UNIVERSITÀ DEGLI STUDI DEL PIEMONTE
ORIENTALE "AMEDEO AVOGADRO"

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DOCTORATE (PH.D.) IN MOLECULAR MEDICINE XXVIII CYCLE

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Introduction

Osteopontin (OPN) is a matricellular protein originally isolated from bone, expressed by various cell types including macrophages, dendritic cells and activated T cells. OPN mediates different biological functions such as bone remodelling, macrophage response, cell migration, adhesion and it is implicated in the pathogenesis of several diseases including artherosclerosis, multiple sclerosis (MS), cancer and chronic inflammatory diseases. OPN costimulates T cell proliferation and it is classified as a T helper cell 1 (T_H1) cytokine because of its ability to enhance IFN-γ and IL-12, and to dicrease IL-10 production.

OPN is a member of SIBLINGs (Small Integrin Binding Ligand N-linked Glycoprotein) protein family, mapped to the human chromosome 4, synthetized as a 32 kDa polipeptide. Due to extensive posttranslational modifications the molecular weight ranges between 45 and 75 kDa. The protein has two calcium binding sites, two putative heparin binding domains (1), and multiple adhesion motifs which allow interaction with many cell types including smooth muscle, endothelial and inflammatory cells. OPN has an RGD (arginine-glycine-aspartate) integrin binding domain, several phosphorylation and glycosylation sites. The RGD domain of OPN mediates interactions with αvβ1, αvβ3, αvβ5, αvβ6, α8β1, and α5β1 integrins (2; 3; 4; 5). In the carboxy terminal, the protein has a cryptic SVVYGLR (SLAYGLR in mice) sequence that becomes exposed upon thrombin cleavage and mediates interactions with α9β1, α4β1, and α4β7 integrins (6; 7; 8).

Data obtained in animal models and humans strongly suggest that OPN may a play role in the pathogenesis of MS (9). Patients with MS display variable clinical course, at onset, approximately 15% of the patients have a primary progressive form (PP), whereas the remainder start out with a relapsing-remitting form (RR), and most of them switch to a secondary progressive form (SP) within 10-30 years. Both genetic and environmental factors are involved in the development / progression of MS and several studies point at a complex picture characterized by interactions between different combinations of scenes that may influence the immune response (10, 11).

In MS context, OPN transcript is abundant in plaques dissected from brains of MS patients, whereas it is absent in the control brain tissue (12). This finding was confirmed in rat experimental autoimmune encephalomyelitis (EAE) by microarray cDNA analysis of spinal cord tissue. OPN protein levels had been evaluated in plasma, serum and cerebrospinal fluid (CSF) of MS patients and the results were correlated with clinical forms of MS. During inflammation thrombin acts on the cleavage site of OPN and generate two OPN fragments (N- and C-terminal). The thrombin cleavage of OPN may be responsible for the functional difference between the two fragments and the full length protein (13). The N-terminal fragment of OPN has adhesion motifs that may explain its higher proinflammatory effect compared to that displayed by the full length protein, and it is highly expressed in atherosclerotic plaques of patients with hypertension. The properties of the C-terminal fragment are less documented. The individual role of the two OPN fragments in EAE and their functional activity has never been investigated in vitro.

Aim:

1, Clonal selection of mouse OPN clones: Our laboratory is running pre-clinical studies in animal models with EAE. The aim of these experiments is to investigate the role of the OPN fragments (N- and C-terminal) in the diseases using the following recombinant OPN proteins: OPN – full length (FL); mutant (MUT) in the site of thrombin cleavage; N-terminal and C-terminal fragments. For this purpose, the corresponding plasmids have been stably transfected in CHO cells and clones expressing high levels of the recombinant proteins have then been selected. Since the initial protein purification showed substantial product degradation, possibly due to the instability of the clones, we decided to perform a new clonal selection.

2, Quantify the levels of human thrombin-cleaved OPN: To evaluate the individual levels of the N- and C-terminal fragments in the human biological fluids, we designed an ELISA assay using monoclonal antibodies specific for different epitopes of OPN. This was required since there is no available commercial ELISA kit for this purpose.

Materials and methods:

1, Clone selection:

Murine OPN FL, N-terminal, C-terminal and MUT recombinant protein were previously produced in our laboratory from cDNA cloned by PCR into pUCOE vector using a 3'-primer adding the six histidins Tag and stably transfected into Chinese Hamster Ovary cells (CHOs). For a new clone selection cells expressing each construct were plated into 96 wells plate (1-2 cells / well) in Pro-CHO medium (SIGMA) with 0.2 mg / ml hygromycin B (Invitrogen). After about 3 weeks, several clones appeared. We tested the supernatants by ELISA and Western blot to assess the presence of the protein. According to the results, clones were selected for further expansion.

