Research Progress of Necroptosis in Digestive Diseases

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Abstract Digestive system diseases refer to organic and functional disorders of the digestive system, which are prone to recurrence and frequently accompanied by multiple complications. Necroptosis is a regulated mode of cell death mediated by death receptors, dependent on receptor protein activation, and could be specifically inhibited by necrostatin-1. Necroptosis is involved in pathological and physiological processes of various diseases, and plays an important role in the growth and development of organisms and the homeostasis of organ tissues. This paper reviewed the research advancement of necroptosis in digestive system disorders, and discussed the relationship between necroptosis and digestive system diseases, aiming to provide theoretical basis for the cure of these diseases.

Key words Necroptosis; Digestive diseases; RIP1; RIP3; MLKL

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Digestive system diseases refer to organic and functional diseases of the digestive system, including the injuries of esophagus, stomach and duodenum, small intestine, large intestine, pancreas, liver and gallbladder, which are prone to recurrence and often accompanied by various complications. They have become one of the main reasons for the current major global public health problems and social disease burden. Necroptosis is a newly discovered form of cell death in recent years, which is closely regulated by signaling molecules such as receptor interacting protein kinase 1 (RIP1), receptor interacting protein kinase 3 (RIP3), and mixed linear kinase domain like (MLKL). The classic signal pathway of necroptosis is that RIP1-RIP3-MLKL forms a death complex, which destroys the integrity of cell membrane and is highly inflammatory. In recent years, the research on necroptosis has become more and more in-depth, and its role in digestive system diseases has also attracted much attention from scholars. Activated necroptosis signaling pathway is involved in the pathogenesis of various digestive tract diseases, and targeting RIP1 or other downstream signaling molecules, such as RIP3 and MLKL, has great therapeutic potential. This paper reviewed the important role of necroptosis in digestive system diseases.

Overview of Necroptosis

Previous studies believe that necrosis is passive, uncontrolled and irreversible, while apoptosis is active and orderly[1]. New studies found that there is still a necrosis mode regulated by gene coding in cells, namely necroptosis, which can be specifically inhibited by a small molecule preparation, necrostain-1 (Nec-1)[2-3]. Necroptosis is a cell death mode regulated by protein molecules, such as RIP1, RIP3 and MLKL[4]. RIP1 interacts with RIP3 for phosphorylation, which further promotes the phosphorylation of downstream MLKL, which is then transferred to the plasma membrane and combined with it, and further mediates the damage of plasma membrane structure and the release of damage-associated molecular patterns (DAMPs), as shown in Fig. 1. Necroptosis is characterized by cell swelling, plasma membrane rupture, cell content loss, a large number of inflammatory cell infiltration and damage of surrounding tissue[5]. Necroptosis can be induced by many factors, including death receptors in TNF superfamily, Toll-like receptor 3/4 (TLR3/4) and type Ⅰ interferon receptor (INF-I). Necroptosis can be divided into three types according to the mode of induction: (1) TNF-α-induced necroptosis, (2) reactive oxygen species (ROS)-induced necroptosis, and (3) ischemia-induced necroptosis, among which the TNF-α-induced necroptosis pathway is the most classic.

TNF-α-induced Necroptosis

TNF-α binds to tumor necrosis factor receptor type I (TNFR1) on the cell membrane and then recruits a series of proteins to form a first-order complex I, which includes TNFR1 associated death domain protein (TRADD), RIP1, TNF receptor-related factor 2/5 (TRAF2/5), apoptosis inhibitor 1 (cIAP1) and cIAP2[6]. RIP1 in complex I is a key factor in regulating cell response and determining cell survival or death. RIP1 ubiquitinates to form stable complex I and activates NF-κB signaling pathway[7]. When RIP1 is deubiquitinated, it is activated and released from complex I, forming a secondary complex (complex II) with TRADD, Fas death domain-associated protein (FADD) and caspase-8[8]. Caspase-8 is the initiator of exogenous apoptosis pathway. When caspase-8 is activated, RIP1 and RIP3 cannot be activated, and the apoptosis signal pathway is activated, which induces cell apoptosis. When caspase-8 is inhibited or knocked out, RIP1 and RIP3 interact through receptor-interacting protein (RIP) kinase homotypic interaction motif (RHIM) to form a RIP1-RIP3 complex[9]. MLKL is an important protein molecule downstream of RIP3. RIP1-RIP3 complex recruits and phosphorylates

