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TITLE:
Host-protective effect of circulating pentraxin 3 (PTX3) and complex formation with neutrophil extracellular traps

ABSTRACT:
Pentraxin 3 (PTX3) is a soluble pattern recognition receptor which is classified as a long-pentraxin in the pentraxin family. It is known to play an important role in innate immunity, inflammatory regulation, and female fertility. PTX3 is synthesized by specific cells, primarily in response to inflammatory signals. Among these various cells, neutrophils have a unique PTX3 production system. Neutrophils store PTX3 in neutrophil-specific granules and then the stored PTX3 is released and localizes in neutrophil extracellular traps (NETs). Although certain NET components have been identified, such as histones and anti-microbial proteins, the detailed mechanisms by which NETs localize, as well as capture and kill microbes, have not been fully elucidated. PTX3 is a candidate diagnostic marker of infection and vascular damage. In severe infectious diseases such as sepsis, the circulating PTX3 concentration increases greatly (up to 100 ng/mL, i.e., up to 100-fold of the normal level). Even though it is clearly implied that PTX3 plays a protective role in sepsis and certain other disorders, the detailed mechanisms by which it does so remain unclear. A proteomic study of PTX3 ligands in septic patients revealed that PTX3 forms a complex with certain NET component proteins. This suggests a role for PTX3 in which it facilitates the efficiency of anti-microbial protein pathogen clearance by interacting with both pathogens and anti-microbial proteins. We discuss the possible relationships between PTX3 and NET component proteins in the host protection afforded by the innate immune response. The PTX3 complex has the potential to be a highly useful diagnostic marker of sepsis and other inflammatory diseases.

Introduction:
The release of neutrophil extracellular traps (NETs), first reported in 2004 (Brinkmann, 2004), is one of the anti-microbial actions of neutrophils. NETs are mesh-like structures that contain DNA as a backbone, with anti-microbial proteins attached (Amulic and Hayes, 2011). NETs trap microbes and form an anti-microbial-protein-rich microenvironment (Medina, 2009).
Pentraxin 3 (PTX3) was reported as one of the NET component proteins (Jaillon et al., 2007). PTX3 is a member of pentraxin family and mainly acts as a soluble pattern recognition receptor (PRR) in the innate immune response (Bottazzi et al., 2010). In NETs, PTX3 may participate in microbial recognition by facilitating the trapping of microbes. The circulating PTX3 level is known to be increased in certain diseases, and PTX3 may predominantly play a critical role in host protection. Interestingly, proteomic identification of the circulating PTX3 interacting proteins revealed that PTX3 formed a complex with NET component proteins (Daigo et al., 2012). This finding implies that the NET component proteins are active in pathogen recognition and clearance by tethering with each other in NETs and bloodstream. PTX3 appears to be a key tethering molecule to enhance the actions of NETs component proteins. In this review, we will discuss the host-protective roles of PTX3 in relation to NETs component proteins.

Source, expression, and function ::: NETs:
Neutrophils are the major player in the innate immune system response against microbial pathogen invasion. One of the anti-microbial activities of neutrophils is the extrusion of NETs (Brinkmann, 2004). NETs are formed upon the activation of neutrophils by factors such as IL-8, lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA), bacteria, fungi, and activated platelets (Brinkmann, 2004; Clark et al., 2007; Fuchs et al., 2007). Neutrophil death as a result of the extrusion of NETs is called “NETosis,” which is a cell death pathway distinct from apoptosis or necrosis (Brinkmann and Zychlinsky, 2007; Steinberg and Grinstein, 2007). The release of NETs has also been reportedly observed without cell death (Yipp et al., 2012). Extracellular formations of this type are also observed in basophils and eosinophils (Schorn et al., 2012). NETs are mesh-like structures that consist of cellular DNA, along with bactericidal proteins, that reside in neutrophil granules and the nucleus. These proteins are connected to DNA fibers, and form a specialized microenvironment which facilitates the capture and killing of bacteria.

