5. aPDIA1 mediated Complement Dependent Cytotoxicity.

Aim of a current project and hypothesis

Analyzing result from previous research PDI imply to be a promising biomarker founded in ascites of ovarian cancer patients that is able to activate immune system and lead to killing of ovarian cancer cells. Controversially to this finding, it has been known that PDI is an intracellular protein and can be found in endoplasmic reticulum. Accordingly has raised a question, how PDI is able to go to extracellular matrix and activate immune system response? There must be a mechanism that is bringing PDI to the cell surface for a certain purpose. Further investigation of literature showed that PDI can be secreted by the cells. It has been known that ER chaperones and oxidoreductases are able to enter retrograde trafficking whenever they are pulled outside of the ER by their substrates. ER oxidoreductases and chaperones are equipped with a C-terminal KDEL motif that serves to interact with the KDEL receptor, a retrieval receptor that reestablishes ER localization for proteins with such a motif. In a cancer setting, proteins of this group can become localized to the plasma membrane or even secreted (12). Further literature studies confirmed that PDI can be found extracellular in the cancer derived exosomes. When PDI is secreted in the extracellular space it is staying on the cell surface due to electrostatic interactions and can be easily released (13), (14) , (15).

Aim of the current project is to analyze PDI as a possible target for immune therapy of ovarian cancer. It is important to understand the possibilities to stimulate immune system to react on ovarian cancer cells and kill them. As it has been seen that PDI is able to trigger immune activating, we hypothesizing that PDI extra added in extracellular space will go to ovarian cancer cells, stay on the cell surface due to electrostatic interactions and as result stronger triggering of immune system response.

As the second aim we would like to understand if smaller fragments of PDI protein can trigger immune system activation the same way as full PDI protein. As it has been known that PDI protein contain four domains, two of which are considered to be active domains, it has been hypothesized that these two active domains, namely domain a and a' must be able to bind to cell surface and trigger immune activation. If the hypothesis is right and at least one of the domains is able to trigger immune response and lead to the killing of ovarian cancer cells, it can give a future step in studding domains and it future application as possible peptide vaccine.

Peptide vaccines are made from one or more short or long amino acid sequences as tumor antigens, combined with a vaccine adjuvant. They belong to the category of defined-antigen vaccines along with vaccines using protein, protein subunits, DNA, or RNA and is one of the most immunogenic approaches. These peptides are becoming recognized by MHC-II molecules (13-18 amino acids) and further represented to by T-helper (CD4+) cells. Most peptide vaccines have been designed to activate the CD8+ cytotoxic T cell arm of the host immune system. T cell response to the vaccine can be measured in the blood or on draining lymph nodes. T cell responses to vaccines may be durable for months or years (16).

To analyze hypothesizes have been applied methods as immunofluorescent staining of the cell surface of ovarian cancer cells and Western blotting technique. Western blotting is a straightforward technic that can also give us information about binding of recombinant protein on the cell surface. A disadvantage of Western blotting is that it can't represent if PDI remain on the cell surface or is taken inside the cells. Western blotting gives general information about translocation of added protein. Immunofluorescent staining is more sensitive and informative technique as it gives detailed picture of localization of added protein.

Methods

Cell lines

The human ovarian adenocarcinoma cell line OVCAR3, SKOV3, OAW42, A2780 were grown in RPMI 1640 and DMEM (for OAW42) media containing 10% fetal calf serum, 2 mM glutamine, and 1% penicillin/streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing CO_2.

Preparing of recombinant proteins

Each domain, namely a, b, b'and a' has been amplified by PCR using the specific primers. Amplified sequences were restricted by enzymes EcoRI (New England Biolabs) and BamHI(New England Biolabs) and subcloned into an expression vector pTrc B (Invitrogen, #V360-20). Plasmid has been transformed into DH5α (Gibco BRL), cells and plated to grow overnight. Single colonies have been picked up from plates and have been sequenced to detect possible mutation occurred during the cloning. Afterwards vectors have been purified using the Perfectprep Plasmid kit (Eppendorf) and transformed into BL21 (CodonPlus(DE3)-RIPL) cells that are more suitable for producing recombinant protein. Purification of recombinant proteins was done using Ni-NTA magnetic agarose beads. The production and purification of recombinant proteins were analyzed by SDS-PAGE.