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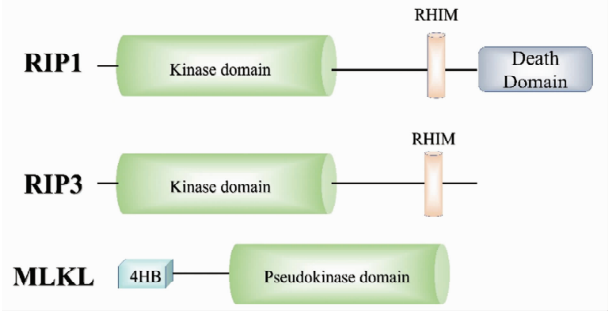
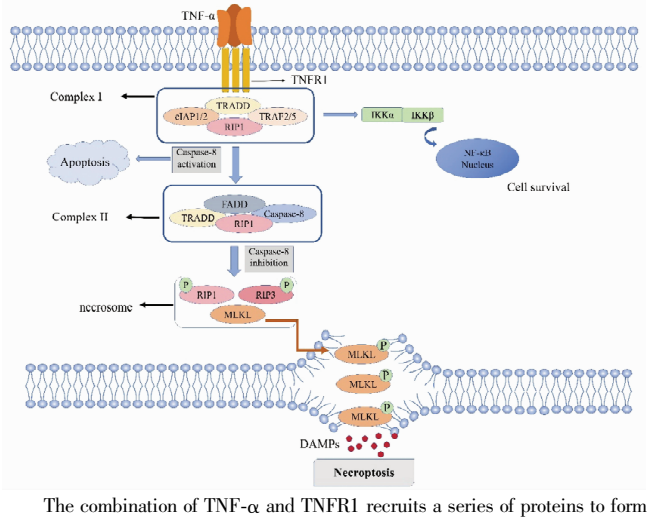
MLKL to form a necrosome, which activates downstream signal molecules of necroptosis[10]. Although the molecular mechanism of plasma membrane rupture induced by MLKL has not been clarified at present, its translocation to the plasma membrane is an indispensable key step to trigger cell membrane rupture and induce necroptosis. After the phosphorylation of MLKL by RIP3, its structure is changed, and it interacts with the amino terminal of phosphoinositide phosphate to form plasma membrane pores which penetrate the plasma membrane, which leads to the swelling of organelles, the release of DAMPs and the induction of necroptosis[11] (Fig. 2).



RIP1 is composed of N-terminal kinase domain, C-terminal death domain (DD) and RIP homotypic interaction motif (RHIM). Its RHIM domain plays an important role in the interaction with proteins with the same RHIM domain, and the death domain participates in the recruitment of protein components of complex 1. RIP3 also contains kinase domain and RHIM domain, but lacks DD. RIP3 is phosphorylated by the interaction between RHIM and RIP1. MLKL contains 4HB domain and pseudokinase domain, and phosphorylation of pseudokinase domain regulates necroptosis.

Fig. 1 Structural diagram of RIPK1, RIPK3 and MLKL proteins



The combination of TNF-α and TNFR1 recruits a series of proteins to form complex I, which includes RIP1, TRADD, TRAF2/5 and CIAP1/2; and when RIP1 is ubiquitinated, NF-κB pathway is activated. When RIP1 is deubiquitinated, RIP1 leaves complex I and forms complex II with FADD and caspase-8. In complex II, the interaction between FADD and caspase-8 can induce apoptosis. When FADD is deleted or caspase-8 is inhibited, RIP1 and RIP3 are phosphorylated through RHIM interaction, and the phosphorylated RIP3 recruits and phosphorylates MLKL, which is transferred to the plasma membrane to form membrane pores, leading to membrane rupture and eventually necroptosis.

