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# Differential expression of pentraxin 3 in neutrophils

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ABSTRACT

Pentraxins belong to the superfamily of conserved proteins that are characterized by a cyclic multimeric structure. Pentraxin 3 (PTX3) is a long pentraxin which can be produced by different cell types upon exposure to various inflammatory signals. Inside the neutrophil PTX3 is stored in form of granules localized in the cytoplasm. Neutrophilic granules are divided into three types: azurophilic (primary) granules, specific (secondary) granules and gelatinase (tertiary) granules. PTX3 has been considered to be localized in specific (secondary) granules. Immunofluorescent analyses using confocal laser microscopic examination were performed to clarify the localization of all three groups of granules within the cytoplasm of the mature neutrophils and neutrophils stimulated with IL-8. Furthermore, PTX3 was localized in primary granules of promyelocyte cell line HL-60. As a result, we suggest that PTX3 is localized not only in specific granules, but is also partly expressed in primary and tertiary granules. After the stimulation with IL-8, irregular reticular structures called neutrophil extracellular traps (NETs) were formed, three types of granules were trapped by NETs and PTX3 showed partial colocalization with these granular components. PTX3 localized in all three types of granules in neutrophils may play important roles in host defense.



## 1. Introduction

Polymorphonuclear neutrophils (or PMNs) are the most numerous leukocytes in mammals, much more numerous than the longer-lived monocytes/macrophages (Witko-Sarsat et al., 2000). PMNs are fast acting and effective phagocytes, which play a very important role in innate and adaptive immunity (Jena et al., 2012). They also stand as a first-line defense against invading pathogens (Borregaard, 2010). Neutrophils have various types of granules, containing lots of antibacterial proteins which can kill microbes and digest tissues (Borregaard, 2010; Kaplan and Radic, 2012). Neutrophil granules have been divided into three major subsets according to differences in protein content and propensity for mobilization (Hager et al., 2010). These subsets are azurophilic (primary) granules, specific (secondary) granules and gelatinase (tertiary) granules. The primary granules contain myeloperoxidase (MPO), neutrophil elastase, azurocidin and others, secondary granules contain lactoferrin and cathelicidin, and tertiary granules contain gelatinase, cathepsin and others (Jaillon et al., 2007). These granules appear in the PMNs sequentially as a result of granulocytic differentiation in the bone marrow, from myeloblast to the segmented stage when maturation is reached (Borregaard, 2010; Papayannopoulos and Zychlinsky, 2009).

Pentraxins are small pentameric innate immunity effector proteins, which are very important in infectious and inflammatory responses (Bottazzi et al., 2006). Pentraxin 3 (PTX3) is the member of the pentraxin superfamily, which expression is induced in response to inflammatory signals. It is characterized by the presence of the carboxil terminal region, called pentraxin domain. PTX3 is the first identified long pentraxin (Mantovani et al., 2008). A variety of cell types, particularly macrophages, polymorphonuclear neutrophils, dendritic cells, endothelial cells and smooth muscle cells can produce PTX3 upon exposure to primary inflammatory signals, such as lipopolysaccharide and other agonists for Toll-like receptor family, IL-1β, and TNF-α, as well as oxidized low-density-lipoproteins (Bottazzi et al., 2006).

Although previous studies demonstrated that PTX3 inside the neutrophil is stored in form of specific (secondary) granules in the cytoplasm (Jaillon et al., 2007), the strict co-localization of PTX3 with other granular proteins in the PMNs is not fully confirmed. In this study, using antibodies for granular components in the three groups of granules, we have investigated the localization of PTX3 and its relation to granules inside the neutrophil and those adhered to degenerated neutrophils called NETs.

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## 2. Materials and methods

### 2.1. Isolation of neutrophils

Human polymorphonuclear leukocytes (PMLs) were isolated from the peripheral blood of healthy individuals using mono-poly resolving medium (DS Pharma Biomedical Co., Ltd., Osaka, Japan). After centrifugation the lower fraction consisted of PMNs, the percentage of neutrophils was evaluated using May–Giemsa staining. There were no immature myeloid elements. Isolated PMNs were suspended at a concentration of $1 \times 10^6$ cells/ml in RPMI 1640 medium with 2% fetal bovine serum and Penicillin Streptomycin (Pen Strep) mixture (Gibco). Freshly isolated cells were collected for immunofluorescent staining and immunoblotting. For immunoblotting cells were placed in a 3 cm dish and incubated, then one group of cells was stimulated using 100 ng/ml of IL-8. After 40 min, the supernatant and cell lysate were collected. Supernatant was also collected for enzyme-linked immunosorbent assay (ELISA), in order to check the level of PTX3 released by activated neutrophils. For immunofluorescent staining of neutrophils without stimulation freshly isolated non-stimulated PMNs were cytospined for 1 min at 1000 rpm and then stained. For immunofluorescent staining with stimulation, PMNs were seeded on glass coverslips treated with 0.001% polylysine and stimulated with 100 ng/ml of IL-8 (R&D Systems Inc., Minneapolis, MI, USA) for 40 min.

### 2.2. Isolation of HL-60 cell culture

HL-60 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and Penicillin Streptomycin (Pen Strep) mixture (Gibco). Cells were maintained at 37 °C in a 5% $CO_2$/95% air atmosphere and were used for experiments during the exponential phase of growth. The percentage of cells was evaluated using the Giemsa staining. Freshly isolated cells were collected for immunofluorescent staining and immunoblotting. For immunofluorescent staining cells were cytospined for 1 min at 1000 rpm and then stained.