Western Blotting for total cell lysate

Western blot was performed to confirm the presence of tumor specific antigens present in analyzed cell lines. OVCAR3, SKOV3, OAW42, A2780 cell extract was prepared under denaturing condition using 8M Urea. Total cells proteins have precipitated in 80% acetone at -20C° overnight. Precipitated proteins were dissolved by 2x Sample buffer. Samples have been loaded on SDS-PAGE gel and transferred on cellulose membrane. Membrane has been blocked by 4% milk diluted in PBST 0,1%. Later membrane was incubated by primary anti PDI antibodies (SIGMA, #MFCD01094568) in concertation 1:1000 diluted in 2%BSA in PBST 0,1% for 1 hour at room temperature. Afterwards membrane has been washed three times by PBST 0,1% and incubated by secondary antibody labeled HRP in concentration 1:5000 diluted in 2% BSA in PBST 0,1%. Membrane has been validated by Versadoc.

Western Blotting for identification of recombinant protein

Cells have been grown on the 96 well plate till it become confluent. Cell culture medium has been changed by fresh one. 1µg of PDI, domains a, b, b' and a' has been added in the medium to each well separately and cells have been incubated for 1,5h at 4C°. Later culture medium has been removed and cells have been washed by serum free culture medium. Afterwards cells have been incubated for 10 min in 1mM H_2O_2 diluted in serum free medium. Cells have been lysate by adding 100µl of 5x Sample buffer. Samples have been loaded on SDS-PAGE gel and transferred on cellulose membrane. Membrane has been blocked by 4% milk diluted in PBST 0,1%. Later membrane was incubated by primary anti 6x-His antibodies in concertation 1:3000 diluted in 2%BSA in PBST 0,1% for 1 hour at room temperature. Afterwards membrane has been washed three times by PBST 0,1% and incubated by secondary antibody labeled HRP (anti mouse) in concentration 1:5000 diluted in 2% BSA in PBST 0,1%. Membrane has been validated by Versadoc.

Immunofluorescent assay for cell surface staining

Cells have been grown on the cover slips in the 96 well plate till it become confluent. Cell culture medium has been changed by fresh one. 1µg of PDI, domains a, b, b'and a' has been added in the medium to each well separately and cells have been incubated for 1,5h at 4C°. Later culture medium has been removed and cells have been washed by serum free culture medium. Afterwards cells have been incubated for 10 min in 1mM H_2O_2 diluted in serum free medium. Further cells have been

washed three times by the blocking buffer (5%BSA in serum free culture medium). Further cells have been incubated at 4C° by primary anti 6x-His antibody in concentration 1:2000 and Cholera Toxin Subunit B (Molecular probes #34775) conjugate by Alexa Fluor 647 for cell membrane staining in concertation 1:1000 diluted in blocking buffer. After incubation with primary anti 6x-His antibodies cells have been washed three times by blocking buffer and incubated with secondary antibodies (anti mouse- cy3) in concentration 1:200. Afterwards cells have been fixed incubating for 15 min in 4%PFA+ 4% Sucrosa. Cells were washed thrice with PBS and once with dH_2O, mounted to a glass slide with a small drop of mountant and the cells on cover slip facing the mountant and sealed with nail polish. The cells were directly analyzed with a Leica TCS-SP confocal laser scanning microscope using sequential scan tool. Laser power and photomultiplier settings were kept identical for all the samples to be able to compare the results.

Immunofluorescent assay for intracellular staining

Cells have been grown on the cover slips in the 96 well plate till it become confluent. The confluent cells on the cover slips were washed twice with pre warmed phosphate-buffered saline (PBS). The cells were fixed with 200µl of 4% PFA + 4% Sucrose on ice for 15 min, and permeabilized the cells for 2-3min 0.2% triton in PBS. The fixed and permeabilized cells were washed once with culture medium and 1:10 primary antibody diluted in blocking buffer (5% BSA in serum free culture medium) was added. After 2hr incubation at 37C°, the unbound antibodies were removed by washing 3 times with blocking buffer. The cells transferred to new well and were subsequently incubated for another 1hr at 37C° with secondary antibody (anti hIgG- cy5) diluted 1:200 in blocking buffer. Performed 2 washes, transferred them to new wells in which the cells were incubated with 1:2000 dilution of propidium iodide (PI: nuclear stain) in PBS. Cells were washed thrice with PBS and once with dH_2O, mounted to a glass slide with a small drop of mountant and the cells on cover slip facing the mountant and sealed with nail polish. The cells were directly analyzed with a Leica TCS-SP confocal laser scanning microscope using sequential scan tool. Laser power and photomultiplier settings were kept identical for all the samples to be able to compare the results.

