ROLE OF INTRACELLULAR HISTAMINE DURING IN VITRO DIFFERENTIATION OF DENDRITIC CELLS.

INVESTIGATION OF SUBPOPULATIONS OF DENDRITIC CELLS.

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Budapest, 2001

INTRODUCTION

It is known that histamine has an important role in different physiological and pathophysiological processes like vasodilatation, increasing blood vessel permeability, smooth muscle contraction, production of gastric acid, immunmodulation and neurotransmission. In these effects, histamine acts through H_1, H_2 and H_3 membrane receptors.

Recently histamine was shown to act not only on surface receptors but intracellularly as well. As an autocrine or intracrine mediator, histamine may have a possible role in regulating cell proliferation, differentiation, hematopoiesis, embryonic development, regeneration and wound healing.

Histamine is produced from histidine by histidine decarboxylase (HDC), which can be found in nearly every cell.

The main aim of our experiments was to investigate the cell differentiation modulating effect of intracellular histamine. We used a model of in vitro differentiation of monocytes towards dendritic cells. Dendritic cells are professional antigen-presenting cells; they efficiently take up and process antigens and activate T-cells through MHC Class II-peptide complexes.

During in vitro differentiation of dendritic cells, we wanted to detect the changes in the level of histidine decarboxylase and histamine by flow cytometry.

Previously we worked out a flow cytometric method for measuring HDC-content of leukocytes. The different leukocyte populations and lymphocyte subpopulations were distinguished by cell surface direct labelling and indirect intracellular labelling was used to detect histidine decarboxylase. Using this method we determined changes in the HDC-content of lymphocyte subpopulations in bacterial infections.

We also investigated the effect of histamine and its analogues on the superoxide production of leukocytes.

During in vitro differentiation of dendritic cells we measured cell surface antigens paralelly with the histamine and HDC-content. We studied how histamine receptor

antagonists and a histamine synthesis blocker influence the expression of differentiation antigens on the dendritic cells during in vitro maturation.
We wanted to characterize small dendritic cell populations in peripheral blood by their cell surface markers.

AIMS OF THE STUDY

Detection of histidine decarboxylase in different leukocyte populations and lymphocyte subpopulations

In our first experiments we wanted to determine the histidine decarboxylase content of different leukocyte populations by flow cytometry. Our question was whether there are detectable differences in the HDC-content of leukocyte populations and lymphocyte subsets from the same donor.

Measurement of HDC-content in lymphocyte subsets in healthy individuals and patients suffering from bacterial infections

Histamine and histidine decarboxylase can be detected in lymphocytes. The immune-modulating effect of histamine is well known. The activation state of lymphocytes can be influenced by histamine released from surrounding cells or intracellular histamine. The aim of our experiments was to find differences between the HDC-content of lymphocytes from healthy individuals and from patients suffering from systemic bacterial infections.

Determination of changes in intracellular histamine- and histidine decarboxylase-content and cell surface marker expression during in vitro differentiation of monocytes towards dendritic cells and macrophages

We wanted to determine how the quantity of intracellular histamine changes during cell differentiation. To this study we used the model of in vitro differentiation of monocytes towards dendritic cells and macrophages. Monocytes cultured in vitro in the presence of M-CSF differentiate to macrophages, in the presence of GM-CSF and IL-4 they differentiate to dendritic cells. Expression of cell surface markers was investigated by flow cytometry paralelly with histidine decarboxylase and intracellular histamine level.

Investigation of the effect of histamine-blocking agents on the expression of cell surface markers during in vitro differentiation of dendritic cells

In our next experiments, we wanted to investigate the influence of compounds which block the effects of histamine on the process of dendritic cell differentiation. Cells were treated with H_1-receptor antagonist triprolidine, H_2-receptor antagonist tiotidine, DPPE, which binds to the intracellular histamine binding site, and α-fluoromethyl-histidine, an irreversible blocker of HDC.

The changes in the expression of cell surface markers were followed by flow cytometry.