### 2.3. Differentiation of HL-60 cell culture to neutrophil-like cells

HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and Penicillin Streptomycin (Pen Strep) mixture (Gibco) and at a density of $1 \times 10^6$ cells/ml was stimulated with 1.25% dimethyl sulfoxide DMSO (Sigma-Aldrich, Missouri, USA) for 7 days to induce differentiation. Differentiated cell morphology was monitored by the Giemsa staining. A stationary state was reached at day 7 and isolated cells were collected for immunofluorescent staining. For immunofluorescent staining cells were cytospined for 1 min at 1000 rpm and then stained.

### 2.4. Immunofluorescent analysis

Freshly isolated PMNs and HL-60 cells were cytospined for 1 min at 1000 rpm, blocked with 4% paraformaldehyde for 5 min and 0.5% Triton X-100/PBS for 5 min. Then specimens were blocked with 10% normal goat serum and stained with primary antibodies: anti-PTX3 monoclonal antibodies PPZ-1228 (Savchenko et al., 2008) at a dilution of 1:100 (Perseus Proteomics Inc., Tokyo, Japan), anti-lactoferrin monoclonal antibodies (EPR4338, GenTex Inc., Irvine, CA, USA) at a dilution of 1:100, polyclonal anti-myeloperoxidase antibodies (Abcam plc., Cambridge, UK) at 1:1000, polyclonal anti-gelatinase antibodies (EMD Millipore Corporation, Billerica, MA, USA) at 1:10,000 and monoclonal anti-azurocidin 1 Z6718 at 1:1000 dilution (Daigo and Hamakubo, 2012). Alexa Fluor 568 goat anti-mouse IgG Fab′ fragment (1:250 dilution, Invitrogen, Carlsbad, CA, USA) was used as secondary antibodies for PTX3 detection. A secondary antibody used for the detection of lactoferrin, gelatinase and MPO was Zenon Alexa Fluor 488 labeling kit goat anti-rabbit IgG Fab′ fragment (Invitrogen) at a dilution of 1:500. Monoclonal anti-azurocidin 1 Z6718 antibody was labeled with Z25102: Zenon Alexa Fluor 488 Mouse IgG2a Labeling kit (Invitrogen).

DNA detection was carried out using blue fluorescent Hoechst 33342 (MW 615.99, H3570), purchased from Molecular Probes Inc. (Carlsbad, CA, USA). Control experiments were carried out by omitting primary antibodies. Immunofluorescent analysis was performed using the photomultiplier of a multi-photon laser scanning microscope (LSM510 META; Carl Zeiss, Jena, Germany).

### 2.5. Immunoblotting

Grown PMNs were collected and lysed in a solution containing 150 mM NaCl, 50 nM Tris–HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM aprotinin for 10 min on ice and centrifuged at 15,000 rpm at 4 °C for 15 min. The supernatants were used with no dilution. Twenty five micrograms of the lysates and supernatants was

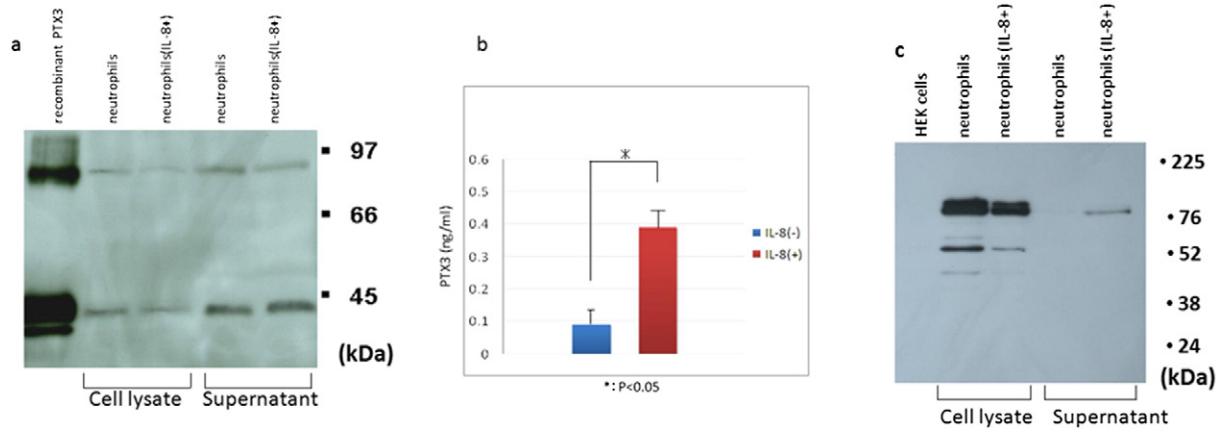


**Fig. 1.** a Expression and secretion of PTX3 protein in neutrophils. Lysates and supernatants revealed bands corresponding to molecular weight of 90 and 40 kDa. Freshly isolated lysates and supernatants showed PTX3 protein presence, with more intense expression in supernatants. After 40 min of IL-8 stimulation expression of PTX3 in cell lysate appeared to decrease in comparison to the cell lysate without stimulation. However, no clear difference was seen in PTX3 expression between supernatants of neutrophil culture with and without stimulation. b PTX3 levels in supernatant of non-stimulated neutrophils and neutrophils stimulated with IL-8. Supernatant of stimulated neutrophils showed 4 times higher PTX3 concentration compared to non-stimulated ones. c Lactoferrin expression in neutrophils. Lactoferrin expression without stimulation was seen in cell lysate only. Forty minutes after IL-8 stimulation lactoferrin expression was seen in supernatant as well as in cell lysate. Lactoferrin expression in cell lysate of non-stimulated neutrophils is more remarkable than in cell lysate of IL-8 stimulated neutrophils. HEK cells — Human Embryonic Kidney 293 cells.