Results

Immunofluorescent intracellular PDI staining

Firstly PDI present in the ovarian cancer cells have been represented by immunofluorescent intracellular staining of the cells. It has been showed that all four cell line express PDI abundantly (Fig. 6).

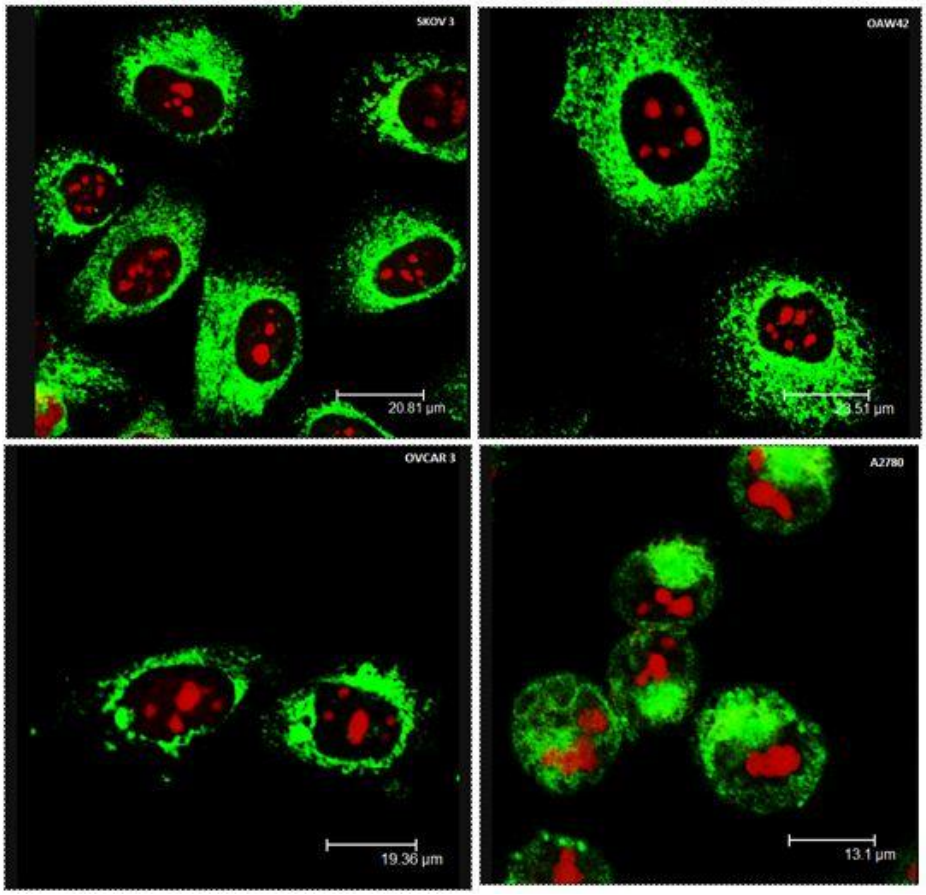


Fig.6. Immunofluorescent assay for intracellular PDI staining in OVCAR3, SKOV3, OAW42, A2780 ovarian cancer cell lines. Green color represents PDI staining. Red color represents PI staining of nucleoli.

Western blotting for total cell lysate

Total PDI in ovarian cancer cell line has been represented by Wester blotting technique. From Fig.7 it can be concluded that all four analyzed cell lines are expressing PDI protein. Fig. 8. represents expression of PDI in the cell line in the colons chart. Expression of PDI in SKOV3, A2780 and OAW42 has been estimated compare to expression in OVCAR3 cell line. Expression of PDI in A2780 cells is slightly lower and in OAW42 is higher compare to OVCAR3. Incubation of the membrane with anti β-actin antibodies represents a control for equal loading of the cell lysate.