2, Detection of mouse OPN by enzyme-linked immunosorbent assay (ELISA):

Quantitative ELISA was performed to evaluate the amount of mouse OPN FL and N-terminal during clonal selection by commercially available ELISA kit (R&D Systems) according to the manufacturer's instruction. 96 wells microplate was coated with 100 µl / well anti-mouse OPN capture antibody (0.8 µg / ml) in PBS and incubated overnight at 4°C. Wells were blocked with 300 µl diluent reagent (1% BSA in PBS) and incubated at room temperature for 1 hour then 100 µl of sample (1:100 dilution) were added into each well and incubated at room temperature for 2 hours. 100 µl / well biotinylated goat anti-mouse OPN was used as secondary antibody (100 ng / ml) and incubated for 2 hours at room temperature. For detection 100 µl streptavidine conjugated with horseradish-peroxidase was added each well. After twenty minutes incubation at room temperature, 100 µl TMB (SIGMA) solution was loaded into the wells and the reaction was stopped with Stop Solution (2 N H_2SO_4) after 5-10 minutes. Each step was followed by three washes with PBS plus 0.05% TWEEN® 20 (SIGMA). The optical density was determined by microplate reader (VICTOR™ X3) set to O.D. 450 nm.

3, Evaluation of OPN protein expression by Western blot:

From each clone, 50 µl of supernatant were loaded onto 10-12% polyacrylamide gel, fractioned by sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membrane. The membrane was saturated with fat-free milk (5%) in TBST (TBS + 0.01% TWEEN® 20 – Sigma) then incubated with Tetra His Antibody (QIAGEN) primary antibody overnight at 4°C. As secondary antibody ECL Mouse IgG, HRP-linked antibody (dilution 1:3000, GE Healthcare Life Sciences) in 5% non-fat milk was added onto the membrane and incubated for 1 hour at room temperature. Each step was followed by extensive washes with

TBST. The reaction was developed with Chemiluminescent substrates (Bio-Rad) and signals were detected with VersaDoc™ Imaging System.

4, Evaluation of antibodies specific for the human N- and C- terminal fragment by Western blot:

We evaluated the specificity of several anti-OPN antibodies by western blot. For the mAb(53), 10 ng of Hu OPN FL (recombinant and commercial – R&D), N- and C-terminal OPN (recombinants) protein were loaded onto 10% polyacrylamide gel. T. For the MAB194P or the MAB197P mAb, the amount of the loaded OPN was 100. The OPN proteins were fractioned by sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membrane. The membrane was saturated with fat-free milk (5%) in TBST (TBS + 0.01% TWEEN® 20 – Sigma) then incubated with 5 μg/ml primary antibody (mAb53/MAB194P/MAB197P) overnight at 4°C. As secondary antibody, ECL Mouse IgG, HRP-linked antibody (dilution 1:3000, GE Healthcare Life Sciences) in 5% non-fat milk was added onto the membrane and incubated for 1 hour at room temperature. Each step was followed by washes with TBST. The reaction was developed with Chemiluminescent substrates (Bio-Rad) and signals were detected with VersaDoc™ Imaging System.

Results:

1. Evaluation of positive Ms CHO OPN clones

The cloning selection was performed on mouse OPN FL, MUT, N- and C-terminal cell line to enhance the quality of the protein production. After several weeks, only few clones grew up from MUT and C-terminal transfected cells in the selective agent for hygromycin resistance. Protein expression of each clone was analyzed by Western blot from the supernatant (Fig. 1.). Three positive clones from MUT (B7; B3; G8) and C-terminal (C9; C4; F10) were selected for further expansion.

