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Review

Molecular Mechanisms of Acute Radiation Intestinal Injury and Its Control

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The intestinal tract is a representative radiosensitive tissue and a risk organ in radiotherapy that limits the prescribed dose to tumors in the abdominal and pelvic regions. High-dose radiation damage to intestinal tissue induces the loss of intestinal epithelial stem cells, and it is now becoming clear that this process consists of two steps: crypt cell death regulated by p53 and exacerbation caused by inflammatory immune responses. Transcriptional regulators that enhance p53 function without enhancing apoptosis are effective in controlling the first step. The common activity of these p53 modulators that promote such functions is that they are able to suppress apoptosis without impairing p21-mediated cell cycle arrest. Regulating the second step can be achieved by agonists of Toll-like receptor signaling pathways that enhance the priming signal of pyroptosis and inhibitors that suppress the activity of the inflammasome. In this review, we outline the molecular mechanisms of each process and discuss strategies for effectively controlling the acute radiation-induced gastrointestinal syndrome.

Key words: radioprotector, p53, p53 modulator, inflammatory response, inflammation control

1. Introduction

Death from exposure to radiation varies with the dose: death from a whole-body exposure of less than 10 Gy is referred to as bone marrow death because it is caused by the hematopoietic tissue damage, whereas death resulting from exposure to 10 Gy or more results in severe damage to the intestinal epithelium that precedes the progression of hematopoietic tissue damage. This process is referred to as intestinal death[1]. Radioprotectors (or Radiation protectors) are drugs that are administered prior to exposure to prevent radiation damage and to confer radioresistance. Radiation mitigators are defined as drugs that promote tissue recovery after radiation injury, but in a broader context, they are also sometimes referred to as radioprotectors. Radioprotectors include conventional radical scavengers that reduce the indirect effects caused by radiation, and agents that activate biological defense mechanisms have recently been successfully developed as radioprotectors. It should also be noted that, regarding the development of radiation mitigators, several hematopoietic agents have been developed and are generally regarded as promising agents.

The most important challenge in developing radioprotectors for radiotherapy is to ensure that they selectively protect normal tissue but not tumor tissue. The only radioprotector approved for clinical use is the FDA-approved antioxidant amifostine (also known as WR-

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2721 or Ethyol), which has been approved for use as a prophylactic agent for dry mouth that selectively protects salivary glands in the head and neck during radiation treatments for cancer[2, 3]. Amifostine is a prodrug in which the thiol group that is required for radical scavenging is covered by a phosphate group. After the phosphate group is removed in the body, it then exerts its radioprotective effects. If the uptake of amifostine and removal of the phosphate group is slower in head and neck cancers than in salivary glands, irradiation at a timing within 10 to 30 minutes of administration would selectively protect the salivary glands. In other words, only when there is a delay in uptake and dephosphorylation of the drug in the tumor will it be selective for radiotherapy.

In addition, research on medical countermeasures against acute radiation syndrome (ARS) has been vigorously pursued in the United States, and since 2015, the following four hematopoietic agents have been approved by the FDA; G-CSF (filgrastim), pegfilgrastim (a sustained G-CSF product), GM-CSF (sargramostim), and Romiplostim (Nplate; a thrombopoietin receptor agonist)[4]. These agents include hematopoietic factors that are effective in treating chemotherapy-induced bone marrow suppression in cancer treatment, but when the cancer is treated by radiation alone, it is rare that the hematopoietic tissue is so extensively irradiated that the treatment must be interrupted. Therefore, protecting organs at risk (OAR) near the tumor, rather than hematopoietic tissue, should be considered in radiation therapy.

2. Protection of the intestinal tract from radiation by p53 regulation

The intestinal tract is a typical radiosensitive tissue and is the OAR that limits the prescribed dose to tumors in the abdominal and pelvic region in radiation therapy. Intestinal tissue damage due to high-dose radiation leads to the loss of intestinal epithelial stem cells. High-dose radiation exposure causes the radiation-induced gastrointestinal syndrome (RIGS), which leads to intestinal death 10 to 15 days after exposure through symptoms such as diarrhea, dehydration, sepsis, and intestinal bleeding[1]. In addition, although fractionated irradiation reduces the extent of normal tissue damage in radiotherapy, acute and late effects such as intestinal bleeding, ulcer formation, stenosis, perforation, and intestinal obstruction are known to occur when the intestinal tract is in close proximity to the irradiation field[5, 6]. Henson *et al.* reported that, in a cohort of patients treated with pelvic radiotherapy, 90% experienced acute RIGS during treatment, 70% of the patients experienced either an incomplete resolution of acute RIGS symptoms or the progression of symptoms originating from acute RIGS, and by 6 months after treatment, 30% had been diagnosed with radiation proctopathy[5].

