Tec Empresarial
P-ISSN: 1659-2395; E-ISSN: 1659-3359

Clinical Implications of Necroptosis Biomarkers in Sepsis

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Abstract

Necroptosis is a cell death process that is attractively unique and separate from apoptosis, as indicated by the involvement of receptor-interacting protein kinases (RIPK) and mixed lineage kinase domain pseudokinase (MLKL). This highlights important biomarkers of necroptosis: RIPK1, RIPK3, and MLKL, which encompass their structural, functional, and contributory roles in disease pathogenesis. We explore the intricate molecular mechanisms that underpin necroptosis, emphasizing the activation and complex interactions among RIPK1, RIPK3, and MLKL. The clinical relevance of necroptosis biomarkers is thoroughly assessed, particularly in the context of sepsis, where elevated levels of RIPK1, RIPK3, and MLKL are strongly associated with disease severity and patient prognoses. Techniques for the detection and quantification of these biomarkers are reviewed, along with current therapeutic strategies aimed at modulating necroptosis. Furthermore, we evaluate the impact of various therapeutic agents, such as estradiol, on the levels of these biomarkers and their potential to alter the course of disease progression. The review concludes with a forward-looking perspective on future research directions and the potential for innovative therapeutic interventions targeting necroptosis.

Keywords: *Necroptosis, Receptor-Interacting Protein Kinases, Mixed Lineage Kinase Domain-Like Pseudokinase, Sepsis, Estradiol.*

INTRODUCTION

Necroptosis is caspase-independent programmed cell death regulated by signal transduction pathways. Necroptosis is characterized by organelle swelling and increased cell volume, destruction of membrane cell structures, and release of cellular contents. Molecules known to activate the necroptosis pathway are RIPK1, RIPK3, MLKL, while molecules that inhibit the necroptosis pathway are caspase-8.[1,2] Necroptosis is known to have similarities with necrosis in terms of its morphological features and is similar to apoptosis because most of its

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functions are regulated by intracellular proteins. However, unlike apoptosis which depends on caspase activation, necroptosis in its process does not depend at all on caspases.[2,3]

50% of the dead neutrophils showed apoptotic morphology after 24 hours, while the corresponding results for sepsis neutrophils were only 5-10%. Anti-apoptotic pathways acquired during sepsis, such as the preservation of Mcl-1 and Annexin A1, as well as the release of PBEF, IL-10, inhibition of MNDA translocation from the nucleus to the cytoplasm, limitation of the Caspase-3, -8, and -9 pathways, and increased expression of PD-L1.[4]

Necroptosis is a pro-inflammatory form of cell death. It releases damage-associated molecular patterns (DAMPs) that can exacerbate inflammation.[5] Necroptosis also plays a critical role in defending against certain pathogens, including viruses and bacteria. Studying necroptosis helps understand how pathogens evade immune responses and how this process can be manipulated to enhance immune defence.[6]

LITERATURE REVIEW

Molecular Mechanisms of Necroptosis

Sepsis can induce necroptosis. Necroptosis is defined as a caspase-independent programmed necrotic cell death that can be regulated through signal transduction pathways.[7,8] In general, necroptosis is characterized by organelle swelling, the increase in the number of cells, cell fusion, plasma membrane merging and cell content fusion.[2]

Necroptosis is influenced by the activation of toll-like receptors (TLR) or tumor necrosis factor receptor 1. (TNFR 1). When this process occurs, receptors interacting protein kinases 1 and 3 (RIPK1 and RIPK3) are activated and will interact to form necrosomes.[9] There are two conditions that are the basis for necroptosis, cells must have RIPK3 and MLKL and inactivation of the caspase-8 molecule.[9]

Necroptosis was first investigated when it was inhibited with a specific chemical inhibitor of RIPK1, necrostatin-1 (Nec-1), and its analog, Nec-1s. The discovery of Nec-1 spearheaded deeper research into the mechanisms of necroptosis and its relevance to diseases. Necroptosis is also known as an important cell death mechanism.[9,11]