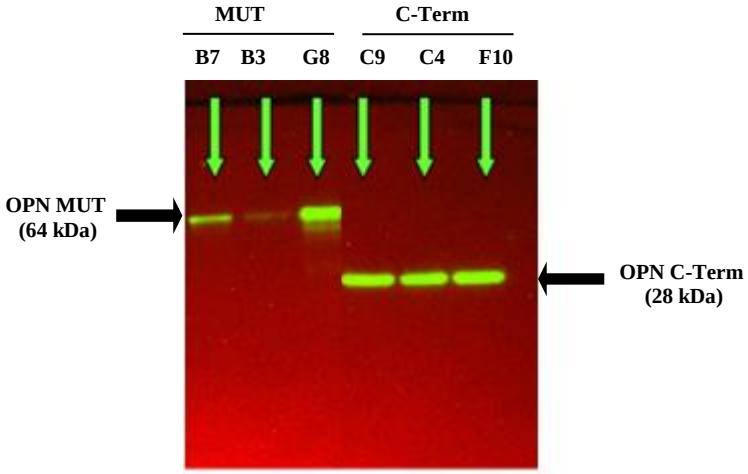

Figure 1. Western blot result shows the expression of MUT and C-terminal OPN. The molecular weight of MUT is ~64 kDa, while C-terminal is ~ 28 kDa.

We had 35 clones from FL and 51 from N-terminal OPN. Their expression was first evaluated by ELISA assay. Nine positive clones were selected both from FL and N-terminal OPN (Table 1.), and their protein expression was determined by Western blot. The results revealed two stable clones from FL OPN (Fig. 2.), while all clones from N-terminal showed bands of inappropriate MW. Further investigations are necessary to identify stable clones from N-terminal OPN.

FL clones	
	pg/ml
D2	149,7131
D8	299,54179
F11	673,49615
E5	889,8205
G6	1171,8962
G2	2196,9532
B8	2289,2624
C3	2986,5025
F7	4385,3712

N-terminal clones	
	pg/ml
D11	233,43514
B3	383,70002
G5	433,78254
E6	450,32394
F3	500,18877
C3	622,80605
E3	628,93812
F7	674,20576
C6	940,24469

Table 1. ELISA results of MS CHO full length and N-terminal protein expressing clones.

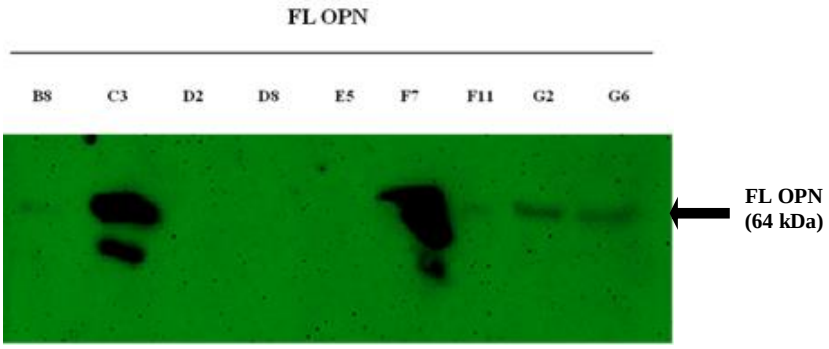

Figure 2. Western blot result of MS OPN FL. The G2 and G6 clones produce the protein at the correct molecular weight (~64 kDa), while the C3 and F7 clones showed bands of inappropriate MWs. For further expansion the G2 and G6 clones were chosen.

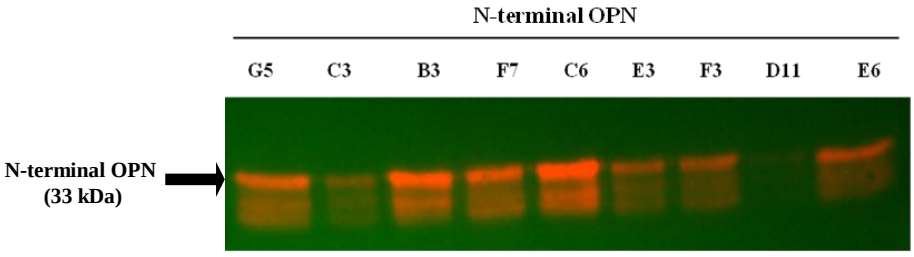

Figure 3. Western blot result of MS OPN N-terminal. In all cases we observed several signals besides the expected molecular weight (~33 kDa).

2. Development of ELISA assay for detection thrombin-cleaved OPN fragments

Our aim is to set up an ELISA assay which can be used to determine the amount of the thrombin-cleaved OPN fragments, N- and C-terminal, in human biological fluids. For this purpose, we purchased three monoclonal antibodies specific for different sites of the protein. The mAb(53) antibody recognizes an epitope mapped to the thrombin-cleavage site, that is destroyed upon thrombin cleavage. Therefore, is strictly specific for OPN FL protein and doses not bind to the N- and C- fragments (14; 15) (Fig.4).

