SUMMARY OF THE THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INVESTIGATION OF THE ROLE OF INTRACELLULAR BLOOD COAGULATION FACTOR XIII SUBUNIT A IN THE PHAGOCYTOSIS OF MONOCYTES/MACROPHAGES

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1. INTRODUCTION

The role of factor XIII in hemostasis

The role of factor XIII (FXIII) in hemostasis is well known several decades. The active form of FXIII (FXIIIa) a transglutaminase acts as an enzyme by catalysing the formation ε(γ-glutamyl)lysyl crossbridges between the fibrin monomers, which stabilize the fibrin polymer formed in the blood coagulation cascade. It is also known that the α_2-antiplasmin, which is the main inhibitor of fibrinolysis is crosslinked to the α-chain of fibrin polymers by FXIIIa which protect the fibrin polymer from the rapid fibrinolytic degradation. FXIII circulates in the blood as a heterotetramer (A_2B_2) consisting of two globular A subunits (FXIII-A) surrounded by two strand-like B subunits (FXIII-B). FXIII is activated by thrombin in proteolytic way, which mainly regulated by Ca^{2+} and fibrin(ogen). It has been shown that in the human body FXIII-B is synthesized in the hepatocytes while FXIII-A is produced mainly by megakaryocytes/platelets and monocytes/macrophages. Hepatocytes synthesize only trace amounts of FXIII-A. Recently, expository descriptions have verified that articular chondrocytes can also synthesize FXIII-A.

Role of factor XIII beyond hemostasis

In the last three decades it has become evident that the role of FXIII is not restricted solely to the area of hemostasis. Data accumulated in the literature have demonstrated its participation in wound healing and in maintaining pregnancy. However the FXIII-A was showed in platelets decades ago and it was identified in the monocyte/macrophage cell line more than 15 years ago the intracellular role of FXIII-A is almost unknown. On the basis of the results of experimental and clinical investigations it can be supposed that megakaryocytes, monocytes/macrophages cannot be simply viewed as sites of synthesis for the A subunit of plasma FXIII, but FXIII-A has some intracellular functions in these cells:

a.) FXIII-A is present in the monocyte/macrophage cell line from the very early stage of their bone marrow development, via circulating blood monocytes, to the final stage of macrophage differentiation in both tissues and serous cavities.

b.) FXIII-A has no signal sequence and when expressed in baby hamster kidney cells, it is not secreted into the culture medium, i.e. FXIII-A can be released from cells only at times of cell death or injury.

c.) FXIII-A shows characteristic cytoplasmic localization in macrophages, namely it is accumulated around cytoplasmic vacuoles and in pseudopods, but it is not present in phagocytic vacuoles; by means of immunoelectronmicroscopy it can be demonstrated in association with microfilaments, but can never be detected in secretory vesicules.

d.) Major cytoskeletal proteins such as myosin, actin and vinculin, are substrates of activated FXIII-A

In taking the above mentioned facts into consideration, it is reasonable to suppose that FXIII-A in monocytes/macrophages can be activated at cellular level and consequently, may have certain intracellular function(s) and it may act as a cellular transglutaminase participating in cytoskeletal remodeling. Phagocytosis is the most characteristic function of monocytes/macrophages in which the cytoskeletal system is deeply involved. Therefore, our work was designed to determine whether or not cellular FXIII-A could be implicated in the phagocytic processes of monocytes and macrophages.

2. OBJECTIVES

The aim of our work was to investigate the possible intracellular role of FXIII-A in monocyte/macrophage cell line.

To determine whether or not FXIII-A could be involved in the phagocytic processes we intended to answer the following questions:

- how do the mRNA expression and protein production of FXIII-A change during monocyte/macrophage differentiation?

- how do the Fcγ and complement receptor-mediated phagocytosis change during monocyte/macrophage differenciation?

- is there any correlation between the changes in phagocytic activity of monocytes/macrophages and alterations in FXIII-A mRNA expression?

- how does the inhibition of FXIII-A influence the phagocytic activity of monocytes/macrophages?

Monocytes from patients with FXIII-A deficiency were examined

- to detect any alterations in phagocytic activity of the cells.

- to establish whether the FXIII-A could be implicated in the receptor binding capacity of the cells or internalization of bound particles during the phagocytosis.

2. MATERIALS AND METHODS

3.1. Separation and culture of monocytes

Monocytes were isolated from the human buffy coat of healthy volunteers. The monocytes were cultured in a RPMI 1640 medium supplemented with 10% heat inactivated human AB serum.