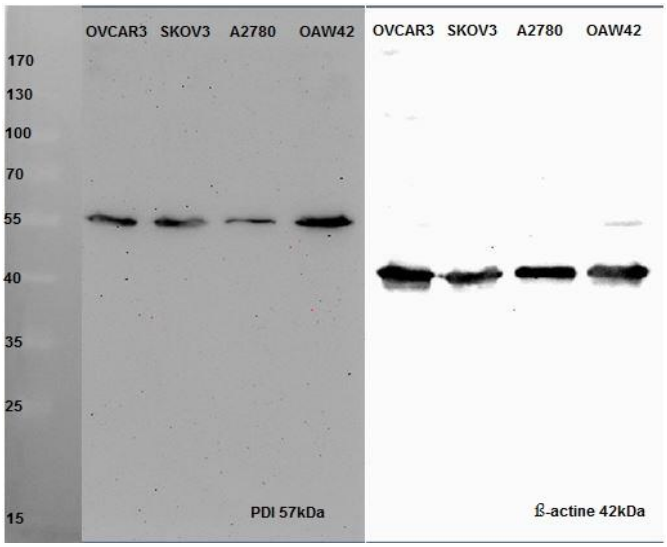


Fig.7. Western blotting for total cell lysate of ovarian cancer cells.

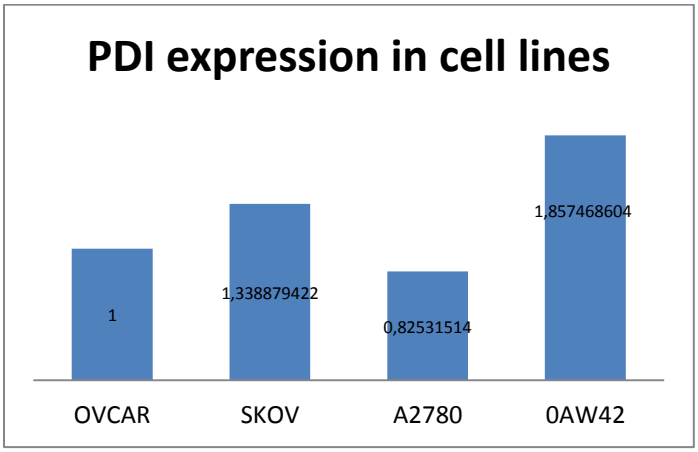


Fig.8. Graphic representation of PDI expression calculated from the bend intensity of the Western blotting membrane. Expression of OVCAR3 cell line was estimated as 1.

Immunofluorescent staining of cell surface PDI

Immunofluorescent staining of PDI on the cell surface of ovarian cancer cells didn't show abundant staining (is not represented). It can be explained due to the facts that PDI is secreted mostly during stress conditions and because PDI is remaining on the cell surface by electrostatic interaction with other proteins, and is getting easily lost during washing steps of immunofluorescent staining (15).

Recombinant PDI protein but also each of recombinant domains were added to the cells. To remain recombinant protein on the cell surface and not to get lost during the staining steps cells were incubated shortly with low concentration of H_2O_2, which make sulfur bridges between PDI protein that was attached to membrane and cell membrane. As result it has been see abundant binding of PDI protein to the cell surface in ovarian cancer cell lines OVCAR3 (Fig. 9) and A2780 (Fig. 11). Cell line SKOV3 (Fig. 13) showed binding of PDI on the cell surface , however this binding was less strong than the binding of OVCAR3 cell line.

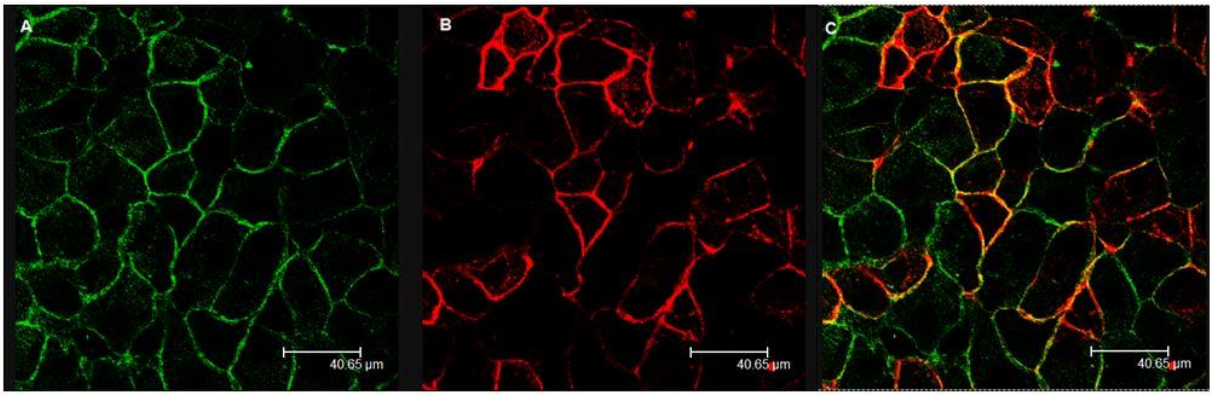


Fig. 9. Immunofluorescent staining of cell surface PDI in OVCAR3 cell line. **(A)** Anti 6x-His staining. **(B)** Cholera Toxin Subunit B cell membrane staining. **(C)** Merge of the picture A and B.

