Analysis of gene expression of mast cells and basophils

Ph.D. thesis

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Introduction

Innate immunity provides the first line of protection upon pathogen invasion, and it mainly involves cells of myeloid lineages. These cells can mediate rapid clearance of invading pathogens by phagocytosis, and they are rapidly recruited to local tissues to the site of infection via various chemokine receptors, leading to a complex sequence of inflammatory responses, including vasodilatation and increased vascular permeability. Abnormalities in the development of myeloid cells may cause an abnormal increase in the number of mature myeloid or blast cells resulting in chronic or acute myelogenous leukemia.

Mast cells and basophils belong to the myeloid cell population, known as the main effector cells of allergic reactions, were discovered by Paul Erlich at the end of the XIX century. Sharing many functional and morphological characteristics they were considered as circulating or tissue-bound forms of the same cell type for a long time.

Mast cells are round or elongated cells and have one, sometimes two or many non-lobed nuclei. Their intracellular granules are stained purple with aniline dye. This staining is caused by heparin, the highly acidic constituent of the granules. Mature mast cells are able to proliferate and survive for months.

The basophilic granulocytes are only less than 1% of the peripheral blood leukocytes; unable to proliferate in mature form and their lifetime is quite short: only approximately 60 hours. Their most characteristic feature is the lobed nucleus, the round or oval shaped cytoplasmic granules and their staining by basic dyes. Though basophils and mast cells have very similar, metachromatic staining, they can be distinguished by the position of the stained granules, since they only cover the nucleus of basophils.

Similar to monocytes, eosinophil and neutrophil granulocytes, basophils and mast cells are also differentiated from cord-blood, peripheral blood or bone marrow derived $CD34^+$ progenitors. However, unlike granulocytes, mast cells mature at the periphery.

Bone marrow-derived hematopoietic stem cells differentiate to multipotent progenitors (MMPs), and common myeloid (CMPs) and lymphoid progenitors (CLPs) are derived from them. CMPs then differentiate to megakaryocyte/erithrocyte progenitors (MEPs) and granulocyte/monocyte (GMPs) progenitors. It is still debated that from which progenitor mast cells originate, and whether mast cells or eosinophils are more closely related to basophils.

Basophils have long been in the shadow of mast cells, as they act together in several processes, have similar morphologic properties and similar granule contents, and both of them have high-affinity IgE receptor, FcεRI on their cell surface. Their examination was also

hindered by many factors: they circulate in very low amount and mature in peripheral blood, and as basophil-specific surface marker is still lacking, due to this fact their isolation is from peripheral blood was and still is difficult. Isolation of high-quality RNA in larger amount from basophil samples is also problematic. Moreover, a basophil-free animal model was missing for a long time. Recently, as their role in several important immune processes is getting more obvious, importancy of this cell type increases, which long relegated to the periphery of interest. Both mast cells and basophils were known about their important role in allergy and infections, where they have significant effector functions by releasing histamine and leukotrienes. Both cell type have immunmodulator function, both of them influence Th2 responses. As main Th2 producing cell types they promote naive T cell-Th2 cell altering and B cells maturation into IgE-producing plasma cells. Both play a role in leukocyte recruiting to inflamed tissues. Novel properties of mast cells were discovered in mouse, that they are involved in humoral immune memory. it was previously known that mast cells are involved in Th1 processes, but as for basophils, it was only recently verified.

There is more and more information on the role of different pathogens in cancer, and it has been long known that chronic inflammationmay lead to cancerogenesis. As recently increasing number of reports about the relationship of inflammation and cancer were published, it seemed worthwhile to analyse basophils and mast cells in this context as well.

Mast cells

Mast cells contains several preformed mediators and they can quickly syinthetize other molecules after activation, by which they promote IgE-mediated inflammatory reactions, late-phase hypersensitivity and anaphylactic processes. They support B cells maturation to antibody-producing plasma cells, they play a role in the immune response against parasites, stimulate eosinophil chemotaxis, activation and proliferation, they promote phagocytosis, and several tumor-related processes: tumor development, tumor-inducated angiogenesis, tissue remodeling processes and adaptive immune response against tumors.