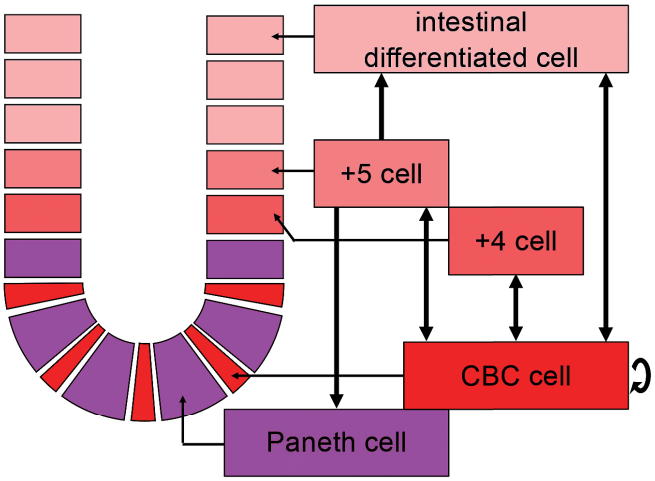

Fig. 1. Structure of the intestinal crypt. Intestinal epithelial cells are replaced by new ones as CBC stem cells actively divide, with the upper differentiated cells reaching the surface and are then shed from the tips of the villi. The direction of the arrow indicates the direction of differentiation and reversibility. The downward arrows mean that the upper differentiated cells serve as replacement CBC stem cells, when CBC stem cells are lost and they are able to receive niche factors.

Figure 1 shows the structure of the intestinal crypt. The epithelial surface of the intestinal tissue is composed of crypts and villi, and two types of intestinal epithelial stem cells are known to exist: crypt base columnar (CBC) cells, which are actively dividing, and +4 cells, which rarely divide[7, 8]. CBC cells constitute the crypt basement by residing between differentiated secretory cells called paneth cells, and niche factors that are secreted by these neighboring cells maintain CBC cells in an undifferentiated state[9, 10]. Paneth cells express stem cell growth factors such as EGF, Dll4, and Wnt3, and provide a niche for CBC cells, primarily by expressing Wnt[10]. Since CBC cells are actively dividing while paneth cells are not, the intestinal epithelial cells that originate from CBC cells differentiate when they leave the paneth cells and are no longer able to receive niche factors. CBC cells actively divide, causing old differentiated cells to move to the upper crypts and villi with differentiation and subsequently shed from the tips of the villi, replacing them with new cells within a few days. There are also secretory progenitor cells, such as +4 and +5 cells, whose descendants are usually only secretory cells, but are reserve cells that function as alternative CBC cells by dedifferentiating, when CBC cells are severely damaged by radiation or other insults and they are able to receive niche factors from paneth cells[11-14]. The cells of the intestinal crypts are known to contain marker genes that are highly expressed specifically in each cell: *Ascl2*, *Lgr5*, *Olfm4*, and *Ephb3* in CBC cells; *Bmi1*, *Hopx*, *Lrig1*,

Tert, and *Mex3a* in +4 cells; *Dll1* in +5 cells; and in more differentiated cells *Alpi* and *Krt19*[15-26]. By examining the expression levels of marker genes in samples obtained from mouse intestinal epithelia that have been damaged by radiation or other agents, it is possible to estimate the percentage of cells that are severely damaged and the percentage of cells that are protected from the damage by administering a protective agent.

The tumor suppressor gene product p53 is involved in the progression of radiation cell death in bone marrow and the intestine, but its involvement is quite different in the two tissues. In the bone marrow, p53 acts to promote radiation-induced apoptosis, while in the intestinal epithelium it functions as a resistance factor against nonapoptotic mitotic death. In fact, caudal half-body irradiation studies indicated that mice that specifically lack *Trp53* in the intestinal epithelium are more radiosensitive, whereas Super p53 mice with two extra copies of *Trp53* are radioresistant[27]. These findings indicate that there are cell deaths that can be inhibited by p53 in RIGS.