The necroptosis pathway is composed of various stages of post-translational modifications. Therefore, the mechanism of necroptosis regulates Necroptosis is included in several groups. However, necroptosis mediated by tumor necrosis factor receptor 1 (TNFR1) is the most studied and widely used pathway to describe the necroptosis mechanism.[9,12,13]

Polyubiquitin linkage is a transformation pathway after synthesis that can attach ubiquitin proteins to this pathway component, NF-κB (NEMO), also known as IKKγ, which can specifically tighten the ubiquitin sequence on RIPK1, potentially leading to the configuration of the kinase agent IκB (IKKα along with IKKβ) being bound to the RIPK1 factor. Additionally, the The kinase complex activated by β (TAK),

including TAK1 and the TAK1/2 binding proteins (TAB1/2), also binds RIPK1. The binding of these two complexes (TAK and IKK) activates. Thus, the formation of complex I promotes cell survival by activating the NF-κB pathway.[17]

The next step in the necroptosis mechanism is the formation of complex II. This complex forms in the absence of cIAPs and TAK1, and inhibition of NEMO activity due to translational inhibition or activation of two deubiquitinases, cylindromatosis (CYLD) and A20. This results in the hydrolysis of the ubiquitin chain and the deubiquitination of RIPK1.[9,18] There are two types of complex II, complex IIa and complex IIb, which are distinguished based on the composition of complex II and the activity of the proteins involved. Complexes IIa and IIb are known to induce apoptotic cell death.[2] Both complexes can also induce necroptosis if caspase-8 is inactive or absent.[12,15]

RIPK1: Structure and Function

RIPK1 is a protein consisting There are 671 amino acid units that have a molecular mass of approximately 76 kDa. These molecules are equipped with a serine/threonine enzyme category at the N-terminal (the first 300 amino acid units), as well as one category of death. at the C-terminus (last 112 amino acids), and an intermediate domain (ID) located between the KD and DD.[21–23]

Area protein kinases play a crucial role in the existence of basic units and become a key element in initiating the processes of cell death.[24] With the help of the kinase area, RIPK1 collaborates with the TRAF2 protein while Necrostatin-1, which most recently acts as a modifying inhibitor for RIPK1's task.[25] When RIPK1 is presented in a mortal amount without the influence of kinase function, it can trigger NF-kB molecules.[21]

The death domain (DD) of RIPK1 shares similarities with other receptor domains such as Fas, TRAILR2 (DR5), TNFR1, and TRAILR1 (DR4). This alignment allows DD to bind with these receptors, as well as TRADD and FADD in conjunction with the signalling of TNF-alpha Receptor 1. Enhanced delivery of RIP can trigger programmed cell death and activate NF-kB, but increased delivery of only the DD from RIP can inhibit NF-kappaB activation by TNF-alpha Receptor 1.[21,24]

The intermediate domain (ID) is essential for NF-kB activation and signalling dependent on the RHIM. Through the ID, RIP can interact with proteins like TRAF2, NEMO, RIPK3, ZBP1, and OPTN, among others, depending on the cellular context.[24,26]

RIPK1 is a crucial mediator of TNFR1 signalling and is implicated in various human diseases due to its roles in inflammation and cell death. Its activation leads to necroptosis and apoptosis, influencing conditions like rheumatoid arthritis, Crohn’s disease, amyotrophic lateral sclerosis (ALS), Alzheimer’s disease (AD), and multiple sclerosis (MS). RIPK1's involvement in neurodegeneration, inflammation, and autoinflammatory syndromes.[30]

RIPK3: Structure and Function

RIPK1 and RIPK3 proteins share almost 50% of their amino acid sequences and have very similar structural features. RIPK3 consists of 518 amino acids in humans, has an N-terminal kinase domain similar to RIPK1, a RIP homotypic interaction motif (RHIM), and a unique C-terminus that lacks a death domain.[31]

This highlights the multifaceted roles of RIPK1 and RIPK3 in both cell death and inflammation.[41]

RIPK3 is a protein with an N-terminal kinase domain that allows it to phosphorylate itself and other substrates. It also has a C-terminal domain containing a receptor-interacting protein homotypic interaction motif (RHIM), which helps it associate with other proteins to form oligomers. When activated, RIPK3 autophosphorylates and subsequently phosphorylates and activates a pseudokinase called MLKL, which is crucial for membrane permeabilization during necroptosis.[42]