3.2. Patients

Thirteen FXIII deficient patients were involved in this study: three patients (all females) from Poland, one from Germany (male) and nine patients (five males and four females) from Israel (three unrelated Palestinian Arab families). Mutations in the FXIII-A gene which resulted in FXIII deficiency were identified in cases of German and Palestinian Arab patients.

3.3. Separation of monocytes from patients and healthy volunteers

Mononuclear cells were isolated from heparinized blood samples of patients and healthy volunteers (n=21) following informed consent. Platelet-depleted mononuclear cells were obtained by centrifugation through Ficoll-Hypaque. Monocytes were purified by adherence to a glass surface.

3.4. Binding and phagocytosis of IgG-sensitized red blood cells

Erythrocyte antibody (EA) binding and phagocytosis via the Fcγ receptor (FcγR) were tested with sheep red blood cells (SRBC) sensitized with an IgG fraction isolated from rabbit anti-SRBC serum. In order to prepare the EA, the SRBC were sensitized with a subagglutinating quantity of rabbit anti-SRBC IgG. Cells separated from patients and healthy donors were allowed to adhere to a cover glass surface then incubated with EA. Binding and endocytosis of the EA were evaluated with a Diaplan microscope (Leitz, Wetzlar, Germany) by examining 300 cells. The numbers of all uptaken particles/cells were counted (Uptake Index, UI). After hypotonic lysis of the cell-associated SRBC, the number of phagocytosed erythrocytes/cells was determined (Phagocytic Index, PI). The number of erythrocytes bound to cell

surface/cells (Binding Index, BI) was defined as the difference between the uptake and phagocytic indices: UI-PI=BI.

3.5. Binding and phagocytosis of yeast particles

The baker's yeast *(Saccharomyces cerevisiae)* were treated with IgG- and fibronectin-depleted human AB serum. Monocytes were placed on a cover glass and the adherent cells were incubated with complement-coated or uncoated heat-killed yeast conjugated with fluorescein isothiocyanate. The binding and phagocytosis of particles were determined by using the fluorescence quenching method. Uptake of particles/cell (UI) was determined with an Axioplan fluorescence microscope (Zeiss Oberkochen, Germany) by examining 300 cells. The numbers of all uptaken particles/cell were thus determined (UI). After staining with 0.2 % trypan blue, which completely quenches the fluorescence of noningested particles, the number of phagocytosed yeasts/cell (PI) was also determined. The number of particles bound to cell surface (BI) was calculated.

2.6. Inhibition of phagocytosis

The effect of monodansylcadaverine, which inhibit the development of crossbridges catalysed by transglutaminases, on FcγR and CR mediated phagocytosis was tested. Monocytes/macrophages were incubated in MDC solution prior to phagocytosis tests and the assays were carried out as described in the sections 3.4. and 3.5.

2.7. Immunofluorescent detection of FXIII-A and fluorescent image analysis

For detection of FXIII-A, cells from the monocyte/macrophage culture were spun down onto glass slides using a Cytospin 3 cytocentrifuge (Shandon, Pittsburg, UK). Cells were fixed in paraformaldehyde. FXIII-A was detected by an indirect immunofluorescent reaction on samples incubated with rabbit polyclonal antiserum to FXIII-A and subsequently anti-rabbit IgG conjugated with biotin. The reaction was visualized by FITC labeled streptavidin. The relative amount of FXIII-A was determined at single cell level by measuring fluorescence intensity values using an Axioplan fluorescence microscope connected to a black and white intensified charge-coupled device (CCD) IMAC camera (Sony, Japan), an image analysis

processor and a computer system (ISIS fluorescence imaging system, Metasystems, Germany) applying the method developed and published by ourselves.

2.8. Changes in mRNA expression of FXIII-A during culturing

Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was used to quantitative determination of mRNA expression of FXIII-A. Total RNA from monocytes/macrophages was isolated by High Pure RNA Isolation Kit. Two-step quantitative RT-PCR was applied. The same amount of RNA was reverse transcribed to cDNA then amplified by specific primers and probe for FXIII-A designed and developed by the Applied Biosystems. PCR amplification was performed in triplicate wells. Normalization was performed using the expression of 18S rRNA as an internal control.