Mast cell activation basically takes place in two ways: the most important mechanism is by FcεRI/IgE crosslinking, the other is through non-IgE signals, such as TLRs and their ligands (LPS and nucleic acids), anaphylatoxin C3a and C5a, and activation by certain cytokines and chemokines.

Molecules stored in granules of mast cells are serotonine, histamine, heparin, tryptases and chymases. Other molecules are de novo synthesized after activation, such as lipid mediators: PAF, prostaglandines (PDG2) and leukotrienes (LTB_4 and LTD_4). In addition, Th1 (IFNγ, IL-2, IL-3, GM-CSF and TNFα), Th2 (IL-4, IL-5, IL-6, IL-10, IL-13, IL-33 és GM-CSF), Th17-

related (TGFβ, IL-6, IL-1β és TNFα) cytokines, chemokines (CCL3, CCL5, MIP-1β, MCP-1, MIP-2, CXCL8) and angiogen factors (VEGF, NGF, FGF) can release from mast cells.

Basophils

Basophils, similarly to mast cells, as main effectors of hypersensitivity reactions, contain inflamatory mediators and histamine. They can *de novo* synthetize other molecules, typically they produce Th2 cytokines, they promote B cells into mature Ig-producing plasma cells, they are important in bacterial and helminth infections, play a role in recruiting other cells into the place of inflammation, and in humoral immune memory as well.

The importance of basophils in AML is significant. However, data are not available about the function of basophils in tumor tissues, but since they are able to migrate from the bloodstream into the tissue, it is likely that such studies will be cerried out in the future.

The well known receptor of basophils is the IgE/antigen-binding high-affinity FcεRI. Activation through TLR acts synergistically with IgE-mediated activation and cytokines promote Th2 differentiation. Their cytokine receptors are CD123 (receptor of IL-3), CD125, CD116 (receptors of IL-5 and GM-CSF), IL-18 receptor, as well as ST2 (receptor of IL-33, promoting the Th2 differentiation). Basophils have many chemokine receptors as well. Different complement proteins, adhesion proteins, "homing" -, death-, and some receptors with role in angiogenesis are also expressed on their surface.

Amongst molecules stored in basophils are histamine, serotonin, basophil proteases. Lipid mediators found in basophils are PAF and LTC4. Moreover the production of Th2 cytokines (mainly IL-4, IL-13 and TSLP), chemokines (IL-8, CCL3, CCL4, CCL5) and angiogenic factor (VEGF) also characterizes them.

microRNAs

MicroRNAs are a group of small, evolutionarily conserved, non-coding RNA families, whose members negatively regulate gene expression at post-transcriptional level. They bind to the complementary sequences in the 3' untranslated region of their target mRNA inducing their degradation or inhibition of translation. MicroRNAs are formed in a special way: the primary microRNAs are encoded in the DNA of a cell, they are transcribed in the nucleus, and the Drosha enzyme processes and exports them to the cytoplasm where the RNase Dicer further processes them. Finally, the mature single-stranded microRNAs are 19 to 23 nucleotide-long. MicroRNAs were considered important factors of cell development and differentiation, but soon their importance was confirmed in a variety of other normal and pathological biological processes, such as apoptosis, signal transduction, organ development; or a number of human

diseases, like congenital malformations, cancer (including hematological disorders), inflammatory diseases, asthma and infection. MicroRNAs have decisive importance in normal hematopoiesis, while they regulate almost the whole hematopoiesis, however their abnormal expression is associated with solid tumors and hematopoietic malignancies.

Aims

It has long been known among researchers and clinicians that abnormal inflammatory processes, particularly if they are chronic, result in tumor formation in the body. For example there is evidence that inflammatory mediators, which can cause acute inflammation and after that, when normally regulated, they can promote development, invasivity and angiogen activity of certain tumors. Therefore, it can be useful to investigate main inflammatory cells from this point of view. There is a plethora of information about the inflammatory mediators (e.g. histamine) play significant role in tumor processes. While it is fairly unknown which other, previously unknown molecules play a role in the tumor-related functions of inflammatory cells.

Our aims were

- to identify new genes and microRNAs, which contribute cell functions of mast cells and of hardly examined basophils in tumor processes.

- to find protease and protease inhibitors, whose expression previously had been unknown in mast cells.