The NET component proteins ::: NETs:

Using a proteomic approach, Urban et al. identified 24 NET-associated proteins (Urban et al., 2009). These proteins are; nuclear components such as core histones; granular components such as neutrophil elastase (ELANE), lactotransferrin (LTF), cathepsin G (CTSG), myeloperoxidase (MPO), proteinase 3 (PRTN3), azurocidin 1 (AZU1), lysozyme C (LYZ), neutrophil defensins, and cytoplasmic proteins. In other proteins, histone H1, bactericidal permeability-increasing protein (BPI), pentraxin 3 (PTX3), and cathelicidin anti-microbial peptide (CAMP) are also defined as NET component proteins (Brinkmann, 2004; Jaillon et al., 2007; Lauth et al., 2009). Essentially all of these proteins possess anti-microbial activity.

Genome ::: PTX3:
Breviario et al. identified PTX3 as one of the IL-1β -induced genes in human umbilical vein endothelial cells (HUVECs) (Breviario et al., 1992). The human PTX3 gene is located on chromosome 3q band 25, consists of 1861 base pairs, and is translated into 381 amino acids (Breviario et al., 1992). PTX3 belongs to the pentraxin family, which included the acute phase proteins C-reactive protein (CRP) and serum amyloid P-component (SAP). As PTX3 has a longer N-terminal domain, it is classified as a member of the long-pentraxin subfamily. Unlike the more common short pentraxins CRP and SAP, the PTX3 gene is highly conserved across species (Garlanda et al., 2005). The PTX3 gene consists of three exons, among which the first and second exons encode the signal sequence peptide and the N-terminal domain, and the third exon encodes the C-terminal domain. In the promoter region of the PTX3 gene, a number of potential enhancer binding sequences (Pu-1, AP1, NF-κB, SP1, and NF-IL6) are located (He et al., 2007).

Structure ::: PTX3:
After the processing of the signal sequence of the translated 1–17 amino acids, the mature PTX3 consists of two domains, i.e., the N-terminal domain (18–178 a.a.) and C-terminal domain (179–381 a.a.). The PTX3 C-terminal domain is a pentraxin-like domain, which is conserved among the pentraxin family with pentraxin signature (His-x-Cys-x-Ser/Thr-Trp-x-Ser). An N-linked glycosylation site (Asn220) is located in the C-terminal domain. In contrast to the C-terminal domain, the PTX3 N-terminal domain is a unique sequence unrelated to other proteins. The PTX3 protein forms an octamer via the inter-molecule disulfide bonds (Inforzato et al., 2008, 2010). Briefly, the N-terminal domain participates in the organization of a tetramer, and the C-terminal domain participates in the dimerization of the tetramer. Interestingly, the N-terminal tetramer formation has two states; a tetramer via the inter-disulfide bonds or non-covalent dimerization of the inter-disulfide-bonded dimer. This results in the asymmetric form of the full-length PTX3 (Inforzato et al., 2010).

Expression pattern ::: PTX3:
PTX3 mRNA expression is induced by primary inflammatory signals in certain cells, such as myeloid dendritic cells (Doni et al., 2003), peripheral blood leukocytes (Alles et al., 1994), mononuclear macrophages/phagocytes (Alles et al., 1994; Goodman et al., 2000), vascular endothelial cells (Breviario et al., 1992; Lee et al., 1993), smooth muscle cells (Klouche et al., 2004), fibroblasts (Lee et al., 1993; Goodman et al., 2000), adipocytes (Abderrahim-Ferkoune et al., 2003), glial cells (Polentarutti et al., 2000), cumulus oophorus cells (Salustri et al., 2004), mesangial cells (Nauta et al., 2005), and synovial cells (Luchetti et al., 2000). Transcriptional activation of PTX3 in response to the pro-inflammatory cytokines TNFα and IL-1β is regulated by NF-κB binding site in the PTX3 promoter (Altmeyer et al., 1995; Basile et al., 1997). Other pathways also regulate PTX3 expression in a cell- and signal-dependent manner. In detail, please refer to the excellent reviews cited (He et al., 2007; Ortega-Hernandez et al., 2009; Deban et al., 2011; Inforzato et al., 2011).
The characteristic PTX3 expression pattern is observed in neutrophils. In mature neutrophils, the PTX3 protein is abundantly present in granules, but PTX3 mRNA expression is not detected. In contrast, PTX3 mRNA expression is observed in progenitor neutrophils, such as promyelocytes and myelocytes/metamyelocytes (Jaillon et al., 2007). As PTX3 protein expression is observed in both neutrophil precursors and mature neutrophils, it is considered that the PTX3 protein is produced during the course of neutrophil maturation and mature neutrophils store it for use-on-demand. Immunostaining revealed that PTX3 is present in neutrophil granules and that it colocalizes with lactoferrin (Jaillon et al., 2007; Savchenko et al., 2011), suggesting that PTX3 localizes to specific granules. The stored PTX3 in neutrophils is released upon E. coli, S. aureus or zymosan stimulation, as well as PMA, ionomycin or TNFα treatment (Jaillon et al., 2007; Savchenko et al., 2011; Daigo et al., 2012). PTX3 release is not induced by IL-1β or latex bead