In the Fig. 10, Fig.12 and Fig.14 is visible that the controls OVCAR3, A2780 and SKOV3 cells, incubated by anti 6x-His antibodies without extra adding of recombinant proteins shows only Cholera Toxin cell membrane staining.

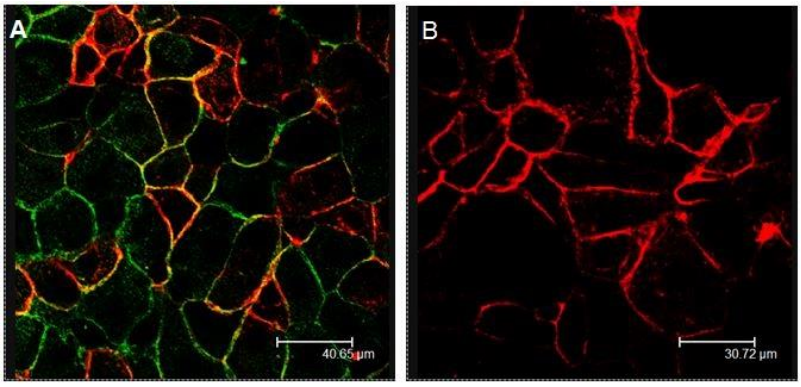


Fig. 10. Immunofluorescent staining of cell surface PDI in OVCAR cell line. **(A)** In green anti 6x-His staining, in red Cholera Toxin cell membrane staining. **(B)** Negative control, OVCAR cells that have been stained without incubation with extra recombinant proeins.

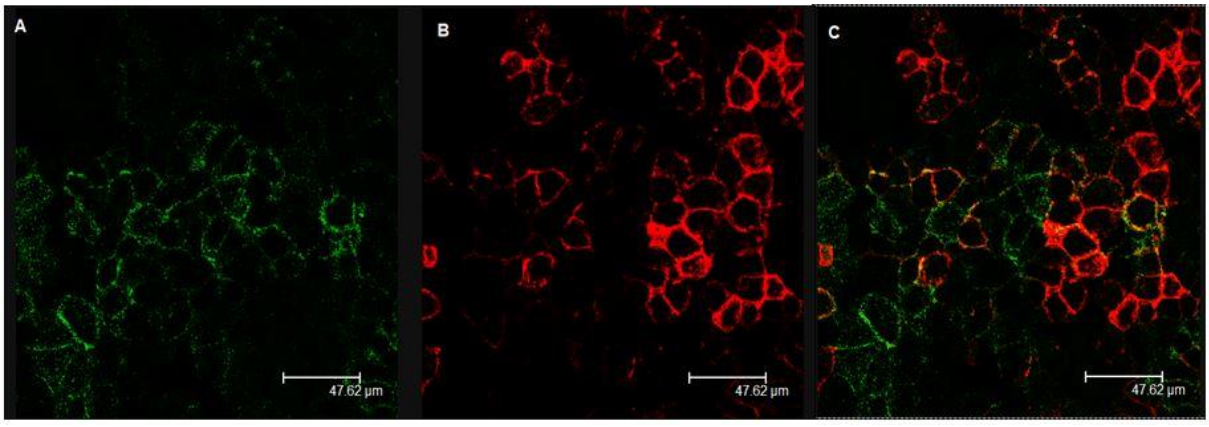


Fig. 11. Immunofluorescent staining of cell surface PDI in A2780 cell line. **(A)** Anti 6x-His staining. **(B)** Cholera Toxin Subunit B cell membrane staining. **(C)** Merge of the picture A and B.