- to validate expression of selected genes by real-time PCR and confirmation of the expression of the functionally relevant genes at protein level.

- to estimate validated gene functions based on publications about the role of certain genes in other cells and tissues.

- to validate expression of selected microRNAs in basophils, which were chosen based on literature data

- to investigate the effects of hIL-3 on basophil differentiation and activation of the expression of theidentified microRNAs.

Methods

Differentiation of BMMC

Balb/c 2–4-month-old female mice were killed by cervical dislocation and both of their femora were removed and flushed with FCS. Cells were washed twice by PBS. $CD117^+$ stem cells were magnetically isolated twice by MACS LS separation columns and the bone marrow stem cells were cultured in complete DMEM medium The culture medium was supplemented with 4 ng/ml recombinant mouse IL-3 and 40 ng/ml recombinant mouse stem cell factor. In some experiments 2 ng/ml human TGFβI and 10 ng/ml IL-9 were also added to obtain the in vitro homologues of mucosal mast cells. Half of the medium was replaced in every 4–5 days. After 20 days the purity of mast cell preparations was checked by flow cytometry for CD117 and by alcian blue-safranin staining.

To investigate gene expression changes during mast cell differentiation, cells were magnetically isolated on the bases of their surface c-kit expression on the 4th and 20th day of culture.

Cord blood-derived mast cells

Mononuclear cells from cord blood samples were separated on Ficoll-Hypaque and then $CD34^+$ stem cells were magnetically isolated according to the manufacturer. Cells were cultured in 10% FCS-containing complete DMEM medium supplemented with 40 ng/ml SCF, 20 ng/ml IL-6 and 3 μM lysophosphatidic acid as it has been recommended to stimulate mast cell development. Half of the medium was replaced in every 4–6 days. After 6 weeks $CD117^+$ mast cells were separated with mouse anti-human CD117 antibody and magnetically labeled goat anti-mouse IgG.

Isolation of peritoneal mast cells

Balb/c 2–4-month-old female mice were killed by cervical dislocation and the peritoneal cavity was flushed twice with 15 ml ice-cold PBS. Cells were then washed by PBS and mast cells were separated twice by the magnetic by MACS LS separation columns. The purity of the isolated cell population was checked by alcian blue-safranin staining and flow cytometry.

Toluidine blue and alcian blue-safranin staining

Small aliquots of mast cell cultures were cytocentrifuged, fixed in ice cold methanol for 10 min and stained with 0.1% toluidine blue (pH 1) or alcian blue-safranin for 45 min at room temperature. The ratio of positive cells (in case of toluidine blue the metachromatic cells) was counted and cultures containing > 90% mast cells were used for further studies.

Flow cytometry

Cells were washed twice with PBS, labeled at room temperature for 25 min and then analyzed by a FACSCalibur flow cytometer. The following antibodies were used in our experiments: anti-human CD117 PE, antimouse CD117 APC, anti-mouse B220 PE, anti-mouse CD11b PE, mouse anti-human tryptase, mouse anti-human chymase and anti-mouse IgG FITC. For intracellular FACS analysis, before labeling cells were fixed in 2% PFA for 20 min and permeabilized by 0.1% saponin.

RNA isolation, quality determination and real-time PCR

RNAs from cell samples were prepared by RNeasy columns, the quality and quantity of total RNA was determined with an Agilent 2100 Bioanalyzer. Only those samples were used either for microarray or for real-time PCR experiments that gave >8.0 for RNA integrity number, showed a clear gel image and no DNA contamination was observed on the histogram.

One microgram RNA was reverse transcribed by 1U MuLV reverse transcriptase with random primers at 42 ◦C for 55 min. MuLV was then inactivated at 95 ◦C for 5 min. The real-time PCR reactions were carried out with 1.5µl cDNA in each well and in 25µl final volume in an ABIPrism 7000 instrument according to the manufacturer.

Microarray experiments and ingenuity pathway analysis

For microarray experiments the Agilent Mouse Oligonucleotide 22K chips were used. Gene expression data transferred from GeneSpring 7.3 were further analysed by Ingenuity Pathway Analysis software.

Immunoblotting

Cells were denatured in lysis buffer and protein concentration was determined by Bradford method. The 15 µg protein/sample was denatured and reduced in β- mercaptoethanol at 100°C for 5 min, applied to10% SDS-PAGE and then transferred to P-Immobilon membrane. The full-range Rainbow marker was used as molecular weight marker.