Study of dendritic cell subpopulations in peripheral blood

Dendritic cells are present in immune organs like the spleen, lymph nodes and tonsils. The precursors of dendritic cells can be found in peripheral blood as well, but their detection raises difficulties as they are present only in a small amount and no exclusively specific marker for these cells is available.

Based on coexpression analysis of cell surface several subpopulations of mononuclear phagocytes have been described. Heterogeonous analytical approaches were used in these studies, so the comparison between subsets was difficult.

The aim of our study was to identify and characterize peripheral blood dendritic cells as subsets of mononuclear phagocytes by their combinations of cell surface markers by flow cytometry. Directly stained whole blood samples were used in our experiments, as this approach is close to the in vivo situation.

In pathological states the quantity and ratio of dendritic cell precursors can change. By determining the normal values, our study can be a starting point for further phenotype and functional investigations of changing dendritic cell subsets in different diseases.

Effect of histamine and histamine-agonists on the NADPH-oxidase activity of leuocytes

In granulocytes and monocytes, NADPH-oxidase enzyme activates upon stimulation and superoxide anion is formed from molecular oxygene. From superoxide anion reactive oxygene intermediers are produced. At the site of inflammation, these free radicals can induce the release of histamine from mast cells. Furthermore, leukocytes can synthetise histamine, as we showed previously. The purpose of the study was to determine whether histamine has an effect on the oxidative burst activity of human leukocytes.

METHODS

Most of our measurements were performed by **flow cytometry**.

To determine histidine decarboxylase content in different leukocyte populations, heparinized blood samples were used. Blood samples were from healthy donors and from patients attending Szent László Hospital of Infectious Diseases, suffering from systemic bacterial infections.
The indirect flow cytometric method for detecting intracellular histidine decarboxylase was developed on melanoma cell lines by our laboratory. We introduced the method of measuring HDC by flow cytometry on leukocytes as well. After cell surface labelling samples were fixed, permeabilized and labelled by chicken anti-HDC antibodies raised against epitopes 3-14 and 318-325. FITC-labelled rabbit anti-chicken secondary antibody was used. Measurement and data analysis was performed by Coulter Epics Elite (software: Elite 4.01), and Becton Dickinson FACSCalibur (software: Cell Quest 3.1) flow cytometers.

In our next experiments **the changes in intracellular histamine and histidine decarboxylase-content and expression of cell surface markers during in vitro dendritic cell differentiation were determined.**

Leukocytes were gained by leukapheresis from healthy volunteers, high purity monocyte suspension was obtained according to a standard elutriation protocol. The cells were cultured for 7-14 days in Macrophage-SFM medium in the presence of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and rekombinant human interleukin-4 (IL-4) to get dendritic cells, and in the presence of rekombinant human macrophage colony-stimulating factort to get macrophages.

Cells were treated by compounds blocking the effects of histamine: triprolidine (H_1-receptor antagonist), tiotidine (H_2-receptor antagonist), N,N-diethyl-2-[4(phenylmethyl)-phenoxy]-ethanamine (DPPE, intracellular histamine binding site blocker), α-fluoromethyl-histidine (irreversible HDC blocker).

Treated and control cells were labelled with fluorescent antibodies on the day of elutriation, on the 1st, 2nd, 3rd, 5th, 7th, 9th, 12th, 14th day of culture.

10^6 cells were incubated with combinations of antibodies against cell surface antigens. The following markers were investigated: CD80 (B7.1) and CD86 (B7.2) costimulatory molecules, CD14 (LPS-receptor), CD16 (Fcγ receptor III), CD33 (pan-myeloid marker), CD45 (common leukocyte marker), CD123 (IL3-receptor α-chain), HLA-DR (MHC II), CD11c (β_2-integrin α-chain) and CD40 (costimulatory molecule). Two-and four colour measurements were performed.

Indirect detection of histidine decarboxylase was performed as described previously. To measure intracellular histamine by flow cytometry, cells were fixed in the mixture of carbodiimide-paraformaldehyde, permeabilized in saponine, labelled with rabbit anti-histmine antibody and secondary goat anti-rabbit antibody.

Samples were measured within a few hours after labelling. A total of 10000 leukocytes were measured per sample by FACSCalibur flow cytometer (Becton Dickinson). The analysis was made by CellQuest 3.2 software.

