The Biochemistry Of Apoptosis

Programmed Cell Death

Apoptosis is defined as programmed cell death. Apoptosis recently is not considered as the only cell death pathway since various cell death pathways are discovered. More accurately programmed cell death is defined as cell death that is dependent on genetically encoded signals or activities within the dying cell. Therefore, the designation programmed refers to the fixed pathway followed by dying cells, regardless of the mechanism or of whether the characteristic features of apoptosis accompany the process. Acute cell breakdown due to the direct action of a damaging stimulus is the conceptual converse of programmed cell death since it requires no cellular activity and is prevented only by the absence of the damaging stimulus. Autophagy has been well documented as a mechanism of programmed cell death occurring during the process of normal embryonisc development. The dependence of pyroptosis on the activation of caspase-1 also indicaes that it is a program of cell death. Furthermore, increasing genetic data indicate that oncosis requires an intrinsic molecular program.

The Neutophil and NETosis

Neutrophils are the first line of defense of the immune system against infection. Among their weaponry, they are able to mix and extrude their DNA and bactericidal molecules creating NET-like structures in a unique type of cell death called NETosis. NETosis is a unique form of cell death that is characterized by the release of decondensed chromatin and granular contents to the extracellular space. This process is important in order to control extracellular infections limiting collateral damage. Its ‘unique’ function has been implicated in several human diseases including sepsis and autoimmune disease. Human neutrophils constitute the first line of defense of innate cellular immunity. As the most abundant subtype of leucocytes in peripheral blood, they constitute approximately 70% of these cells. They are terminally differentiated cells with a life span of 12 to 15 hours, whom after this time period undergo apoptosis: this life span can be extended after exposition to several substances like cytokines. These cells have an approximate diameter of 12 to15 ?m and a nucleus with several lobules.

They also have a cytoplasm rich in different granules abundant of antimicrobial peptides and enzymes necessary to synthesize several substances, including arachidonic acid derivates with either inflammatory properties like tromboxans and leucotriens, or negative regulators of inflammation such as prostaglandins, lipoxins, protectins, and so forth. These substances can be produced entirely within the neutrophils or in conjunction with other cells using transcellular pathways to produce lipoxins. Neutrophils can produce chemokines, cytokines from the tumor necrosis factor (TNF) superfamily, angiogenic and fibrogenic factors, and pattern recognition molecules such as pentraxins, collectins, and ficolins. In addition, they have an enzymatic complex known as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase type 2 (NOX2) responsible for the production of reactive oxygen species during the respiratory burst.

NETosis as One of Cell Death Pathway

The researchers firstly did not consider NETosis as an active process that related to cell death based on several observations. Stimuli that induce NET did not promote the release of cytoplasmic markers like lactate dehydrogenase and activated cells excluded vital dyes for the least two hours after stimulation ruling out necrosis as an associated phenomenon. Using time-lapse video microscopy, it is evidenced that NETs were formed by motile cells. Furthermore NETs were formed as early ten minutes after activation and this time course is not compatible with apoptosis. It was considered that a process that leads to nuclear disintegration with DNA extrusion into the extracellular space should inevitably lead to cell death. In 2007, further investigation by Dr. Brinkmann's group concluded NETosis was a cell death pathway. This process was later described in other granulocyte lineage cells, but was not found in monocites or basophiles. Considering these findings, the term ETosis appeared and describes the process of cell death that leads to extracellular traps formation, using NETosis specifically when these ETs are produced by neutrophils (NETs).