Blots were blocked with 10% milk powder in PBS for 1 hour and then incubated with the primary antibody in PBS containing 1% milk powder and 0.1% Tween-20 for 2 h at room temperature. After excessive washings HRP-conjugated secondary antibodies were applied for 45 min and the signals were developed by ECL Plus Western Blotting Detection System. tubulin was used as housekeeping control. The following antibodies were used: rabbit polyclonal anti-N-terminal HtrA1 antibody (1:250 dilution), mouse anti-human HtrA1 (0.5 μg/ml), rat anti-tubulin antibody (1:1000 dilution), anti-rat HRP (1:10,000), anti-mouse HRP(1:10,000), anti-rabbit HRP (1:8000).

ELISA assays

Histamine ELISA was carried out by using a commercially available (Beckman Coulter) kit. To detect secreted HtrA1 protein in cell culture supernatants, an in-house developed ELISA was used. Maxisorp plates were coated with rabbit polyclonal anti-HtrA1 N-terminal antibody (0.5 μg/well in 100 μl) overnight and then blocked with 1% BSA + 0.5% Tween-20 in PBS. As a capturing antibody monoclonal anti-human HtrA1 was used at 1 μg/ml and then HRP-conjugated anti-mouse antibody was applied (1:10,000 dilution). The reaction was developed by tetramethylbenzidine, stopped by $2NH_2SO_4$ and then analyzed at 450 and 540 nm.

Confocal laser scanning microscopy

Aliquots of human mast cell cultures were cytocentrifuged and fixed in 2% paraformaldehyde for 10 min. Specimens were washed three times in washing buffer. Then specimens were incubated with non-immune mouse serum to block the aspecific binding sites for 45 min at room temperature. Cells were labeled with mouse anti-human-HtrA1 primary antibody (5 μg/ml) and incubated for 60 min at room temperature, then washed three times in PBS. Cells were incubated with FITC labeled anti-mouse secondary antibody for 40 min at room temperature and stained with Daunorubicin. The mounted slides were analyzed in a Bio-Rad MRC 1024 confocal laser scanning microscope equipped with a krypton/argon mixed gas laser as light source. Excitation was carried out with laser with 480 nm . All negative controls (primary antibody was omitted) demonstrated negligible background fluorescence.

Statistical analysis

Our results were statistically evaluated by using Student's t-test, one-way ANOVA and Tukey HSD post hoc tests and Microsoft Excel or Statistica version 7 softwares.

Human blood samples

Peripheral blood samples were obtained from normal adult healthy blood donors of the Hungarian National Blood Transfusion Service. During the entire investigation period we followed the guidelines and regulations of the Helsinki Declaration in 1975, and the experiments were approved by the Hungarian Science Ethical Committee.

Immunomagnetic separation of basophils from human peripheral blood samples and the in vitro stimulation of human granulocytes by IL-3

To isolate basophils from peripheral blood, we used a two-step procedure, where the first step is a gradient based separation by HetaSep^{TM}, and then, basophils were negatively isolated by using EasySep Human Basophil Enrichment Kit and standing magnet. The purity of isolated basophils was assessed by flow cytometry (FSC/SSC) using a FACSCalibur flow cytometer and by May-Grünwald staining.

Basophils purified to near homogeneity (>99% pure) were cultured in complete RPMI medium (supplemented with 10% FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin) for 100 minutes in the presence or absence of 10 ng/µl hIL-3, using 3-5x10^5 cells per well in flat-bottom 96-well plates. For the analysis of cluster formation upon hIL-3 treatment Nikon Diaphot TMD-EF microscope was used.

Staining basophils by May-Grünwald solution

Small aliquots of basophil samples were cytocentrifugated and stained by May-Grünwald solution.

Isolation and detection of miRNAs by human basophil granulocytes and the effect of hIL-3 on miRNA expression

Single primer reactions with TaqMan ”MicroRNA Cells to Ct Kit” were used for both isolation and detection of miRNAs, according to the manufacturer’s instructions. Gene expression analysis was assessed using ΔΔCt method. Delta-delta Ct values were normalized using RNU48 as an endogenous miRNA control.