Therefore, to protect OARs, it is necessary to change the way p53 is regulated according to its role. We previously reported that sodium orthovanadate is an effective p53 inhibitor that suppresses the apoptosis-promoting activity of p53 for radiation myelopathy[28, 29] and that 5-chloro-8-quinolinol (5CHQ), a p53 modulator, enhances the anti-cell death activity of p53 for RIGS (Fig. 2)[30]. While the p53 inhibitor sodium orthovanadate does not show any protective effect against RIGS after single caudal half-body irradiation[31], 5CHQ is one of the few protective agents that shows efficacy against RIGS and is a valuable seed compound for promoting the development of intestinal protective agents.

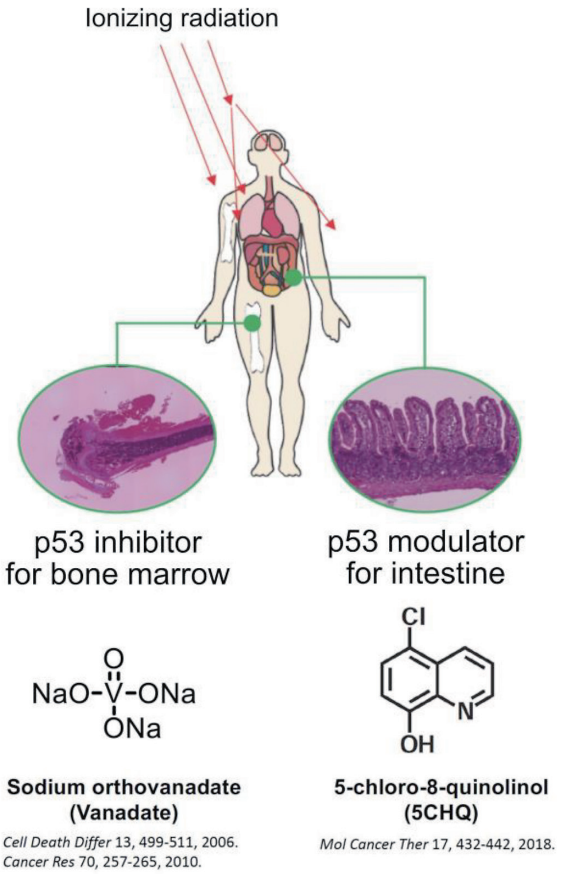

Fig. 2. Two types of p53 regulators that protect radiosensitive tissues. There are two types of p53 regulators: p53 inhibitors and p53 modulators. p53 inhibitors are effective for treating bone marrow suppression caused by whole-body exposure, while p53 modulators are effective for treating intestinal injury caused by local exposure.

In mouse intestinal epithelium, 5CHQ enhances the expression of *Cdkn1a*, which encodes p21, and suppresses the expression of *Bbc3*, which encodes Puma, in a p53-dependent manner[30]. In addition, the expression level of *Lgr5*, a marker gene for CBC stem cells, is decreased by RIGS after caudal half-body irradiation, but 5CHQ suppresses this effect[30]. *Cdkn1a* is known to suppress nonapoptotic mitotic death in RIGS by arresting the cell cycle in a p53-dependent manner[27], and *Bbc3* acts as an inducer of radiation-induced apoptosis in mouse intestinal epithelial stem cells[32]. Research in C57BL/6 background *Cdkn1a* knockout mice, *Bbc3* knockout mice, double knockout mice in both genes, and *Trp53* knockout mice has shown that *Bbc3* contributes to the promotion of the radiation-induced cell death of intestinal tissues, as *Bbc3* knockout mice show a prolonged survival after irradiation, and *Cdkn1a* contributes to the protection of intestinal tissues based on the fact that *Cdkn1a* knockout mice show reduced survival time[33].

Several p53 modulators that confer radioresistance to cells have been reported in addition to 5CHQ. CHIR99021, an inhibitor of GSK-3, a serine/threonine kinase, that specifically inhibits the acetylation of lysine 120 in the DNA-binding domain of p53 and PUMA induction by inhibiting the phosphorylation of the acetyltransferase Tip60, one of the phosphorylation targets of GSK-3. On the other hand, it does not inhibit p21 induction, effectively suppressing the intestinal death caused by abdominal irradiation[34]. Auranofin, a gold-containing anti-rheumatic drug, stabilizes p53 by inhibiting proteasomal degradation through the inhibition of HAUSP7, a deubiquitinase that is involved in regulating the stability of p53[35]. This agent, like 5CHQ, induces the p53 accumulation and p21 by itself, but not PUMA, and does not induce the acetylation of lysine 120 in p53, which is required for PUMA induction, and the resulting reversible cell cycle arrest by activating the p53-p21 pathway that inhibits abdominal irradiation-induced intestinal death and an improved survival[36]. In addition, antitumor effects were obtained in mouse abdominal tumor models and