stimulation (Jaillon et al., 2007). The released PTX3 localizes to NETs and plays a non-redundant role in pathogen resistance. Thus, PTX3 in neutrophils plays a distinctive role in the innate immune response due to its rapid secretion, as well as by its unique pattern of ready-to-use expression and storage.

Circulating levels ::: PTX3:
As the pentraxins CRP and SAP are well-known acute phase proteins, PTX3 may also be an acute phase biomarker. Under physiological conditions, the circulating PTX3 level is as low as approximately 2 ng/mL (Yamasaki et al., 2009). Recently, many studies on the circulating PTX3 level in clinical trials have been reported. These reports indicate that the PTX3 levels are significantly increased in certain infectious, cardiovascular, kidney, and female reproductive system diseases as well as other disorders (summarized in Table 1). In most cases, the PTX3 level correlates with both the severity and survivability of the disorder. In these diseases, the increases can reach up to 10~100 times the control level in severe inflammatory and infectious diseases such as sepsis. In the case of sepsis, the plasma PTX3 dramatically increases to a level of up to 100 ng/mL (Muller et al., 2001) and the increase correlates with mortality (Mauri et al., 2010). Although not included in Table 1, there are other infectious diseases, such as severe dengue virus infection (Mairuhu et al., 2005) and meningococcal disease (Sprong et al., 2009), in which the PTX3 levels are also increased. The PTX3 plasma concentration is increased in patients with acute myocardial infarction (Peri et al., 2000). During pregnancy, the serum PTX3 level slightly increases as the pregnancy progresses (Larsson et al., 2011). A higher PTX3 level is observed in preeclampsia (Cetin et al., 2006; Rovere-Querini et al., 2006). Finally, the serum PTX3 level is reported to be a biomarker for lung carcinoma (Diamandis et al., 2011). Thus, the circulating PTX3 level increases non-specifically in various infections and inflammatory disorders. For the purpose of diagnostic measurement, the dynamics of the PTX3 complex, such as the NET component proteins should be monitored (more details are discussed below).

Function ::: PTX3:
PTX3 has been postulated to play a variety of roles in innate immunity, inflammatory regulation, and female fertility (Bottazzi et al., 2006; Garlanda et al., 2009; Inforzato et al., 2011; Cieslik and Hrycek, 2012). PTX3-knockout and transgenic mice studies have indicated that the predominant role of PTX3 occurs in host protection in the case of lung injury, infection, vascular damage, as well as certain other disorders (summarized in Table 2). Briefly, the resistance against pathogens such as Aspergillus fumigatus, Paracoccidioides brasiliensis, and Klebsiella pneumoniae has been reported (Garlanda et al., 2002; Diniz et al., 2004; Soares et al., 2006). In addition to its anti-pathogenic activity, PTX3 also has been shown to play a role in protecting against severe inflammatory reactions in animal models of sepsis (Dias et al., 2001), seizure-induced neurodegeneration (Ravizza et al., 2001) and acute myocardial infarction (Salio et al., 2008). In addition, PTX3 participates in extracellular matrix deposition. PTX3 is involved in the organization of hyaluronan in the viscoelastic matrix of cumulus oophorus (Scarchilli et al., 2007). It is considered that these functions of PTX3 are exhibited synergistically along with the binding of specific ligands (the details are provided in section “Ligands”).
Of note, among the studies in PTX3-knockout and transgenic mice, there are some reports of an opposite effect of PTX3 on host-protection. In an intestinal ischemia and reperfusion model, Souza et al. reported an increased injury and lethality in the PTX3-transgenic mice that seemed to be associated with elevation of the TNFα concentration and aggravation of the inflammatory response (Souza et al., 2002). They also reported the suppression of tissue injury and lethality after ischemic and reperfusion in PTX3-knockout mice. PTX3 administration to these PTX3-knockout mice reversed this suppression (Souza et al., 2009). Other groups have also reported an adverse effect of PTX3 in acute ischemic lung injury (Chen et al., 2012) and ventilator-induced lung injury (Real et al., 2012). In the case of Klebsiella pneumoniae infection, faster lethality was observed when a higher inoculum was administered to PTX3-transgenic mice, but the lethality was conversely delayed when a middle or low inoculum was administered (Soares et al., 2006). Taking these bi-phasic functions of PTX3 in host-defense into account, more detailed accounts of the disease-specific mechanisms of PTX3 need to be elucidated to achieve useful clinical applications.