Fig. 2 Schematic diagram of TNF-α induced necroptosis

Necroptosis and Digestive System Diseases

In recent years, with the deepening of the research on necroptosis, its role in digestive system diseases has attracted increasing attention. Numerous studies have shown that necroptosis is related to many digestive tract diseases, including liver injury, nonalcoholic liver disease, liver cancer, acute pancreatitis, ulcerative colitis, gastric ulcer and gastric cancer.

Necroptosis and acute liver injury

Acute liver injury (ALI) refers to large-scale necrosis of liver cells, inflammatory cell infiltration or severe loss of liver function in a short period of time caused by many factors. Necroptosis is considered to be an inflammatory cell death mode highly related to hepatocyte damage. In the ALI mouse model induced by lipopolysaccharide (LPS), the expressions of p-RIP1, p-RIP3 and p-MLKL increased significantly[12]. The expression of RIP1, RIP3 and MLKL and their phosphorylation in the liver of mice with acute liver injury induced by CCl_4 increased significantly[13]. Yi *et al*.[14] explored the effect of excessive acetaminophen (APAP)-induced ALI by inhibiting RIP1 activity, and found that compared with wild-type control, the hepatocyte mortality induced by APAP decreased due to RIP1 kinase deletion, and RIP1 might be a potential therapeutic target for acute liver injury caused by excessive APAP. Specific inhibition of necroptosis may be a new therapeutic idea for ALI.

Necroptosis and nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) refers to a clinicopathological syndrome which is not caused by alcohol and other clear liver damaging factors, but characterized by excessive deposition of fat in liver cells. The pathogenesis of NAFLD is not clear, and hepatocyte death is an important mechanism of NAFLD progress. It was found that the characteristic factors RIP3 and p-MLKL of necroptosis in the serum of patients with NAFLD increased at the level of gene and protein expression, and the liver injury index γ-GT increased significantly, indicating that necroptosis participated in the development of NAFLD[15]. Nonalcoholic steatohepatitis (NASH) is a serious clinical manifestation of NAFLD. Excessive fat accumulation in the liver can lead to increased inflammatory reaction and gradual fibrosis of the liver. Excessive fat accumulation in the liver can lead to increased inflammatory reaction and liver fibrosis. In NASH mouse model, specific knockout of MLKL could reduce the degree of liver fibrosis[16]. In the experiments of NASH mice induced by high-fat diet, inhibiting the expression of RIP1 and the formation of necrosome could significantly improve NASH symptoms, including relieving fatty degeneration, liver inflammation and fibrosis[17]. In methionine-choline deficiency diet model, compared with wild-type mice, the liver fibrosis of RIP3 knockout mice was also significantly reduced[18]. All the above studies show that inhibiting the death of hepatocytes by inhibiting the expression of genes related to necroptosis may be a new strategy for developing drugs for NALFD.

Necroptosis and liver cancer

Liver cancer is one of the common digestive tract tumors, and hepatocellular carcinoma (HCC) is the most common type of liver cancer. Its incidence is insidious, and its early symptoms are not

obvious. When the liver cancer progresses to the middle and late stage, patients will have liver pain, digestive tract symptoms and liver enlargement. Related studies report that necroptosis plays an important role in HCC. The study of Wang *et al.*[19] confirmed that the expression of RIP1 increased in hepatocellular carcinoma, and predicted the poor prognosis of patients with hepatocellular carcinoma. RIP1 is expected to be developed as a new biomarker of poor prognosis of HCC patients. Han *et al.*[20] found that RIP3 mRNA was highly expressed in peripheral blood mononuclear cells of HBV-related HCC patients, and the combination of RIP3 mRNA level determination and AFP detection could greatly improve the diagnostic accuracy of HCC in healthy people. The mRNA level of RIP3 is a promising biomarker to supplement AFP detection in early diagnosis of HCC. miRNAs of gene related to necroptosis may be used as an auxiliary means for HCC diagnosis, providing new ideas for treating HCC and improving the prognosis of patients.