Table 1. Representative p53 regulators and their radioprotective effects

Agents/Compounds		Characteristics/Mechanisms of action/Effects	References
p53 inhibitors	Sodium orthovanadate	Inhibition of p53 transcription-dependent and - independent apoptotic pathways, bone marrow protection, as well as tyrosine phosphatase inhibitor	28, 29
	Pifithrin α	Inhibition of DNA-binding and transcriptional activities of p53, bone marrow protection	42
	Pifithrin μ	Inhibition of p53 transcription-independent apoptotic pathway, bone marrow protection	43
	Ex-RAD	Inhibition of p53-dependent apoptosis, bone marrow protection	44, 45
p53 modulators	5-chloro-8-quinolinol	Activation of p53/p21 pathway with PUMA suppression, bone marrow and intestinal protection	30
	CHIR99021	Inhibitor of serine/thereonine kinase GSK-3, p21 induction with PUMA suppression, intestinal protection	34
	Auranofin	Inhibition of p53 proteasomal degradation, activation of p53/p21 pathway without PUMA induction, intestinal protection	35, 36
	RG7112	Activation of p53/p21 pathway through transient disruption of p53-Mdm2 interaction, intestinal protection	38

human malignant colon organoids with the drug alone and further antitumor effects were observed when combined with radiation. Pant *et al*. generated knock-in mice in which the p53-Mdm2 negative feedback loop was disrupted by mutating a p53 response element in the Mdm2 P2 promoter[37]. These mice have normal p53 levels and activity under normal conditions, are characterized by increased p53 activity compared to wild-type mice after stimulation by DNA damage, and are resistant to intestinal death caused by caudal half-body irradiation. In these mice, they also confirmed that the knockout of *Trp53* only in *Lgr5*-positive stem cells or the knockout of *Cdkn1a* (*P21*) results in the loss of resistance to intestinal death[38]. In that report, they also reported that the pharmacological transient enhancement of p53 activity in wild-type mice using RG7112, an Mdm2 inhibitor, could suppress intestinal death[38].

One of the issues in radiation protection by p53 regulators is the concern that carcinogenesis is promoted by suppressing the elimination function of p53 against oncogenic mutant cells. However, in various mouse models, it has been shown that the suppression of the three representative DNA damage responses of p53, apoptosis, cell cycle arrest, and senescence, does not affect the p53-mediated inhibition of carcinogenesis, and that the p53-mediated inhibition of carcinogenesis is a delayed response that is independent of these three functions[39-41]. These results suggest that the temporary enhancement of wild-type p53 activity by pharmacological means may be an effective strategy for alleviating the problem of intestinal damage in patients who are undergoing radiotherapy in the clinic. Table 1 shows a list of p53 inhibitors and p53 modulators.

3. Protection of the intestinal tract against radiation through the regulation of immune mechanisms

Genetic deletion of various inflammation-related genes in mice has recently revealed the importance of innate immune responses in RIGS. The molecular structural patterns that elicit inflammatory responses are classified into two major groups: pathogen-associated molecular patterns (PAMPs), which are groups of molecules characteristic of exogenous pathogens, and damage-associated molecular patterns (DAMPs), which are endogenous immunogenic molecules that leak from damaged cells[46]. In RIGS, the leakage of self-nucleic acids as the latter DAMPs is of particular importance.

Concerning pattern recognition receptors (PRRs), which are activated by DAMPs as ligands, it is important to consider the intracellular and extracellular distribution of DAMPs and the DAMPs recognition pathway due to the intracellular location of PRRs. The activation of intracellular PRRs triggers pyroptosis, an inflammation-induced cell death of macrophages and other cells responsible for immunity[47]. Those DAMPs that cannot be degraded or removed after extracellular leakage are taken up by surrounding cells by endocytosis and activate TLR3, TLR7, and TLR9, which are Toll-like receptors (TLRs) in endosomes. The agonists of each are double-stranded RNA (dsRNA) for TLR3, single-stranded RNA for TLR7, and CpG-DNA for TLR9[48].