After the stimulation of a population of neutrophils using LPS, IL-8, interferon gamma, PMA, monosodium urate cristals, and microorganisms, only a portion of the population will generate NETs. Among these stimuli mentioned, microorganisms or PMA are the most potent agents inducing NETosis in approximately 30% of the population, suggesting the importance of different activation pathways in this process. EM analyses have remarked the chain of events that occurs during NET formation. First of all, after activation, neutrophils present a flattening of their cellular structure with the visualization of multiple cytoplasmatic vacuoles. Secondly, the distinction between euchromatin and heterochromatin is lost as well as their characteristic nuclear lobulations and a space between the inner and outer nuclear membrane is formed. Thirdly, the nuclei increased its size to occupy most of the cytoplasm and the integrity of the nuclear and granular membranes is lost allowing the components of NETs to mix. All this process is carried out while the cytoplasmatic membrane remains unharmed. In the last stage, neutrophils die releasing the ET and expressing death cell indicators such as phosphatidylserine.

NET formation requires two events: the production of ROS and chromatin unfolding. ROS production is performed by the enzyme NOX2 and is responsible for the oxidative burst that will ultimately kill the phagocytized organism in the phagolysosome. The intracellular steps leading ROS to create NETs are not completely understood, nonetheless it is known that the protein kinase C (PKC) pathway is important given the fact that PMA (an activator of the former) is one of the most potent inducers of NET formation known to date. The activation of the PKC pathway leads to the assembly of the NOX2 complex in the phagosome membrane and the electron transport inside it generates superoxide anions (O2?). At the same time, the increase in the negative charge generated in the process creates a favorable gradient for the hydrogen ions (H+) to enter the phagosome and mix with superoxide anion generating hydrogen peroxide (H2O2). This compound, unlike other ROS, is electroneutral and can diffuse back to the cytoplasm where it turns the cellular balance toward generalized activation through the oxidation of the tyrosine phosphatase superfamily in a specific cysteine residue highly conserved in this protein superfamily leaving such enzymes inactive in a reversible or irreversible fashion depending on the degree of oxidation. Other enzymes that are target of peroxidation through cysteine residues are caspases, which therefore block apoptosis. Several evidences convince that ROS production is required in NET formation; H2O2 is a potent inducer of NET generation at physiologic concentrations, the inhibition of NOX2 blocks the their production, adding catalase to neutrophil cultures by converting H2O2 to water blocks NET formation, in patients with chronic granulomatous disease (CGD), a disease caused by the deficiency of any of the

NOX2 components, there is an immunosuppressive syndrome leading to life-threatening infections and to the absence of NET formation under EM. Additionally, in order to reestablish NOX2 activity, gene therapy restores NET formation efficiently, which clears the infection.

The Role of Gasdermin D in The Generation of NETs

The host defense against extracellular pathogen is provided by neutrophil extracellular traps (NETs) and concomitant cell death (NETosis). In the other hand, pyroptosis that is characterized in macrophage enables defense against intracellular pathogen. Gasdermin D (GSDMD) provides the connection between those both cell death pathways. Pyroptosis is initiated byinflammasome-activated inflammatory caspases such as capase-1, caspase-4, caspase-5, and caspase-11. These capases will cleave GSDMD to stimulate plasma membrane pores. In macrophage, GSDMD will be cleavaged by active caspase-1 to elicit GSDMD-p30 pores and lead to pyroptosis during signalling by canonical inflammasome which is protein complexes that assemble upon sensing a broad variety of danger signals and activate inflammatory caspases. The example of inflammasomes are nod-like receptor (NLR) pyrin domain-containing 3 (NLPR3) and NLR CARD domain-containing (NLRC4). Most of the agents induce pyroptosis by binding to a specific sites of NLR, TRIM or PYHIN families therefore triggering the activation of caspase-1 within macromolecular complexes is called cannonical inflammasomes. Pyroptosis also can be triggered by noncannonical inflammasome which confers host defense againts Gram-negative bacteria in the cytosol. Caspase-11 (murine) or caspase-4/5 (human) can directly recognize cytosolic bacterial lipopolysaccharide (LPS) from Gram-negative bacteria leading to caspase-4/5/11 activation and GSDMD cleavage, GSDMD-p30 plasma membrane pores, NLPR3 signalling and cell lysis.