Ligands ::: PTX3:
The multiple host-protective functions of PTX3 arise from the capacity for the recognition and binding to ligands. The reported PTX3 ligands are classified as follows: (1) complement

components; (2) Fungi, bacteria, microbial components, and viruses; (3) selectin P; (4) extracellular matrix proteins and (5) growth factors (Presta et al., 2007; Mantovani et al., 2008; Deban et al., 2009; Moalli et al., 2011). Some of these ligands bind to PTX3 in a PTX3-domain specific manner, while others require full-length PTX3 for binding (Deban et al., 2009; Bottazzi et al., 2010).
Taking these results, it is clear that the protective effects of PTX3 are realized in coordination with specific PTX3 ligands. Therefore, we carried out a proteome-wide identification of PTX3 ligands and complexes in septic patient serum and plasma. PTX3 and its complex component proteins were immunoprecipitated by anti-PTX3 antibody-crosslinked magnetic beads, and the isolated fractions were subjected to shotgun proteomics analysis for label-free relative quantitation via spectral counting (Daigo et al., 2012). The identified proteins included the known PTX3 ligands such as C1q, ficolins, TSG-6, and Iα I, as mentioned above. Additionally, the ficolin-binding proteins of mannan-binding lectin serine protease 1 and 2 (MASP1 and MASP2) (Ma et al., 2009), and the TSG-6 binding proteins of the versican core protein (VCAN) and thrombospondin-1 (THBS1) (Salustri et al., 2004) were included in the proteins that were identified. As these proteins were identified in pooled normal human plasma with artificially spiked recombinant PTX3, these appear to be stable circulating PTX3 complexes. Nevertheless, the disease-specific dynamics of these binding levels need to be investigated further, as do the specific functions of these PTX3 complexes in sepsis.

NET component proteins as PTX3 ligands: a newly recognized protective role:
In the effort to identify the PTX3 ligand in septic patient fluids, a novel finding is that the NET component proteins were included (Daigo et al., 2012) (Table 3). A detailed investigation revealed that azurocidin 1 (AZU1) and myeloperoxidase (MPO) directly bind to PTX3. AZU1 and MPO belong to the NET component proteins (Urban et al., 2009) and exert bactericidal activity (Watorek, 2003; Klebanoff, 2005). AZU1 preferably binds to the PTX3 N-terminal domain, with a pattern of calcium ion dependency. In contrast to AZU1, MPO binds to both the PTX3 N-terminal and C-terminal domains, and does not require calcium ions. Further investigation of the PTX3-AZU1 interaction revealed that the AZU1 binding affinity to PTX3 was 22 ± 7.6 nM, and that AZU1 and PTX3 are partially co-localized in NETs (Daigo et al., 2012).
From these results, it is suggested that PTX3 may enhance the bactericidal efficiency of AZU1 and MPO in terms of both pathogen recognition and AZU1 and MPO binding (Figure 1). The mechanism by which PTX3 localizes in NETs has not yet been determined, but it is possible that PTX3 localization arises from an interaction with histones or the basic proteins AZU1, MPO, and defensin, along with a simultaneous association between these basic proteins and DNA (Figure 1). It is not clear at present whether the PTX3-AZU1 and PTX3-MPO binding events in the bloodstream take place within or outside of NETs. Either or even both of these are possible, and these complexes may be active in pathogen recognition and also involved in clearance. In septic patients, the plasma levels of AZU1 are increased, but do not significantly correlate with mortality (Berkestedt et al., 2010). As useful biomarkers of sepsis not yet available (Pierrakos and Vincent, 2010), the binding levels of PTX3-AZU1 and PTX3-MPO in septic plasma have the important potential to fulfill this purpose.

Conflict of interest statement ::: Conclusion:
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.