2.9. SDS-PAGE Electrophoresis and Western blot analysis

Cells were lysed in distilled water and the cytosolic protein concentration was measured. Denatured recombinant FXIII-A, equal amounts of cytosolic protein from monocytes/macrophages at different stages of differentiation, as well as high molecular weight and biotinylated standards, were submitted to SDS polyacrylamide gel electrophoresis. Proteins on the gel were visualized by staining with Coomassie blue. For immunoblotting, proteins were transferred to nitrocellulose membrane. The nitrocellulose membrane was incubated by antibody against FXIII-A, then labeled with biotynilated rabbit IgG and avidin-biotinylated peroxidase complex. Antibody was visualized with DAB (ABC/DAB reaction).

3.10. Statistical analysis

Differences between the data obtained by experiments performed on monocytes from healthy controls and FXIII deficient patients were determined by Student's *t* test. Values of $P<0.05$ were considered as statistically significant. The results of quantitative RT-PCR experiments were statistically analyzed with the comparative Ct (threshold cycle) method. Normalization was performed using the expression of 18S rRNA as an internal control and the mRNA level of FXIII-A on the first day was used as the basis for comparative results (calibrator).

3. RESULTS

4.1. Phagocytosis via Fcγ and complement receptors and inhibition of phagocytosis during culturing

The monocytes elutriated from the human buffy coat of healthy volunteers were cultured for 4 days and the phagocytic functions of monocytes/macrophages via FcγR and CR were measured in the presence and in the absence of MDC. Both the FcγR and the CR mediated phagocytosis increased during culturing and demonstrated the highest level on day 3. Strong inhibition of Fcγ (55-70%) and complement receptor mediated phagocytosis (20-70%) was observed after MDC treatment.

4.2. Expression of factor XIII-A protein at single cell level during culturing

To examine the production of FXIII-A at single cell level, image analysis of immunofluorescently labeled monocytes/macrophages was performed. On the first day, cells with relatively low fluorescence intensities dominated and then the proportion of the cells with higher fluorescence intensity rapidly increased, reaching a plateau on the third day. The distribution of cellular fluorescence intensity values was very similar in the cell population removed from the culture on day 4.

4.3. Phagocytic functions of monocytes from factor XIII deficient patients

In order to study the phagocytic functions of cells lacking FXIII-A, the binding and phagocytosis of EA and phagocytosis of complement uncoated and coated particles of monocytes from FXIII-A deficient patients were examined. Although there was no statistically significant difference between the binding of EA by monocytes from patients and controls, the mean of binding indices obtained by the experiments carried out on the patients' monocytes showed a lower value than that of control cells. However, the phagocytosis of EA by cells from FXIII-A deficient patients was significantly lower than that of normal cells. The phagocytic activities of the cells from patients via complement and lectin-like receptors were also significantly reduced compared to healthy controls.

4.4. FXIII-A mRNA expression during monocyte/macrophage differentiation

To investigate the changes in FXIII-A synthesis of monocytes/macrophages at mRNA level, real-time quantitative RT-PCR analysis was performed. The expression of FXIII-A mRNA increased rapidly during culturing, reaching the highest level on the 3rd day and decreased markedly on day 4. The magnitude of the FXIII-A gene expression was more than 70-fold on day 3 compared to the first day level.

4.5. Western immunoblot analysis of monocytes/macrophages

In order to confirm changes in the expression of FXIII-A protein, Western immunoblot analysis of electrophoretically separated proteins was also carried out. The synthesis of FXIII-A protein increased from the 1st day of culturing to the 2nd and 3rd days and that subsequently, the synthesis of FXIII-A decreased considerably on the 4th day.

5. DISCUSSION

Circulating blood monocytes within the mononuclear phagocyte system represent an intermediate cell population differentiated from bone marrow precursor cells. They are able to differentiate into macrophages after migration from the vasculature into various tissues and body cavities. Maturation of macrophage from monocyte *in vitro* culture conditions resembles, in many respects, the *in vivo* process. It has been demonstrated that FXIII-A is present in various cell types, including monocytes/macrophages. Although FXIII plays a pivotal role in hemostasis and becomes activated by the proteolytic action of thrombin in the presence of Ca^{2+}, it has also been described to be activated by non-proteolytic (thrombin-independent) pathways. In addition, it has been reported that actin, myosin, and vinculin, which are involved in cytoskeletal remodeling, can serve as substrates for FXIII-A. In a previous report on a non-phagocytic FXIII-A-negative myelomonocytic cell line (DD), it has been demonstrated that the phagocytic ability of DD cells can be restored by phorbol ester treatment in parallel with the reexpression of FXIII-A. Therefore, it is reasonable to suppose that intracellular FXIII-A may possess certain cellular function(s) associated with cytoskeletal rearrangement, including phagocytosis. However, no data have been presented either on the change of FXIII-A production during monocyte/macrophage differentiation, or about the relationship between the FXIII-A content and phagocytic activity of monocyte/macrophage cell line.