The role of GSDMD in NETosis has been revealed as well as in pyroptosis. Several biochemical processes including reactive oxygen species (ROS) production can be activated through activation with microbe or mitogens like phorbol 12-myristate 13-acetate (PMA). ROS signalling releases a macromolecular complex called azurosome from granules which translocate neutrophill elastase (NE) to nucleus. NE will process histines that coincides with chromatin expansion in the whole cell before the plasma membrane rupture and NET release. The mechanism of NETs liberation by neutrophil lyse is revealed after small molecule that can block NET formation is found. This small molecule called LDC7559 binds to GSDMD leading to pyroptosis and NETosis inhibition.

Sollberger et al (2018) screened a library of 182.710 small molecules to identify PMA-induced NETosis inhibitors. The NETosis inhibitors are isolated from healthy volunteers. A compound class of NET formation are identified based on the pyrazolo-oxazepine scaffold which potently inhibited PMA-induced NETosis. lDC7559 was not toxic to peripheral blood mononuclear cells (PBMCs) and efficiently inhibited PMA-induced NET formation with IC50 of 5.61 ?M. This compound do not block NADPH oxidase, NE, or myeloperoxidase (MPO) activity, suggesting that it acted downstream of NOX2 and the azurosome. LDC7559 decreases ROS generation after the initial peak in human primary neutrophils despite its reduction is not significant to affect NETosis. This compound does not block phagocytic activity of human primary neutrophils which is in line with our findings that it inhibits NET formation specifically. LDC7559 is really effective in delaying NET formation although in very low concentration applied to PMA-treated neutrophils.

The analysis by mass spectometry (MS) showed that LDC7559 binds specifically to GSDMD which was the most abundant protein. In contrast, LDC7559 did not block the activity of NOX2 and MPO. The effect of LDC7559 in inhibiting GSDMD-dependent process was tested on pyroptosis in human primary monocytes, the monocytic cell line THP-1 and murine immortalized bone marrow-derived macrophages (BMDMs). The cannonical inflammasomes depends only partially on GSDMD to cause lysis because deficiency in this gene delays, rather than abolishes, IL-1B release. In the up to date study, LDC7559 treatment inhibited IL-1B release upon activation of cannonical inflammasomes by LPS transfection in THP-1 cells. LDC7559 is not only active in human, but also in murine. In immortalized murine BMDMs, LDc7559 decrease IL-1B release upon LPS delvery to the cytoplasm.

The cleavage of GSDMD occurs during NET formation and then localize to the plasma membrane of neutrophils. PMA-treated neutrophils showed reduced full-length GSDMD levels. Neutrophils isolated from chronic granulomatous disease (CGD) patient is used as control because CGD patient carry mutations in NOX2 leading to the failure of NETs formation upon PMA stimulation. Levels of full.length GSDMD remains intact in PMA-treated neutrophils isolated from CGD patient. GSDMD is not cleaved upon PMA stimulation because NOX2 is in the upstream of GSDMD activation.The staining of primary neutrophils for GSDMD using an antibody that recognize both full-length and cleaved GSDMD shows that GSDMD localizes primarily to the cytoplasm in nonstimulated cells. GSDMD is also found in NETs and show a Strong signla in remnants of NET-forming neutrophils. An analysis using high-resolution total internal reflection fluorescence (TIRF) microscopy shows that GSDMD accumulates in TIRF zone during NETosis progression. This indicates that GSDMD localizes to the plasma membrane. LDC7559 is able to reduce GSDMD membrane localization suggesting that it inhibits GSDMD cleavage or membrane intergration in neutrophils. Meanwhile, NE can not be found in the TIRF zone of either naive or PMA-treated neutrophils indicating that the translocation of GSDMD to the membrane is specific.