Takemura *et al*. reported that TLR3-mediated immune responses are important in the pathogenesis of RIGS[49]. Their whole-body irradiation studies of mice lacking *Tlr2*, *Tlr3*, *Tlr4*, *Tlr5*, *Tlr7*, and *Tlr9* alone revealed that only the *Tlr3* knockout mice were radioresistant. These mice showed resistance to RIGS by significantly reducing crypt

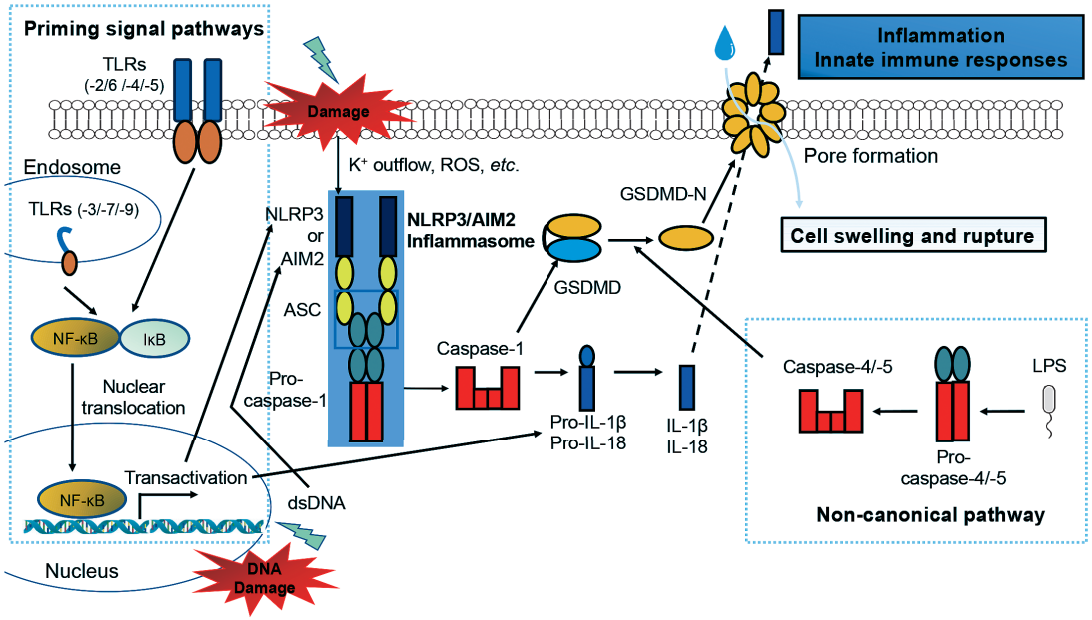

Fig. 3. The role of inflammasomes in pyroptosis. The inflammasome is a platform for the activation of inflammatory caspases in pyroptosis, a form of lytic programmed cell death. The priming signal pathways and the non-canonical pathway are also shown.

cell death. They also found that RIGS consists of a two-step pathological process: p53-dependent crypt cell death resulting in RNA leakage, and p53-independent intestinal epithelial cell death induced by the leaked dsRNA through activating the TRIF-RIPK1 pathway, which can only be utilized by TLR3 in TLR family members. In addition, an inhibitor of TLR3-dsRNA binding was also found to improve RIGS by reducing crypt cell death. Of note, it is known that the TRIF-RIPK1 pathway induces necroptosis. Indeed, a study using necrostatin-1, a specific inhibitor of RIPK1, had a mitigating effect that improved mouse survival only when administered post-irradiation (24-72 hours after total-body irradiation), not pre-irradiation[50], which is consistent with the two-step model of RIGS.

Pyroptosis studies began with the discovery by Friedlander *et al.* that cell death and the rapid release of intracellular contents occurred when primary mouse macrophages were treated with the lethal anthrax toxin[51]. Subsequent studies on the cell death of macrophages infected with *Shigella flexneri* led to the discovery again by Zychlinsky *et al.* in 1992[52]. That cell death was initially considered to be apoptotic because of its similar features to apoptosis, such as caspase-dependence, DNA damage, and nuclear condensation. However, in 2001, D'Souza *et al.* re-defined the term as pyroptosis, derived from the Greek words 'pyro' (fire/heat) and 'ptosis' (fall), to denote caspase-1-dependent inflammatory programmed cell death[53].