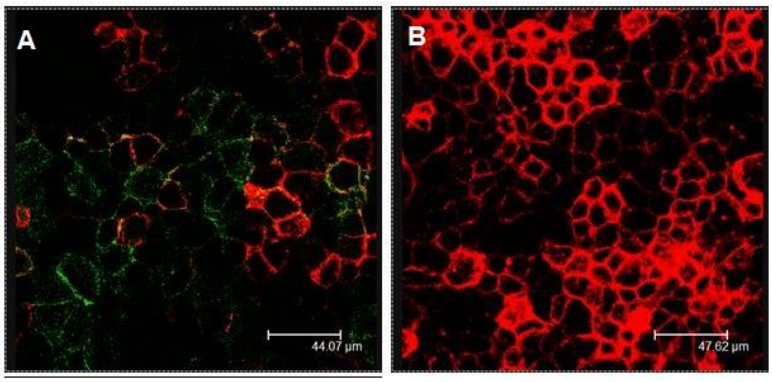


Fig. 12. Immunofluorescent staining of cell surface PDI in A2780 cell line. **(A)** In green anti 6x-His staining, in red Cholera Toxin cell membrane staining. **(B)** Negative control, A2780 cells that have been stained without incubation with extra recombinant proeins.

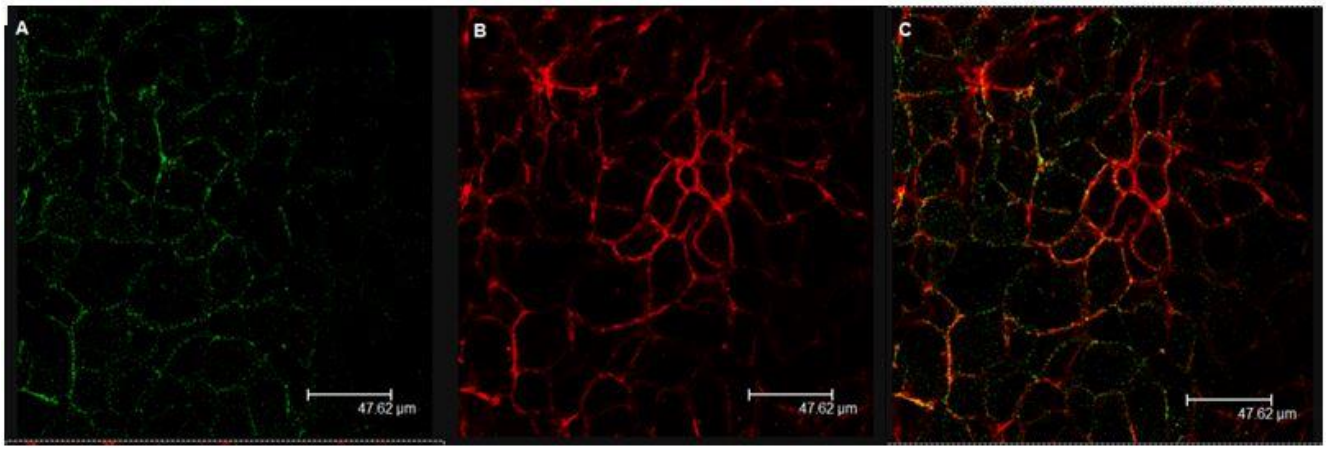


Fig. 13. Immunofluorescent staining of cell surface PDI in SKOV3 cell line. **(A)** Anti 6x-His staining. **(B)** Cholera Toxin Subunit B cell membrane staining. **(C)** Merge of the picture A and B.

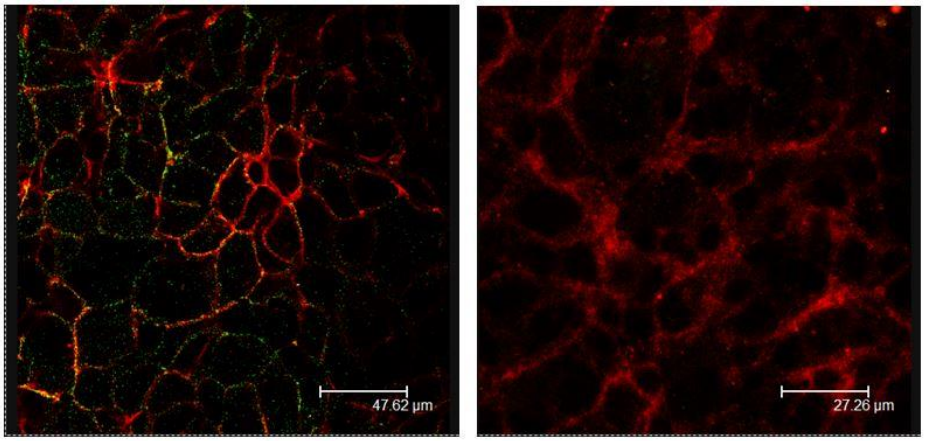


Fig. 14. Immunofluorescent staining of cell surface PDI in SKOV3 cell line. **(A)** In green anti 6x-His staining, in red Cholera Toxin cell membrane staining. **(B)** Negative control, SKOV3 cells that have been stained without incubation with extra recombinant proeins.

