Regulated Cell Death Pathways and Immunohistochemical Markers for Their Detection

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Abstract. Regulated cell death pathways play essential roles in numerous pathological conditions. Their molecular mechanisms involve distinct signaling cascades and specific markers that can be detected through immunohistochemistry (IHC), making tissue-level identification possible. This review summarizes the key features of each pathway and highlights the main IHC markers currently used to detect them: GPX4 and ACSL4 for ferroptosis, RIPK1, RIPK3, and MLKL for necroptosis, and caspase-1, GSDMD-N, IL-1β, and IL-18 for pyroptosis. Accurate detection of these markers in histological samples may enhance diagnostic precision and support the development of targeted therapeutic strategies.

Keywords. Ferroptosis, Necroptosis, Pyroptosis.

Introduction

Programed cell death is a biological process to eliminate potentially damaged cells and maintain tissue homeostasis, having physiological or pathological functions (1).

Traditionally, cell death has been categorized as either non-lytic and non-inflammatory, such as apoptosis, or lytic and pro-inflammatory, like necrosis, which can trigger inflammation in adjacent tissues (2).

Advances in technology have led to the identification of novel forms of regulated cell death: pyroptosis, ferroptosis and necroptosis, each with distinct molecular mechanisms and functional consequences (3,4).

Understanding these processes may contribute to improve therapeutic targets for cancer, neurodegenerative and infectious diseases (5).

Recent therapeutic approaches have begun to target specific cell death pathways to modulate disease outcomes. Inhibitors of apoptosis or ferroptosis have been used as treatments for degenerative conditions, and cancer (6,7). However, for these strategies to be effective, it is necessary to accurately identify the specific type of cell death involved.

Therefore, this review aims to provide an overview of the most recently discovered major types of regulated cell death, their molecular mechanisms, and immunohistochemical markers that can be used to detect and distinguish them in paraffinized tissue samples.

Ferroptosis

Ferroptosis is a form of cell death that occurs independently of caspases, driven by iron-dependent lipid peroxidation. Morphologically, it is characterized by shrunken mitochondria, increased membrane density, lack of chromatin condensation, but an overall preserved cell size (3).

During normal mitochondrial respiration, reactive oxygen species (ROS) are continuously generated as byproducts of oxidative phosphorylation. Under physiological conditions, these ROS are neutralized by antioxidant systems, particularly the glutathione (GSH) system and the enzyme glutathione peroxidase 4 (GPX4), which detoxifies lipid hydroperoxides and prevents membrane damage (8).

However, when the cellular antioxidant capacity is compromised — either due to GSH depletion or inhibition of GPX4 — lipid peroxides accumulate (9). Free iron, especially in its ferrous form (Fe^{2+}),

amplifies this process by catalyzing the conversion of hydrogen peroxide into highly reactive hydroxyl radicals (10). These radicals initiate and propagate the peroxidation of polyunsaturated fatty acids (PUFAs) within membrane phospholipids. As this lipid damage spreads, it disrupts membrane integrity, leading to irreversible cell damage and death through ferroptosis (11).

Ferroptosis has been associated with ischemia/reperfusion injury, and neurodegenerative diseases like Alzheimer's and Parkinson, and notably cancer cells that are resistant to therapy exhibit sensitivity to ferroptosis (12). Thus, pharmacological modulation of this process - either through promoting or inhibiting - may offer promising new therapeutic opportunities for treating ferroptosis-related conditions (13).

In routine pathology, Prussian blue staining is widely used to detect iron accumulation in biopsy tissues, which can be indicative of ferroptosis-related processes (8). However, to specifically identify cells undergoing ferroptosis, immunohistochemical markers are essential. GPX4, ACSL4, and CD71 are markers that collectively provide evidence for the presence of ferroptosis in tissue samples (14).

GPX4 is an antioxidant enzyme that detoxifies lipid peroxides; its downregulation during ferroptosis reflects impaired oxidative defense. ACSL4 promotes lipid peroxidation by incorporating polyunsaturated fatty acids into membrane phospholipids, increasing susceptibility to ferroptosis. CD71, the transferrin receptor, is upregulated in response to cellular iron uptake and is associated with increased iron accumulation in cells undergoing ferroptosis. Higher expression of ACSL4 and CD71 with reduced GPX4 levels suggests iron-mediated oxidative stress and lipid peroxidation, culminating in cell death via ferroptosis (14,15).