Distinct from pyroptosis, NETosis is caspases-independent. Several inhibitors can inhibit pyroptosis but can not affect NETosis. Fragments with size between 25 and 3 kDa resembling the active GSDMD N terminus is found in the mixture of lysates of HEK293T cells that express full-length GSDMD and neutrophil lysates. There are three main proteases of neutrophils like NE, proteinase 3 (PR3), and cathepsin G (CG) and they have random target recognition. Although there are some proteases of neutrophils, only inhibiton of NE is sufficient to decrase cleavage of GSDMD. Caspase-4 and NE are able to process GSDMD into a fragments of smaller size. On the other hand, CG and PR3 are less efficient at cleaving GSDMD. The LDC7559 compound does not inhibit NE therefore it is not interfering with GSDMD processing and not a protease inhibitor.

NE and caspase-4 form an N terminus of similar size due to similar region of cleavage sites. Caspase-4 cleaves GSDMD at amino acid 275 and the first 243 amino acids of GSDMS are required to induce pyroptosis. Five potential NE cleavage sites in the region between amino acids 243 and 282 are identifed. Construct with single point mutations in these sites are still cleaved by purified NE because cleavage sites are abundant. Expression of the GSDMD N terminus corresponding to all but the shortest of the five putative NE-induced fragments induce lysis in HEK293T cells. The fragment ranging from amino acid 1 to 255 are the most effective. Lysis induced by these N termini could be inhibited by LDC7559. Deletion of D275 decrease the sensitivity of GSDMD to caspase-4. In addition, deletion of amino acids 279 to 282 makes the protein insensitive to caspase-4. Effects of protein folding probably cause this insensitivity.

These deletions also make GSDMD less sensitive to NE leading to a suggestion that NE cleaves GSDMD at various positions between amino acids 275 and 282.

The role of GSDMD in NETosis has been revealed. Neutrophils isolated from GSDMD mutant mice have significantly lower NET formation upon PMA activation. LDC7559 does not affect neutrophil lysis upon addition of fetergen digitonin, indicating that teh effects of this compound are not simply stabilizing the cell membrane. LDC7559 is considered to give effect intacellularly since its wash out in different time do not give significant different. LDC7559 blocks a ROS-dependent NET formation pathway but do not inhibit NET formation in response to nigericin which is ROS production-independent. In addition, PMA or nigericin treatment do not induce IL-1B release from neutrophils therefore confirming that the cell death observed occur Independent of inflammasome activation. LDC 7559 and GSDMD act downstream the oxidative burst. LDC7559 might decrease ROS production upon PMA treatment. Cells treated with LDC7559 have the NETosis blocked even if the LDC7559 is added 30 minutes after PMA induction which is a time point when neutrophils had already mounted an oxidative burst. During NET formation, ROS production facilitates NE release from granules through dissociation from a multiprotein aggregate. NE then degrades actin and enters the nucleus where it processes histones. Actin degradation is significantly decreased after LDC7559 treatment and both MPO and NE remain in granules, indicating that GSDMD has a role upstream of NE mobilization from granules. NE inhibitor and LDC7559 reduce processing of GSDMD during PMA-induced NETosis. LDC7559 also decrease processing of histone H3 which is an indicator of NE activity during NETosis.

GSDMD and NE engage in a feed-forward loop in which the protease activates GSDMD. Activated GSDMD, in turn, forms pores in the granule membrane, thus enhancing NE release into the cytoplasm and allowing further GSDMD cleavage in reiterative process. This enables the translocation of NE to nucleus where it processes histones and allows nuclear expansion.The other function of GSDMD is upon completion of NETosis, cleaved GSDMD forms pores in the plamsa membrane allowing NET release. Classical NETosis requires ROS production and the activation of MPO to release NE into the cytoplasm. Kambara et al and Sollberger et al reported that GSDMD-deficient neutrophils are longer-lived than wild types (WT) cells, which is an important aspect of neutrophil bilogy because these cells are notoriously short-lived. Upon neutrophil aging, NE is released from damaged granules and processes GSDMD into an active fragment. GSDMD activation might be detrimental in infections where nuetrophil phagocytosis is effective and beneficial when NETs are required.

Reference

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