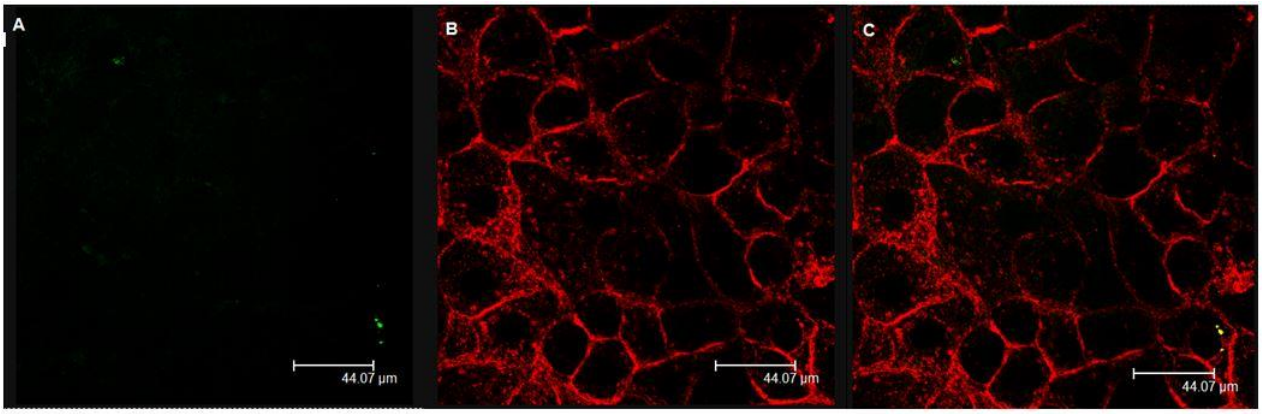


Fig. 15. Immunofluorescent staining of cell surface PDI in OAW42 cell line. **(A)** Anti 6x-His staining. **(B)** Cholera Toxin Subunit B cell membrane staining. **(C)** Merge of the picture A and B.

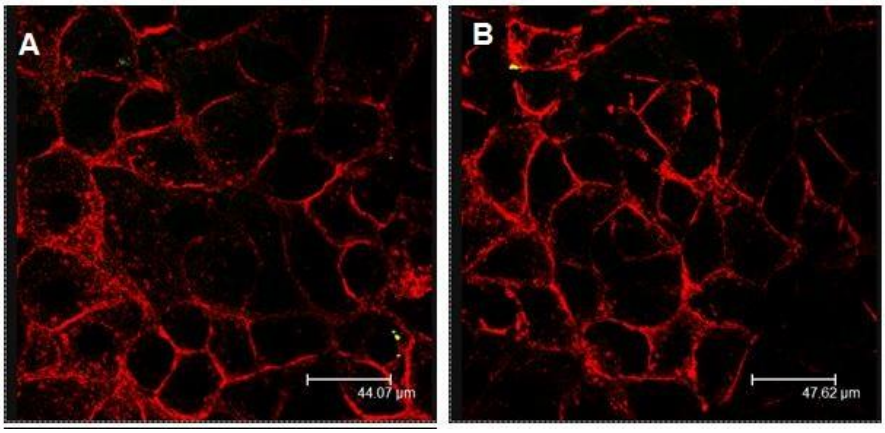


Fig. 16. Immunofluorescent staining of cell surface PDI in OAW42 cell line. **(A)** In green anti 6x-His staining, in red Cholera Toxin cell membrane staining. **(B)** Negative control, OAW42 cells that have been stained without incubation with extra recombinant proeins.

Unfortunaly ovarian cancer cellines OAW42 (Fig.13) didn't show PDI binding on the cell surface. Recombinant domains a, b, b' and a' also didin't show binding to the cell surface.

Western blotting for identification of recombinant protein

Performed Western blotting (Fig. 15) experiment showed that to identified PDI bend on the blot is quit difficult, as anti 6x-His antibody are binding aspecifically to some protein of OVCAR 3 cells. As a size of PDI is 57kDa, aspecific protein is masking the PDI bend. On the Fig. 15 is visible a bend for domain B' of the size of 35kDa that is not present in the control sample. This bend is referring to the dimer forming of b' domain.