Histidine decarboxylase was detected by western blotting as well. HDC mRNA was measured by competitive RT-PCR.

To investigate subpopulations of mononuclear cells heparinized whole blood samples from healthy individuals were used. This method has several advantages: no separation or sorting steps are required which may lead to selective cell losses, the cells are not disturbed or activated so cellular phenotypes are close to the in vivo situation and finally quantitative cell counts can be performed as the basis for quantitative clinical studies.

Whole blood samples were incubated with combinations of fluorochrome (FITC, PE, PerCP, APC) labelled antibodies against cell surface markers. Data for at least 100000 leukocytes per sample were acquired by FacsCalibur flow cytometer. Four-colour analysis was performed by CellQuest 3.1.f and Paint-A-Gate softwares. Leukocyte populations were defined by cell surface marker expression.

To investigate the effect of histamine and its agonists on oxidative burst whole blood leukocytes and separated mononuclear cells were used.
Separated mononuclear cells were gained by Ficoll-gradient-centrifugation.
NADPH-oxidase activity was measured by the intracellular oxidation of dihydrorhodamin-123 using a FACScan flow cytometer.

RESULTS

Detection of histidine decarboxylase in leukocytes

We introduced the method of measuring histidine decarboxylase by flow cytometry on leukocytes. We could detect the presence of HDC in the leukocyte populations analysed (granulocytes, monocytes, T_h-, T_c-, B-lymphocytes, NK cells). Quantitative differences were found between the HDC-content of lymphocyte populations. In healthy individuals B-lymphocytes contained the highest amount of histidine decarboxylase, smaller amount can be detected in helper, cytotoxic T-lymphocytes and NK-cells.

HDC-content of lymphocytes in patients suffering from bacterial infections

The histidine decarboxylase-content and distribution of lymphocyte subpopulations in patients suffering from systemic bacterial infections differed from that of healthy individuals. In these patients the highest amount of HDC was found in helper or cytotoxic T lymphocytes.
The HDC-content of T-lymphocytes was more homogenous in healthy donors than in patients suffering in bacterial infections, as in the latter, a subpopulation appeared where higher HDC-levels could be detected.

We do not know whether the subset with higher HDC content can be connected with the activation state or differentiative or proliferative capacity of T-lymphocytes.
These data suggest that local histamine production may play an important role in the activation of peripheral lymphocytes during the immune responses to bacterial infections.

Determination of changes in intracellular histamine- and histidine decraboxylase content and cell surface marker expression during in vitro differentiation of monocytes

During in vitro differentitaion of dendritic cells, the amount of histidine decarboxylase and the intracellular histamine content increased simultaneously, with a peak on the fifth day. Similar changes were found during in vitro differentiation of monocytes towerd macrophages.
Changes in HDC-content were detected by flow cytometry and western blotting. Parallel changes in histidine decarboxylase and histamine were measured by flow cytometry.
Increase in the amount of HDC mRNA preceeded elevation of HDC-protein level, as shown by our RT-PCR results.
In vitro differentiation of monocytes towards dendritic cells was followed by the measurement of expression of ten cell surface markers: CD80, CD86, CD14, CD16, CD33, CD45, CD123, HLA-DR, CD11c and CD40. The changes were used as starting points to describe further changes observed during treatment with histamine blockers.

Effect of histamine-blockers on the expression of cell surface markers during dendritic cell differentiation

In vitro differentiating dendritic cells were treated by agents blocking the effect of histamine and the expression of cell surface markers were investigated.
All histamine-blockers examined decreased the rate of elevation of the surface expression of CD40.

H_2 receptor antagonist tiotidine (and to a smaller extent, H_1 receptor-blocker triprolidine) decreased the expression of CD45.

These data demonstrate that locally produced histamine influences cell surface expression of CD40 and CD45.

N,N-diethyl-2-[4(phenylmethyl)-phenoxy]-ethanamine (DPPE), which blocks intracellular histamine binding sites, was able to downregulate six (of the ten examined) differentiation antigens, including CD40, CD86, CD45, CD33, HLA-DR and CD11c. The peak of the inhibition was around the fifth day, similar to the highest point in the elevation of HDC expression.