Necroptosis and acute pancreatitis

Acute pancreatitis (AP) is an inflammatory disease of the pancreas, usually with mild self-limitation. It is characterized by the damage of pancreatic acinar cells, the activation of pancreatic enzymes and the triggering of other digestive enzymes, which leads to self-digestion, hemorrhage and edema of pancreatic tissues. Studies have proved that the degree of acinar cell necrosis is positively correlated with the severity of pancreatitis. Necroptosis is the main mode of acinar cell death in mice with severe experimental pancreatitis, and the treatment with Nec-1 can reduce the severity of pancreatitis[21]. In AP, the expression level of RIP3 and p-MLKL is positively correlated with necroptosis, and the decrease of RIP3 and p-MLKL expression levels is beneficial to reducing acinar cell death[22]. Shen *et al.*[23] proved that the expression of RIP3 and p-MLKL in the pancreatic tissue of AP mice induced by caerulein increased significantly. Michittra *et al.*[24] explored the role of RIP3 and MLKL in mouse AP model, and found that compared with normal mice, RIP3 and MLKL knockout mice were more vulnerable to caerulein damage, mainly manifested as severe pancreatic edema, increased inflammatory cell aggregation and acinar cell death. In addition, some studies show that the phosphorylation levels of RIP1, RIP3 and MLKL are significantly increased and the signal of necroptosis is activated in *in-vitro* acute AP model; and specific inhibition of this signaling pathway can reduce the severity of AP[25]. Therefore, inhibiting the necroptosis mediated by RIP1-RIP3-MLKL may become a potential therapeutic target of AP.

Necroptosis and inflammatory bowel disease

Inflammatory bowel diseases are related to autoimmunity, including Crohn's disease (CD) and ulcerative colitis (UC). Necroptosis signal transduction pathway plays an important role in the occurrence of intestinal diseases. In the UC model constructed by dextran sodium sulfate (DSS), the expression of RIP1 and RIP3 increased[26]. In the *in-vivo* CD model induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS), intestinal mucosal damage, intestinal interstitial congestion and focal infiltration of inflammatory cells and necrotic substances were observed, and the expression of p-RIP3 increased. In the *in-vitro* model of Caco-2 cells induced by TNF-α + Z-VAD-fmk, the number of Caco-2 cells decreased; the cells showed irregular shape; the organelles swelled and the plasma membrane ruptured; the morphological damage of cells increased; and the expression of p-RIPK3 and p-MLKL increased. All the above results confirmed that necroptosis could promote the death of intestinal cells[27]. Moreover, some studies have proved *in vivo* and *in vitro* that RIP3 upregulated the expression of p-MLKL, increased the release of inflammatory factors such as IL-8, IL-1β and IL-33, and decreased the expression of E-cadherin, occludin and zonulin-1, thus changing the permeability of cell membrane[28]. Xiong *et al.*[29] found that the expressions of p-RIP3, p-RIP1 and p-MLKL in the colon of UC mice increased, while the expression of caspase-8 decreased, which promoted the occurrence of necroptosis in enterocyte and destroyed the intestinal epithelial structure. Yang *et al.*[30] established an acute colitis mice model by DSS, and found that the structure of colon tissue was seriously damaged, and the expressions of p-RIP1, p-RIP3 and p-MLKL increased significantly. However, after drug intervention was given to inhibit the necroptosis signaling pathway, the release of IL-1β, TNF-α and IL-6 was inhibited, and the injury of colonic crypt was alleviated, thus alleviating intestinal inflammation. In addition, Lee's clinical study confirmed that the expression of RIP3 and p-MLKL in UC patients increased, and the treatment with RIP3 inhibitor GSK'872 could significantly reduce the expression of inflammatory factors in peripheral blood mononuclear cells of UC patients, which inhibited the process of cell necroptosis, and thus improved the symptoms of colon[31]. Therefore, related research targeting at necroptosis may provide new ideas for the diagnosis and treatment of inflammatory bowel disease.