Real-time PCR amplifications were carried out with sequence-specific primers from TaqMan microRNA Assays (RNU48, hsa-miR-16, hsa-miR-155, hsa-miR-223) in ABIPrism 7000 instrument. For adjusting technical features, a pilot experiment was carried out as suggested by manufacturer (data not shown).

In order to investigate the effect of hIL-3 treatment on miRNA expression of miR-223, miR-155 and miR-16, after 10 ng/ml hIL-3 treatment for 100 minutes, real-time PCR experiments were carried out.

Target prediction and pathway analysis of detected miRNAs

To screen potential targets of hsa-miR-223, hsa-miR-155 and hsa-miR-16, multiple miRNA analysis was performed using DIANA-mirPath software, where pathways were identified by at least three of the algorithms. This multiple miRNA analysis identifies pathways in which these three miRNAs together play a role.

Results

Model system for the differentiation of mucosal mast cell homologues

To detect novel genes that are differentially expressed during *in vitro* mouse mucosal mast cell differentiation, cells were cultured in the presence of SCF + IL-3 + IL-9 + TGF-βI and magnetically separated at two time points by their surface c-kit expression. The first one (day 4) represented a stage when mainly mast cell progenitors were found in cell culture. Fully maturated mucosal mast cell homologues were investigated on the 20th day. The purity of the isolated samples was > 95% as verified by FACS analysis. Alcian blue-safranin staining showed that mucosal mast cell precursors were negative on the 4th day and expressed Mcpt1 at a low level, while on the 20th day all cells showed a blue staining and there was a pronounced increase in Mcpt1 production, confirming the presence of mature mast cells committed to the mucosal type. Furthermore, mast cells cultured in IL-3 + SCF + IL-9 + TGF-βI for 20 days showed a lower c-kit expression than cells maintained in medium that lacked IL-9 and TGF-βI and they expressed a much higher the level of mature mucosal mast cell-specific Mcpt1 mRNA. These results are in good agreement with our prior assumption that this model system represents the differentiation of mucosal mast cell homologues.

Selection of protease/protease inhibitor genes differentially expressed during mucosal mast cell maturation

To identify genes that change their expression level during mucosal mast cell differentiation, samples isolated on day 4 and 20 were compared in dual-channel microarray experiments and genes showing a > 2.0-fold upregulation at $p < 0.05$ level (with Benjamini–Hochberg false discovery rate multiple testing correction) were filtered out. Since mast cells are rich sources of proteases, out of the 240 significantly upregulated genes proteases/inhibitors were selected.

As expected, the list contains many well-known, mast cell specific protease genes (*Mcpt5, Mcpt6, Mcpt1*) and genes (tryptase gamma1, also known as transmembrane tryptase) that can be characterized by a less understood function. However, we were also able to identify some protease/inhibitor genes (e.g. *Spink2* or *HtrA1*) that have not been described in mast cells yet. Microarray results were validated by real-time PCR for *Prss11* (*HtrA1*), *Spink2*, *Tpsg1*, *Mcpt6* and for *Mcpt1* as positive control.

HtrA1 (Prss11) is specific for mature mucosal mast cells

The expression level of selected protease/inhibitor genes was examined in different mast cell populations. Mature mucosal mast cells were differentiated in the presence of IL-3, SCF, IL-9 and TGFβI in the culture, while immature mast cells were cultured in the lack of IL9 and TGFβI. Connective tissue type mast cells were magnetically isolated from the peritoneal cavity of Balb/c mice. When comparing the expression level of the selected protease/inhibitor, genes *HtrA1* turned out to be specific for mature mucosal mast cells. Since changes in the mRNA-level do not always correlate with protein levels, HtrA1 expression was investigated by Western-blotting, too. HtrA1 protein was almost undetectable in immature mucosal mast cells (maintained in only IL-3 + SCF) even after 20 days of culture. Furthermore, mature mucosal mast cells isolated on day 20 contained more Prss11 protein than progenitor cells on the 4th day, thus our microarray results were validatedat protein level.