In this study we have demonstrated the upregulation of FXIII-A protein at single cell level during the course of monocyte/macrophage differentiation. The FXIII-A-related fluorescence intensity was 10-fold higher in the dominant subgroup of cells on the 3rd day of culturing than that measured in the cell population removed from the culture on the 1st day. Data obtained by quantitative RT-PCR experiments showed that there was a more than 70-fold increase in the expression of the FXIII-A gene and that the highest relative quantity of FXIII-A mRNA was also recorded on the 3rd day. These findings were in accordance with the results of Western immunoblot analysis indicating a parallel increase of FXIII-A protein synthesis. In parallel, the phagocytic activity of cells increased and the phagocytosis of EA and complement-coated yeast particles reached their peak levels on the 3rd day. The parallel increase in phagocytic activity and FXIII-A synthesis showed in this study raise the possibility of an association between the two processes and this assumption was supported by two series of experiments:

1. MDC, an inhibitor of FXIII-induced crosslinking, strongly depressed phagocytic activity of monocytes/macrophages.
2. Fcγ receptor, complement receptor and lectin-like receptor mediated phagocytosis of monocytes from FXIII deficient patients that lack FXIII-A and have no transglutaminase activity were significantly lower compared to that of controls.

Fcγ and complement receptor mediated phagocytosis differ from each other not only by the receptors responsible for the binding of particles, but also in certain steps of the signaling pathway. The fact that both Fcγ and complement receptor mediated phagocytosis changed almost parallely with the expression of FXIII-A, and that both of them were significantly decreased in normal monocytes/macrophages in the presence of MDC and in monocytes from FXIII deficient patients, suggests that FXIII-A acts on certain common pathways of phagocytic mechanisms. In contrast, the inhibition of transglutaminase activity did not result in a decrease in the binding of EA and complement-coated particles and there was no significant difference between the binding of EA by monocytes from healthy controls and that of FXIII deficient patients. Therefore, the reduced FcγR-mediated phagocytosis is probably not due to a decrease in the binding capacity of patients' monocytes, but to the impaired internalization of the bound particles. Following the binding of ligands to the membrane of monocytes/macrophages, receptor-clustering, permitting multipoint attachments, commonly occurs. This phenomenon is based on cytoskeletal rearrangements which result in capping or even co-capping of CR and Fcγ receptors and consequently, endocytosis. The FcγR-mediated phagocytosis through a complex signal transduction leads directly or indirectly to the polymerization of actin. The cytoskeletal rearrangement is an essential event in CR-mediated endocytosis as well. Biochemical studies demonstrated that cytoskeletal proteins, such as myosin, actin and vinculin, can serve as substrates for FXIII-A and in our previous immunoelectronmicroscopical studies we also showed that FXIII-A was localized in close association with cellular microfilaments.

In conclusion, our work suggests that FXIII-A plays a role in receptor mediated phagocytosis. However, further studies are required in order to explore the details of its involvement in cytoskeletal reorganization or other steps of the phagocytic process.

6. SUMMARY

The aim of our work was to investigate the possible intracellular role of FXIII-A in monocyte/macrophage cell line. On the basis of data accumulated in the literature we have suggested that the FXIII-A may have certain intracellular function(s) and it may act as a cellular transglutaminase participating in cytoskeletal remodeling. Phagocytosis is the most characteristic function of monocytes/macrophages in which the cytoskeletal system is deeply involved. Our experiments were carried out in cultured human blood monocytes and in monocytes from patients with FXIII-A deficiency.

1. Both the FcγR and CR-mediated phagocytosis increased during culturing, which peaked on day 3. The phagocytic activity of the cells could be markedly inhibited with monodansylcadaverine, an inhibitor of the transglutaminase-induced crosslinking of proteins.

2. The FcγR, CR and lectin-like receptor-mediated phagocytosis was found to be strongly diminished in monocytes of FXIII-A deficient patients.

3. The phagocytic functions of cultured cells showed a change in parallel with the alterations in FXIII-A mRNA expression, and protein synthesis.

4. Our results suggest that FXIII-A plays a role in the Fcγ and complement receptor-mediated phagocytic activities of monocytes/macrophages.

Our results can contribute to better understanding of the intracellular role of FXIII-A. However, further studies are required in order to explore the details of its involvement in cytoskeletal reorganization or other steps of the phagocytic process.

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