The formation of inflammasomes plays an important role in pyroptosis (Fig. 3)[54]. The inflammasome contains a key adaptor molecule, ASC (Apoptosis-associated Speck-like protein containing Caspase recruitment domain), which binds to intracellular PRRs via its Pyrin domain, and at the same time, ASC binds to procaspase-1 via its own Caspase recruitment domain. The mechanism by which inflammatory cytokines such as IL-1β and IL-18 are secreted extracellularly has long remained unclear because they lack secretory signaling sequences, but the discovery of Gasdermin D (GSDMD), a plasma membrane pore forming factor that is cleaved and activated by caspase-1[55] explains this dilemma regarding pore formation during pyroptosis is now considered to be the major secretory mechanism of inflammatory cytokines. After the discovery of GSDMD, a new definition of pyroptosis as cell death via pore formation by a group of GSDM molecules was proposed[56].

In RIGS, the AIM2 inflammasome, which is formed via the PRR, AIM2 (Absent in melanoma 2), that recognizes double-stranded DNA (dsDNA), is known to regulate cell death and tissue damage in the intestinal epithelium[57]. In that study, the knockout of either caspase-1, AIM2, or the adaptor molecule ASC, a molecule within the same inflammasome platform, suppressed radiation-induced intestinal death, suggesting a close relationship between RIGS and inflammatory cell death. Furthermore, the study also showed that intestinal resistance is maintained in mice in which caspase-1 is specifically lacking in the

intestinal epithelium, indicating that inflammasome-mediated cell death occurs in the intestinal epithelium in RIGS. It has also been reported that NLRP3 (NACHT, LRR and PYD domains-containing protein 3) knockout mice have also been reported to show resistance in a 9.5 Gy whole-body irradiation test[58].

The mechanisms responsible for pyroptosis have been investigated in detail in the case of TLR signaling pathways. The process is known to occur via a two-step process in the canonical pathway[47, 54]. The first step of the process requires "priming" by the binding of TLRs to their ligands, which activates NF-κB and induces the expression of several proteins that form the inflammasome complex and precursors of inflammatory cytokines such as pro-IL-1β and pro-IL-18. In the second step of the process, caspase-1 is activated and GSDMD, pro-IL-1β, and pro-IL-18 are cleaved, leading to pyroptosis with cell lysis. The pathway in which intracellular PAMPs directly activate caspase-4/5 without the formation of inflammasomes and lead to pyroptosis is referred to as the non-canonical pathway. The molecular mechanism responsible for the transition from the first to the second step in RIGS and the nature of the target cells are currently poorly understood, but since the knockout of intracellular PRRs such as AIM2 and NLRP3 results in radioresistance, the second step in pyroptosis can be considered to be an exacerbating factor in RIGS.

On the other hand, the first-step priming signals are considered to be signals that contribute to intestinal epithelial cell resistance in RIGS, as various agonists that enhance the pathways confer radioresistance. The full activation of the canonical pathway requires transactivation by NF-κB and others, and this action also appears to cause the activation of factors that confer radioresistance. To date, agonists of TLR2/6[59-63], TLR4[64-68], TLR5[69-71], and TLR9[72] have been reported to exhibit protective or mitigating effects against RIGS.

In addition, if the extent of RIGS-induced damage is severe, the intracellular PRRs, AIM2 and NLRP3, may be activated without proceeding through the first step priming process, thus activating signals that exacerbate the inflammatory response. Under these circumstances, caspase-1, which is responsible for inflammasome activity, may be a potential drug target for therapies that antagonize RIGS. Several inflammatory caspase inhibitors have been designed as potential therapeutics for the treatment of inflammatory diseases. Only a few have been clinically tested due to barriers such as inadequate efficacy, poor target specificity, or severe side effects, resulting in the fact that no synthetic caspase inhibitors have yet been approved[73]. Synthetic caspase inhibitors are classified as peptide-based inhibitors, peptidomimetic inhibitors, and nonpeptidic inhibitors. Inhibitors that target caspase-1, such as Ac-YVAD-CMK[74] and Z-YVAD-FMK[75] for peptide-based inhibitors and VX-765 (Belnacasan)[76], VRT-043198[77], and VX-740 (Pralnacasan)[78] for peptidomimetic inhibitors have been actively studied. For the case of RIGS, VX-765 has actually been reported to suppress intestinal death caused by abdominal irradiation[79, 80]. In addition, although the following might be considered as indirect effects, the efficacy of Micheliolide[81], Rosiglitazone[82], Resveratrol[83], which regulate NLRP3, or 5-androstenediol[84], which regulates AIM2, against RIGS has been reported.