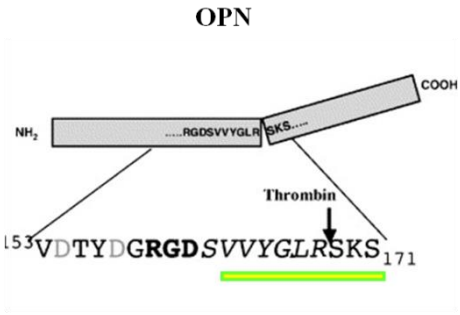

Figure 4. The cryptic epitope of OPN recognized by mAb(53).

Using the OPN epitope map provided by the Maine Biotechnological Service (Fig.5), we have been able to select two monoclonal antibodies specific for the N- and C- terminal portion of the molecule; MAB194P recognizes N-terminal OPN, while MAB197P recognizes C-terminal; both also bind FL OPN (17; 18).

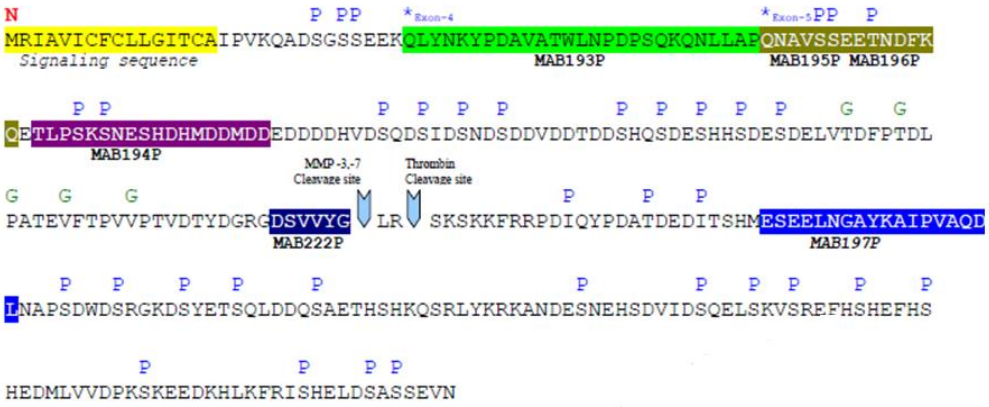

Figure 5. The Human OPN resource map provided by the Maine Biotechnology Services. The mAB194P recognizes FL and N-terminal OPN and the mAB197P recognizes C-terminal and also FL OPN.

The antibodies were tested with our human OPN recombinant proteins to confirm their specificity. Western blot analyses were performed with three different proteins: human (Hu) OPN FL, N- and C-terminal. As a control, we used Hu OPN FL provided by R&D. Results confirmed that mAb(53) recognized FL OPN only (Fig. 6.); MAB194P N-terminal and FL (Fig.7); and MAB197P C-terminal and FL (Fig. 8).

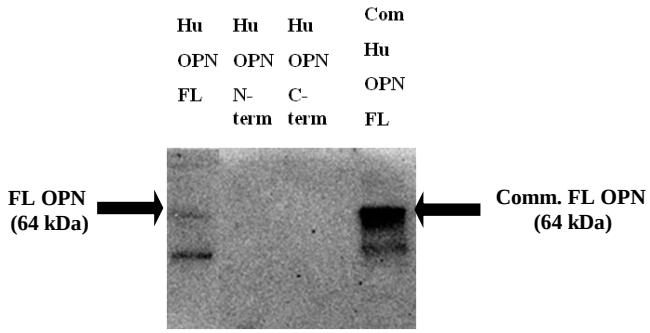

Figure 6. mAb(53) monoclonal antibody recognizes only FL OPN. The sensitivity of the antibody is proven as the amount of protein loaded onto the polyacrilamide gel was 10 ng. The concentration of the mAb(53) was 5µg/ml.

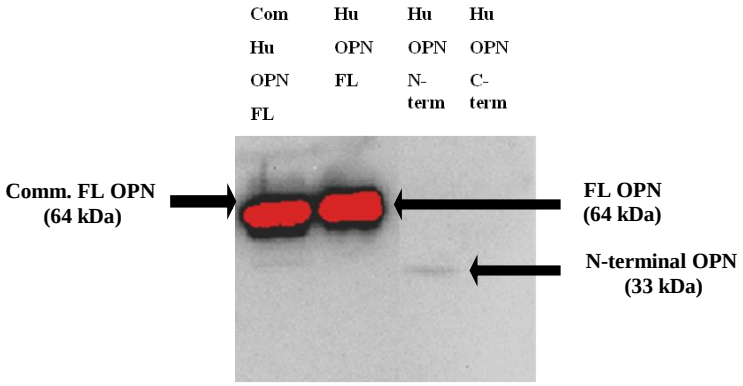

Figure 7. mAB194P monoclonal antibody recognizes FL and N-terminal OPN. The epitope is recognized by the antibody between the 73 and 90 aminoacid. The amount of protein loaded was 100 ng and the concentration of the antibody was 5µg/ml.