The phosphorylation of MLKL by RIPK3 triggers a conformational change in MLKL, exposing its N-terminal helical bundle domain. This domain, usually bound to the pseudokinase domain, facilitates MLKL oligomerization once released.[43] There are varying reports on the number of MLKL subunits involved in oligomers,[44] possibly due to differences between mouse and human systems or variations in activation sites.[45]

The oligomerization of MLKL aids in its translocation to the membrane, leading to membrane permeabilization. Although the exact mechanism is debated, MLKL is known to bind to phosphoinositides,[36] causing membrane leakage, either by forming channels or pores or through interaction with ion channels.[46] This permeabilization allows cellular contents to leak out, resulting in cell death.[47]

MLKL: Structure and Function

MLKL consists of a 4-helix bundle aminoterminal binding domain (4HB), a support region of two helices, and a carboxyterminal pseudokinase domain. The 4HB domain acts as an executor domain due to its activity affecting membrane permeability. The structure of this domain is similar to the HeLo domain, which mediates membrane permeability in the yeast Het-S protein and the plant ortholog MLKL, AtMLKL. The supporting helices have priority tasks including: assisting in the oligomerization of MLKL and transmitting signals from the pseudokinase domain to the 4HB domain (Figure 4).[48,49]

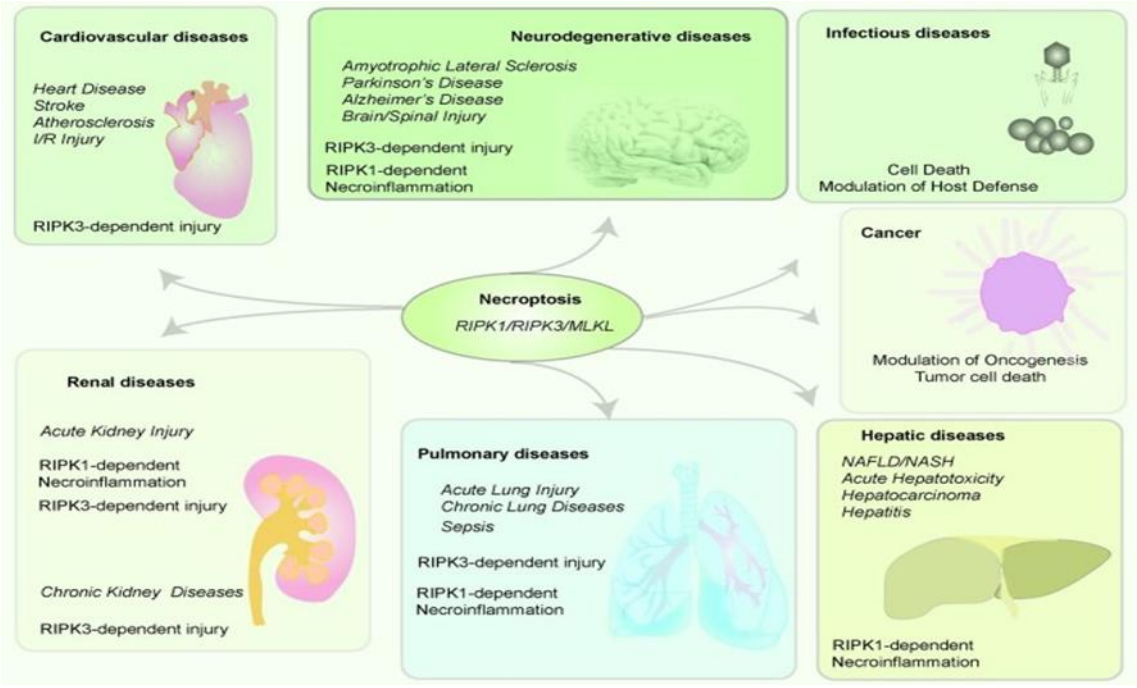

Figure 1 The Central Role of Necroptosis in Human Disease[49]