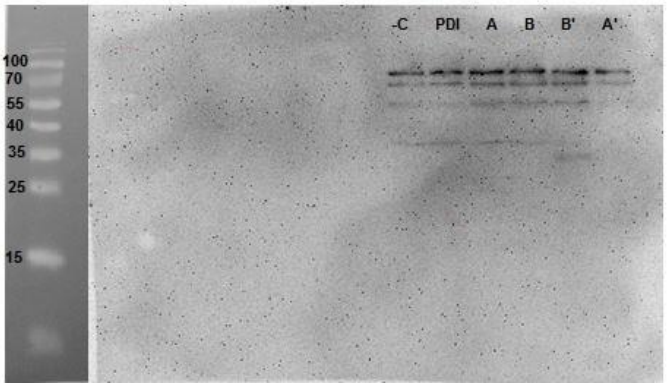


Fig.17. Western blotting for identification of recombinant protein

Discussion

Results of current experiments have showed that added recombinant PDI protein is not remaining in the cell culture medium but is going to the cell surface of ovarian cancer cells. Unfortunately not all analyzed ovarian cancer cells showed this result. OAW42 cell line didn't show binding of PDI on the surface.

Further literature study indicates that surface PDI is involved in integrin-mediated adhesion in platelet. It has been shown that blocking Protein Disulfide Isomerase (PDI) inhibits adhesion of platelets (17) (18). Referring to this studies it can be suggested that also in ovarian cancer cells added recombinant PDI is binding to integrin's on the cell surface. This binding is strongly depended on the expression of integrin's on the cell surface of ovarian cancer cell lines. It has been known that cell line OAW42 is expressing low amount of integrin's, which can be a reason that OAW42 is not showing PDI binding. Cell line SKOV3 is also considered to be low level integrin expressing cell line and as result PDI binding on its surface was less strong.

Unexpectedly any individual domain of PDI is able to bind cell surface. As it has been mentioned before, b' domain is considered to be the primary peptide- or protein-binding domain, it was expected that this domain can bind cell surface. Western blotting experiment showed a bend that correspond to the dimer form of b' domain but immunofluorescent staining protein didn't confirm that b' domain is able to bind cell surface. As immunofluorescent staining is more sensitive technique, it has been concluded that each of PDI domains is not able to bind cell surface of ovarian cell line. However this conclusion do not excluded that two domains expressed together, for example ab, bb', b'a', are able to bind ovarian cancer cells surface.

Further steps of the project will be concentrated to understand which molecule mediates the binding of PDI on the cell surface. One of the hypothesized proteins is transmembrane receptor integrin. Once the mechanism of PDI binding to the cell surface will be clear, the next step will be performing of Complement Dependent Cytotoxicity assay with the aim to understand if extra added PDI is able to activate CDC and lead to the killing of ovarian cancer cells.

One of the further steps of the project will be direct labeling of PDI protein by fluorescent dye. This approach will allow following translocation of PDI to the cell surface or even intracellular in different time moments.

Further it would be important to understand if binding of PDI to normal ovarian epithelial cells is giving the same result as PDI binding on the cell surface of ovarian cancer cells. Moreover is important to understand how specific is binding of PDI to other tissue cells.

Unswering to these questions will give a big step in developing PDI as potential target for the immunotherapy of ovarian cancer.

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List of attended seminars

- Prof. Dr. Yong-Sang Song: “Anticancer strategy targeting cancer cell metabolism in ovarian cancer”
- Dr. Tonino Alonzi: “Regulation of hepatocytes differentiation during the transitions between epithelial and mesenchymal states”
- Prof. Valeria Poli: “Targeting the liver to cure myocarditis: a lesson from a model of STAT3-dependent auto-immune myocarditis”
- Pfor. Antonio Sica: “Myeloid cells as therapeutic target in cancer”
- Prof. Darko Bosnakovski**:** “Proof of principle for cell therapy: from autologous transplantation of tissue specific progenitors to gene corrected patient specific injured pluripotent stem cells”
- Prof. Darko Bosnakovski: “Cell based models for studying molecular mechanism of Facioscapulohumeral Muscular Dystrophy”
- Prof. Darko Bosnakovski**:** “Toward animal model for Facioscapulohumeral Muscular Dystrophy (FSHD)”