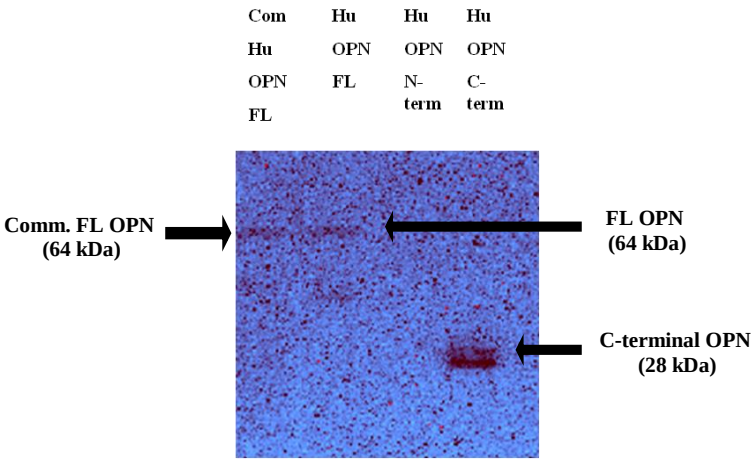

Figure 8. mAB197P monoclonal antibody recognizes FL OPN and C-terminal. The epitope is recognized by the antibody between the 194 and 210 aminoacid. The amount of protein loaded was 100 ng and the concentration of the antibody was 5µg/ml.

Discussion and future prospects:

During my first year of PhD, I performed screening experiments for selection of clones expressing OPN FL, MUT, N- and C-terminal. During the experiment, I was focused on identifying and expanding clones which are producing the recombinant proteins in the correct form. These proteins will be used in future experiments on mouse models.

The second aim of my project is to set up an ELISA assay to evaluate the amount of OPN N- and C-terminal in MS patients sera using combination of three different antibodies. By Western blot analyses, we confirmed the specificity of each antibody. mAb(53) antibody recognizes an exclusive epitope within OPN FL protein, mAB194P recognizes FL and N-terminal OPN, while the mAB197P recognizes FL and C-terminal OPN. The ELISA assay will be optimized by using human CHO OPN clones (FL, N- and C-terminal) provided by our laboratory. In the course of the development it will be crucial to pay attention to set up the optimal concentration of the different antibodies. Since we know that OPN plays role in many different diseases, this novel ELISA assay could be a useful tool to reveal the role of OPN in diseases such as cancer or atherosclerosis.

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Attendances:

Workshop:

- Autoantibodies in autoimmune diseases: pathogenic, predictive and/or protective? – Novara – 21-22/03/2013

Conference:

- National: IRCAD - Novara – 6/12/2012
- International: 5th ICI 2013 – Milan- 22-27/08/2013
  - Poster presentation – Abstract: B7H triggering inhibits the migration of tumor cell lines

Paper under submission:

IL-17 protects T cells from apoptosis and contributes to development of ALPS-like phenotypes.

Seminars:

- Alumina/Zirconia composites for hip joint applications: State of the Art, Market & Future Trends - Dr. Alessandro Alan Porporati

- Glioblastoma stem cell biology and its implications in cancer therapy - Dott.ssa Giuliana Pelicci

- Molecular interactions of histone modification and de novo DNA methylation - Dott.ssa Kyung-Min Noh, PhD

- Cav1 protein in Skin Cancer Pathogenesis - Dott. Franco Capozza

- Interleukin-33: a novel player in chronic intestinal inflammation? - Dott. Luca Pastorelli

- Red blood cells as carriers for magnetically targeted delivery of drugs - Prof. Dr. Hans Bäumler

- Skin cancer in vivo models, what they have and can tell us - Dott. Girish Patel

- "Heparan Sulfate: A versatile target for the development of new drugs - Dott. Giancarlo Ghiselli

- Master class on Genetics & Molecular Medicine - Prof. Steve Ellis