HTRA1 is also expressed in human MC(T) mast cells

As many papers discuss the differences between murine and human mast cells, we also investigated the presence of HTRA1 in the human system. Cord blood-derived mast cells showed a metachromatic staining by toluidine blue after 6 weeks of culture, furthermore, they were uniformly positive for tryptase, but did not contain chymase at all, which is characteristic for MC(T) cells. The appearing MC(T) cells consequently expressed HTRA1, although at varying level. Furthermore, in contrast to many other proteases, HTRA1 did not seem to be stored in the secretory granules of mast cells.

HTRA1 is constitutively released from human mast cells

As HTRA1 is not localized in the secretory granules, it may be constitutively secreted (similar to the MCPT1 chymase) from mast cells. This notion is supported by our ELISA results showing that supernatants of both unstimulated and Ca-ionophore (ionomycin)-treated human MC(T) mast cell cultures did not differ in their HTRA1 content. The degranulating effect of

ionomycin was proven by the elevated histamine concentration in the supernatants of treated cell cultures.

HtrA1 does not influence the TGF-β-induced murine mucosal mast cell differentiation

Ingenuity pathway analysis of our gene expression data showed that HtrA1 is involved in two gene networks, one of them containing genes involved in cell death or cancer, while the other group contains genes participating in cellular-, connective tissue development and function, or skeletal and muscular development. Data suggested that *HtrA1* had only two direct relationships with other genes, one of them with *Tgf-βI* and the other one with *Bmp4* that is also a member of the TGF-β family. Furthermore, the TGF-βI inhibiting activity of HtrA1 was also published. Based on these findings we tested whether HtrA1 has a TGF-β-inhibitory effect on mucosal mast cell differentiation. The two chosen HtrA1 concentrations were based on values found in our human mast cell supernatants (5 ng/ml) and on published data. Surprisingly, HtrA1 did not change either the Mcpt1 expression level or cell surface c-kit expression, thus showing no TGF-β inhibitory effect on the differentiation of murine mucosal mast cells in our model system.

Isolated basophil purity

Isolated basophils from peripheral blood seemed to be near to homogeneity (>99% pure).

Effect of hIL-3 treatment on basophils in in vitro

After 100 minutes of treatment by hIL-3, a pronounced clustering of cells could be observed. Basophils, cultured in the presence of hIL-3, formed clusters, while control cells remained evenly distributed in the culture.

microRNA expression in human basophils

Untreated basophils expressed miRNAs miR-223 and miR-155 and miR-16. While miR-223 and miR-155 have been implicated in modulation of granulocyte differentiation, miR-16 is highly expressed in all native hematopoietic cell lineages as well as in a broad range of tissues. Both miR-16 and miR-223 were highly expressed, while miR-155 expression was lower, albeit pronounced in our healthy human basophil samples.

Effect of hIL-3 treatment on microRNA expression of basophils

All of the investigated miRNAs showed reduced expression after hIL-3 treatment. The most pronounced change in expression among these three miRNAs was observed in the case of mir-223, followed by mir-155 and mir-16.

Target prediction and pathway analysis results of detected microRNAs

Among the 5 algoritms, used by DIANAmirPATH software, in at least 3 we found 'FcεRI signaling pathway' and 'Histidine metabolism', which are in connection with paramount functions of basophil granulocytes.

Conclusions

- Based on microarray experiment of murine mucosal mast cell differentiation, we detected a novel serine-protease, HtrA1 both at mRNA and at protein level, which is specific for murine mucosal and for human tryptase-type mast cells.

- We verified that HtrA1 is not only expressed in mucosal mast cells, but it is specific for this mast cell type, as its expression seemed to be significantly lower in the other murine mast cell type, the connective type mast cells.

- Although it is known from the literature that HtrA1 inhibits Tgfβ signaling pathway, we established by our experiments that HtrA1 can't influence the Tgfβ-inducated mast cell differentiation, but according to our assumptions, it could play a role in mast cell induced tissue remodeling.

- We provide the first data about microRNA expression in basophils. We identified miR-223, miR155 and miR-16 expression in human basophils. The most pronounced expression was in of the miR-223, which microRNA has a key role in myeloid and granulocyte differentiation, and it has been connected with several tumor type.

- We detected that these microRNAs were downregulated after hIL-3 treatment, a cytokine required for basophil differentiation, activation and survival.

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Note: * signed impact factor data means impact factor of the previous year