The necroptosis pathway and its regulatory proteins (RIPK1, RIPK3, MLKL) have been implicated in a variety of human diseases, including cardiovascular, neurodegenerative, infectious, liver, lung, and kidney diseases, and cancer. In diseases such as acute lung and kidney injury, the phenotype is RIPK3-dependent but MLKL-independent. In neurodegenerative, liver, and kidney diseases and sepsis, necroinflammation is RIPK1-dependent, necroptosis-independent.[11]

Detection and Quantification of Necroptosis Biomarkers

In vivo necroptosis induction is indicated by increased mRNA or protein expression of RIPK1, RIPK3, and MLKL. To assess necroptosis occurrence, phosphorylated biomarkers are examined as primary indicators. Tissue and primary cells isolated with elevated expression of (RIPK3 & MLKL) are proven to use antibody-based methods, such as western blot, immunohistochemistry (IHC), and cytometry.[54] Immunohistochemistry is a commonly used method by pathologists to illustrate the distribution and specific quantity of molecules in tissues using antigen-antibody reactions. Researchers favor IHC because it can show test results without damaging the tissue architecture or histological image.[55]

The results of these examinations involve antigen and antibody reactions in specimens when fixed by markers such as fluorophores, enzymes, particulate substances, or visualized isotopes, allowing detection of active substances like proteins, carbohydrates, nucleic acids, and other materials in the tissues. The output is histological imagery.[55]

The limitation of IHC is when subjects have immunodeficiency conditions; the inflammatory response can be mild, leading to rapid organism death in such conditions. This rapid process can occur

without significant inflammatory changes, so IHC examinations sometimes show false negatives.[56]

Another method is ELISA, which describes a method for detecting antibodies quickly, flexibly, and accurately. ELISA is based on immunological work combined with enzymatic reactions; the immunological reaction in the ELISA system involves antigen-antibody binding or vice versa. Enzymatic reactions between enzymes and reactants are used to indicate reactions, which can be measured qualitatively based on color changes in the system. The advantage of this method is its quick reaction and relatively low cost compared to other molecular methods. Based on the reaction system, ELISA is divided into three groups: Direct ELISA, Indirect ELISA, and Sandwich ELISA. These groups are based on competition or inhibition of ELISA. Direct ELISA is one of the simplest types in terms of reactions, requiring only antigen, antibody, enzyme, and substrate. However, this method is considered less effective in distinguishing cell death types due to its limited depiction of direct cell reaction and response.[57]

Currently, determining the types of cell death in cell samples is conducted through Western blot analysis of cell lysate populations and using antibodies against specific intracellular target antigens like Caspase-3, RIP1, RIP3, LC3B, HA2X, or PARP to ascertain which cell death type is dominant and to what extent. Recent developments in three-color flow cytometry assays in the lab target Caspase-3, RIP3, and cell viability, enabling detection and quantification of various cell death forms, including necroptosis, early and late apoptosis, and RIP1-dependent apoptosis simultaneously in single-cell populations.[54]

Therapeutic Implications

a. Necroptosis Inhibitors

Small-molecule inhibitors targeting RIPK1, RIPK3, and MLKL have shown promise in reducing necroptosis and mitigating tissue damage in sepsis. These inhibitors can potentially lower the levels of necroptosis biomarkers and improve patient outcomes.[9,63]

b. Immunotherapy

Modulating the immune response through targeted therapies can influence necroptosis biomarkers. For example, therapies that enhance immune cell infiltration and activity can help in controlling necroptosis and reducing inflammation.[64,65]

c. Hormonal Therapies

Estradiol, a form of estrogen, has been shown to have protective effects against necroptosis. Studies suggest that estradiol can modulate the expression of necroptosis-related genes and reduce the levels of associated biomarkers.[66] This could be particularly beneficial in sepsis management, as estradiol may help in reducing inflammation and tissue damage.

d. Necroptosis in Sepsis

Cell death and inflammation are known to have a close relationship. There are various pro-inflammatory cytokines, such as TNF-α and IL-1β, that can cause cell death. Necroptosis is a

programmed form of cell lysis and has been shown in previous studies that the release of DAMPs can cause inflammation as a response to cell death. However, in addition to DAMPs, other stimuli such as TNF and LPS can activate the NF-κB pathway, which is a potent transcriptional program for inflammation in cells.[14,67]