Necroptosis and gastric ulcer

Gastric ulcer (GU) is a common gastrointestinal disease in the upper digestive tract. The main pathological manifestation is gastric mucosal injury and bleeding, with an incidence of 5% – 10%. The main causes include *Helicobacter pylori* (Hp) infection, excessive ethanol intake and long-term use of non-steroidal anti-inflammatory drugs[32]. Necroptosis is the endogenous inducement of inflammatory reaction. In the mouse model induced by aspirin, the microvilli on the surface of gastric mucosa disappeared; the integrity of cell membrane was destroyed; lactate dehydrogenase was released from cells to the extracellular space; ATP level decreased; and RIP1, RIP3 and p-MLKL proteins were up-regulated, and cells suffered from necroptosis, gastric mucosal injury and inflammatory cell infiltration. After the intervention treatment with Xiaojianzhong Decoction, it could inhibit the occurrence of necroptosis in gastric mucosal cells and reduce gastric mucosal damage[33]. Liu *et al.*[34] stimulated GES-1 cells with ethanol to induce necroptosis, which was mainly manifested by the increase of RIP1 and MLKL expression and the generation of more necrosome, and Nec-1 could effectively inhibit the above changes. Therefore, inhibiting RIP1/RIP3/MLKL pathway may provide a new direction for the treatment of gastric mucosal injury.

Necroptosis and gastric cancer

Gastric cancer (GC) is a gastrointestinal malignant tumor with high mortality, and Hp infection is one of the main causes of GC. Hp infection can induce the expression of TNF-α in gastric

mucosa and the degree of gastroduodenal mucosal damage to increase significantly; and toxic factors released by Hp, such as vacuole A, can also induce necroptosis to damage gastric mucosa[35]. A clinical study found that the expression of RIP1 in gastric cancer patients infected by Hp increased significantly, which may be due to the inflammatory reaction induced by Hp infection, which in turn induced the increase of RIP1 expression[36]. SORI *et al.*[37] found that activating RIP1/RIP3/MLKL signaling pathway could induce AGS cell death. Vetrivel *et al.*[38] found that activated RIP3 can phosphorylate MLKL and induce AGS cell necrosis through *in-vitro* and *in-vivo* experiments, molecular docking and dynamic simulation. Biglycan (BGN) is a proteoglycan, and its significant expression in GC tissue indicates that it plays a carcinogenic role in cancer migration and invasion. When endogenous BGN is overexpressed in HGC-27 and AGS cells, the expression of RIP1 and RIP3 decreases, which inhibits cell necroptosis. The regulation of BGN on necroptosis may be a potential strategy for preventing and treating gastric cancer[39]. RIP1-RIP3-MLKL plays an important role in the pathophysiological process of GC.

Conclusions and Prospects

Necroptosis can determine the ultimate fate of cells and regulate the development of diseases, which is an important link in cell physiological activities. The relationship between necroptosis and digestive system diseases has been reported many times *in-vivo*, *in-vitro* and clinical studies. Understanding different functions of necroptosis in digestive system diseases, clarifying its relationship with digestive system diseases, explicating its activation pathway and targeting necroptosis or its downstream inflammatory cytokines all can provide new ideas for the treatment of digestive system diseases such as liver injury, nonalcoholic liver disease, liver cancer, acute pancreatitis, ulcerative colitis, gastric ulcer and gastric cancer, and also lays a theoretical foundation for the research and development of new drugs.

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