4. Summary

Figure 4 summarizes the molecular mechanism for RIGS and its regulators. The molecular mechanism responsible for radiation acute intestinal injury is outlined as a two-step process that begins with a p53-regulated process, followed by an inflammatory exacerbating process. We also introduced the concept that the enhancement of p53 function that does not enhance apoptosis can be effective in counteracting the first step, and that regulating the priming signal of pyroptosis and the inhibition of inflammasomes activity are effective in counteracting the second step. The suppression of radiation-induced intestinal damage by p53 modulators has shown promising results regarding the selective protection of normal tissues in a mouse model. The issue of whether normal tissue selectivity can be achieved by regulating the second step of the process awaits further research. We hope that further studies on the pathological mechanisms of both processes will lead to the development of more effective methods for controlling radiation intestinal injury.

Conflicts of Interest

The authors declare no conflict of interest.

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Abbreviations

ARS, acute radiation syndrome; OAR, organs at risk; RIGS, radiation-induced gastrointestinal syndrome; CBC, crypt base columnar; 5CHQ, 5-chloro-8-quinolinol; PAMPs, pathogen-associated molecular patterns; DAMPs, damage-associated molecular patterns; PRRs, pattern

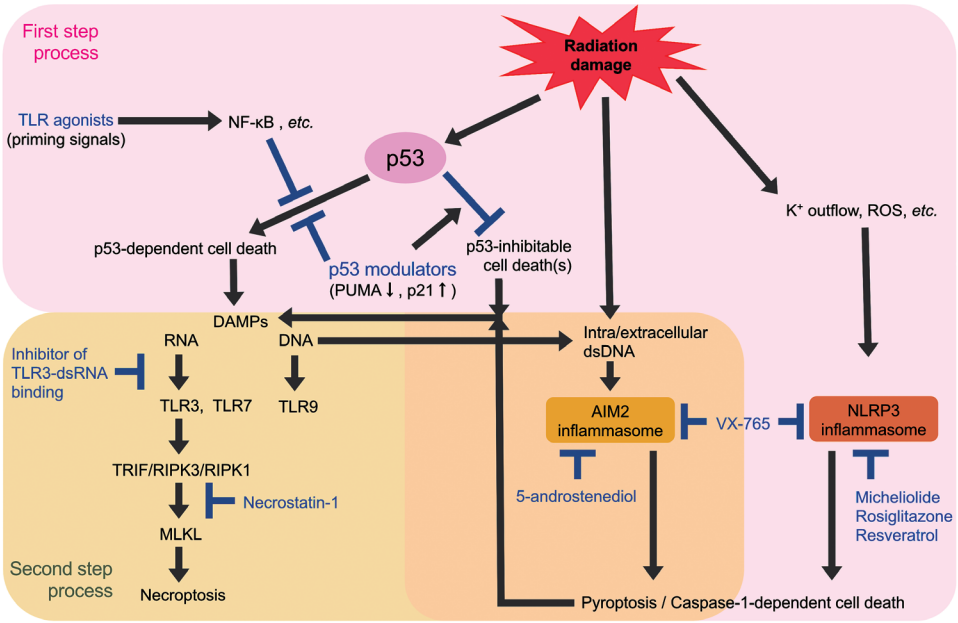

Fig. 4. The role of inflammasomes in pyroptosis. Details of p53-inhibitable cell death(s) are unknown, but the overexpression of p53 results in resistance to RIGS[27]. The role of caspase-1 is not limited to macrophages; the intestinal epithelial-specific deficiency of *Casp1* also confers resistance to RIGS[57]. This death is herein referred to as caspase-1-dependent cell death. A plausible molecule that could act in both the first and second steps could be considered AIM2. In that case, the endogenous dsDNA would act in the first step and the exogenous dsDNA in the second step.

recognition receptors; TLRs, Toll-like receptors; dsRNA, double-stranded RNA; ASC, apoptosis-associated speck-like protein containing caspase recruitment domain; GSDMD, Gasdermin D; AIM2, Absent in melanoma 2; dsDNA, double-stranded DNA; NLRP3, NACHT, LRR and PYD domains-containing protein 3.

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