Necroptosis can directly activate and modulate the inflammatory response when. The relationship between necroptosis and inflammation has been extensively studied in other diseases and is suspected to be a central component of the pathogenesis of diseases caused by necroptosis. Additionally, necroptosis regulators such as RIPK1 and RIPK3 play a significant role in inflammation, independent of cell death. RIPK3 is known to activate inflammasome formation in response to cellular stress or microbial infection, which activates caspase-1 and caspase-11.[68]

Estradiol as Potential Therapy for Sepsis

In terms of renoprotection, estradiol combined with 2ME can offer protection against ischemia-reperfusion-induced kidney injury. to organ dysfunction resulting from sepsis.[70]

One of the symptoms of sepsis is inflammation and organ dysfunction. Previous studies have shown that estrogen/estradiol has the potential to reduce these symptoms. Prior research has indicated that estradiol affects biomarkers at the level of pyroptosis.[70]

First reported in 1975, men are more susceptible to post-traumatic infections. Since then, research on the relationship between sex and disease has become more prevalent.[71] Several studies have indicated that sepsis shows a clear difference between genders, with women having much lower severity levels and mortality rates. In an analysis involving over 20,000 patients across several centers, Consistent with these results, sepsis after injury and organ failure also appears to decrease in women when age is taken into account.[75]

Although serum GPER-1 levels did not show differences between men and women or between those who recovered and those who died, these results are consistent with other studies that show that sex hormone levels in sepsis patients are similar between men and women.[76] Sakr et al.[81] This may explanation conflicting efforts from the frequency and age seen from the visitors. Macrophages produce NOX2 and ROS which are crucial for killing pathogens and fighting infections. When pathogens bind to receptors on the surface of immune cells TLR and FcγR during the phagocytosis process, LC3 can be recruited to the macrophage phagosome, which promotes the occurrence of phagocytosis. To initiate LAP, RUBICON (a protein that binds to Beclin-1 and interacts with a cysteine-rich Run domain) as well as NADPH oxidase are required.[82]

Research by Sun et al.[83] It reveals that the immunity obtained through beta-glucan can help female rats cope with sepsis better than male rats. accelerate the formation of ROS and support the LAP mechanism to strengthen pathogen control. RUBICON functions as an

autophagy inhibitor that preserves the stability of the NOX2 structure in the process of ROS production. Furthermore, laboratory tests show that E2 may be more effective in stimulating trained immunity to support PAP. Sun's findings indicate the high grade of E2 in women may enhance macrophage LAP to destroy pathogens, making women more resistant to sepsis. This may explain the gender difference in sepsis cases.[84–86]

Emerging research on the interaction between necroptosis and other cell death pathways, such as apoptosis and pyroptosis, also holds great potential. Understanding these interactions will shed light on the complex network of cell death mechanisms and how they collectively influence disease pathogenesis. This knowledge could inform the development of combination therapies that target multiple pathways for a more comprehensive approach to disease management.

Finally, ongoing studies exploring the role of necroptosis in various disease contexts, beyond its traditional association with inflammation, will be crucial. Investigating necroptosis in conditions like cardiovascular diseases, metabolic disorders, and autoimmune diseases could uncover novel therapeutic targets and broaden our understanding of the biological processes underlying these conditions.

CONCLUSION

Necroptosis is an essential cell death mechanism with significant implications for various diseases, particularly in inflammatory conditions such as sepsis. Key biomarkers of necroptosis, namely RIPK1, RIPK3, and MLKL, play pivotal roles in both necroptotic signaling and inflammatory response modulation. Understanding the molecular mechanisms of these biomarkers and their interactions provides crucial insights into the pathogenesis of diseases and potential therapeutic targets.

Future research should focus on developing specific inhibitors and modulators of necroptosis biomarkers, understanding their broader roles in other diseases, and refining diagnostic tools to better predict and monitor disease progression. Advances in this field could lead to novel therapeutic approaches that improve patient outcomes by effectively managing necroptosis and its associated inflammatory responses.

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