Our results show intracellular histamine level increases simultaneously with the histidine decarboxylase content during dendritic cell differentiation. Treatment of differentiating dendritic cells with histamine blocking agents disturbs the expression of cell surface markers, thus the process of differentiation. This means elevation of histamine level is not only a passive, secondary process during differentiation, but the produced histamine actively modulates cell differentiation.

Investigation of cell surface markers in peripheral blood dendritic cell subpopulations

By flow cytometric analysis of cell surface markers, we could distinguish six subpopulations of mononuclear cells in blood, of which two small dendritic cell precursor subsets were found. Quantitative changes or phenotypic shifts of dendritic cell precursors in circulating blood can be of pathophysiological relevance.

Effect of histamine and its agonists on NADPH oxidase activity

In granulocytes and monocytes histamine alone could stimulate oxidative burst. H_2-receptor agonist amthamin could had a greater stimulatory effect than histamine.

CONCLUSIONS

Histamine, which can locally influence cytokine production and can modulate the effects of cytokines, has a role in the process of differentiation. Histamine can be detected in nearly every cell, including leukocytes and in vitro differentiating monocytes towards dendritic cells and macrophages. Intracellular histamine and histidine decarboxylase content increases suring dendritic cell differentiation, histamine-blocking agents modulate the process of differentiation. This suggests that elevation of histamine content is not only a passive, secondary process during differentiation, but produced histamine actively influences cell differentiation.

Precursors of dendritic cells appear in peripheral blood, detection and characterization of these small populations can be used for comparison of further investigations where the quantitative and functional changes would be followed in pathological states.

SUMMARY OF RESULTS

1. **We introduced a method for measuring histidine decarboxylase in leukocytes by flow cytometry, and quantitative differences were found between the HDC-content of lymphocyte subpopulations.**

2. **The histidine decarboxylase content and distribution of lymphocytes from patients suffering from systemic bacterial infections differs from that of healthy individuals.**

3. **During in vitro differentiation of dendritic cells, histidine decarboxylase- and histamine content was measured by flow cytometry together with the expression of cell surface markers. HDC- and histamine content increased simultaneously during in vitro differentiation of dendritic cells.**

4. **Cell surface markers of differentiating dendritic cells were investigated after treatment with histamine blocking agents. The histamine blocking agents were found to modulate the process of differentiation.**

5. **By flow cytometric analysis of cell surface markers, we could distinguish six subpopulations of mononuclear cells in blood, of which two small dendritic cell precursor subsets were found.**

6. **In our experiments, histamine increased oxidative burst activity of leukocytes, greater stimulation could be achieved by the use of H_2-agonist amthamine.**

ACKNOWLEDGEMENTS

I should like to thank

- prof. Dr. András Falus, head of the Department of Genetics, Cell- and Immunobiology, the project leader, who helped and supported my research work from the beginning
- Dr. Éva Pállinger, who taught me aki the theory and practice of flow cytometry and gave a lot of useful advice,
- Mr. Tamás Reichenbach, technical support engineer at Becton Dickinson, to whom I could turn any time with any kind of flow cytometry questions
- Dr. Valéria László for her valuable cooperation.
- Most of my experiments were performed at the Institute for Clinical Chemistry and Laboratory Medicine of the University of Regensburg, with the scholarship from the Deutscher Akademischer Austauschdienst.. I'd like to thank prof. Dr. Gerd Schmitz, Dr. Gregor Rothe, and Dr. Evelin Orsó for their help.
- I am grateful to prof. Dr. Erzsébet Ligeti és Dr. Miklós Geiszt who made me acquainted with the joy of research at the Institute of Physiology as a student researcher.
- I'd like to thank Mrs. Angéla Kozák, Mrs Farkas Krisztina Nagy, Mrs. Judit Ördögh and Mrs Mária Balázs for teaching me the methods and for the technical support in the experiments.

- I am grateful to the colleagues at the Department of Genetics, Cell-and Immunobiology for giving me advice and for the friendly atmosphere in the Department.

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