Necroptosis

Necroptosis is a regulated form of cell death that combines features of both apoptosis and necrosis, characterized by a controlled execution mechanism and a pro-inflammatory outcome (16).

Cells undergoing necroptosis are indistinguishable from necrotic cells in histological analysis, but their internal signaling pathways differ, while apoptosis is a caspase dependent process, necroptosis is inhibited by caspase-8 (17).

Necroptosis is triggered by the activation of the death receptor TNFR1. If caspase-8 is inhibited or non-functional, RIPK1 and RIPK3 interact, promoting the phosphorylation and activation of MLKL (Mixed Lineage Kinase Domain-Like protein) (18).

Once phosphorylated, MLKL disrupts membrane integrity, resulting in cell swelling, membrane rupture, and the release of intracellular contents, which leads to inflammation — similarly to necrotic cell death (19). Thus, necroptosis is associated with various inflammatory conditions, atherosclerosis, inflammatory bowel disease, neuroinflammation and autoimmune diseases (20).

The identification of necroptosis in tissue samples relies on detecting key proteins involved in it signaling pathway. Among the most used immunohistochemical markers are Receptor-interacting protein kinase 1 (RIPK1), Receptor-interacting protein kinase 3 (RIPK3) and mixed lineage kinase domain-like protein (MLKL) (21).

MLKL serves as a reliable indicator of active necroptosis, as its phosphorylation is a key event leading to membrane disruption and cell death. Elevated levels of RIPK1 and RIPK3, when found alongside MLKL, strengthen the evidence for necroptotic activity. Additionally, the subcellular localization of MLKL - often translocating to the plasma membrane - can further strengthen the evidence for necroptosis (22).

Pyroptosis

Pyroptosis is a caspase-dependent, highly inflammatory form of cell death that culminates with cell swelling, plasma membrane rupture and release of pro-inflammatory cytokines such as IL-1β and IL-18 (23).

Pyroptosis can be triggered by two distinct pathways: the canonical pathway, which involves inflammasome activation and caspase-1, and the non-canonical pathway, mediated by caspase-4 and caspase-5 (in humans) or caspase-11 (in mice) in response to intracellular lipopolysaccharide (LPS), both pathways use Gasdermin D (GsdmD) as downstream effector (24).

The canonical pathway starts when pattern recognition receptors (PRRs) detect pathogen-associated molecular patterns (PAMPs), or damage-associated molecular patterns (DAMPs). Triggering the activation of an inflammasome complex, which recruits and activates caspase-1. The activated caspase cleaves GsdmD, generating an N-terminal fragment (GsdmD-N), which is the main effector in the pyroptosis process forming pores in the cell membrane, leading to cell death (25,26).

In the non-canonical pathway, caspases-4, -5, and -11 detect LPS, bypassing the traditional PRRs. Once activated, these caspases cleave GsdmD, culminating in cell death, just as in the canonical pathway (27).

To identify pyroptosis using IHC, key markers include caspase-1, GsdmD-N and the pro-inflammatory cytokines IL-1β and IL-18. Caspase-1 is an important activator of the canonical pathway, and GsdmD-N serves as a specific marker, being the primary mediator of membrane rupture. The release of IL-1β and IL-18 further confirms the inflammatory nature of pyroptosis, strengthening the evidence for this form of cell death in tissue samples (28,29).

Table 1. Immunohistochemical markers for different types of regulated cell death.

Type of Cell Death	Key markers (IHC)	Notes
Ferroptosis	GPX4 ACSL4 CD71	ACSL4 and CD71 increase with GPX4 loss indicates ferroptosis.
Necroptosis	MLKL RIPK3 RIPK1	MLKL is the most specific marker; RIPK1/3 are upstream but less specific.
Pyroptosis	Caspase-1 GSDMD-N IL-1β / IL-18	GSDMD-N fragment is a specific indicator; inflammatory cytokines support the diagnosis.

Conclusion

Regulated cell death pathways such as ferroptosis, necroptosis, and pyroptosis are present in a wide range of pathological processes. Their identification through immunohistochemistry is possible and can support the development of new therapeutic strategies.

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