Published in final edited form as:

Adv Exp Med Biol. 2011 ; 691: 589–593. doi:10.1007/978-1-4419-6612-4_62.

A RNA Interference Screen Identifies RIP3 as an Essential Inducer of TNF-Induced Programmed Necrosis

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Abstract

Recent evidence indicates that TNF-like death cytokines can induce apoptotic and non-apoptotic forms of cell death. We have coined the term “programmed necrosis” to describe caspase-independent cell death induced by TNF-like cytokines. Besides an obligate requirement for the protein serine/threonine kinase RIP1 and the production of reactive oxygen species (ROS), relatively little is known about the molecular mechanisms that control TNF-induced programmed necrosis. In order to further illuminate the molecular pathway that governs programmed necrosis, we performed a targeted RNA interference (RNAi) screen. Our screen identified RIP3, a RIP1 family member, as a specific mediator for programmed necrosis, but not apoptosis. Biochemical analyses show that assembly of the pro-necrotic RIP1-RIP3 complex critically regulates induction of programmed necrosis. The physiological relevance of RIP3-dependent programmed necrosis is demonstrated by the failure of RIP3-deficient mice to control vaccinia virus infections.

Introduction

Cell death plays an important role in homeostasis of multi-cellular organisms. TNF-like cytokines and their cognate receptors are important activators of cell death. In recent years, it has become clear that TNF-like cytokines can trigger multiple forms of cell death that are distinct in morphologies and mechanisms. Programmed necrosis (also known as necroptosis) is a caspase-independent, non-apoptotic form of cell death whose molecular mechanism is poorly defined [1]. Until recently, the only identified molecular component of programmed necrosis is the protein serine/threonine kinase Receptor Interacting Protein 1 (RIP1). Morphologically, programmed necrosis is marked by organelle swelling and extensive intracellular vacuolation. The disruption of osmotic balance within the dying cell eventually led to rupture of plasma membrane. The release of cellular “adjuvants” from the necrotic cells promotes inflammation [2]. These features distinguish cell death by programmed necrosis from apoptosis, which is generally considered to be non-inflammatory and tolerogenic.

Identification of RIP kinases as critical mediators for programmed necrosis

RIP1 is a pleiotropic adaptor for TNF receptor signaling. Genetic “knock-out” and biochemical studies indicate that RIP1 is an essential adaptor for TNF-induced NF- κ B

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activation. Interestingly, the kinase function of RIP1 is not required for RIP1-mediated NF- κ B activation. Rather, the RIP1 kinase activity is essential for induction of programmed necrosis [3–5]. We reasoned that since RIP1 can activate multiple downstream signals, additional cues must be involved to turn on its pro-necrotic function. In order to identify the molecular component(s) that control the pro-necrotic activity of RIP1, we performed a targeted RNA interference (RNAi) screen. We focused our screen on kinases based on the assumption that the pro-necrotic function of RIP1 is either turned on by an upstream kinase or mediated through a downstream kinase.

FADD-deficient Jurkat cells were chosen for our screen because these cells undergo programmed necrosis exclusively in response to TNF stimulation [4]. We introduced 21-mer small interference RNAs (siRNA) into FADD-deficient Jurkat cells and found that out of 691 kinases, siRNA against RIP3 along with RIP1 conferred the strongest protection against TNF-induced programmed necrosis. siRNA-mediated silencing of RIP1 and RIP3 specifically inhibited TNF-induced programmed necrosis, but not apoptosis or NF- κ B activation [6]. Thus, RIP3 fulfills the requirement as a specific inducer of programmed necrosis.

RIP3 is recruited to the cytoplasmic signaling complex and interacts with RIP1 via the RHIM

TNFR-1 signaling is mediated by two spatially and temporally distinct complexes. The TNFR-1 associated “Complex I” is made up of the receptor, RIP1, TRAF2 and TRADD. Complex I is a transient complex and is rapidly internalized within the first hour of stimulation. Upon internalization, TNFR-1 dissociates from Complex I to allow binding of additional adaptors including FADD and caspase-8 to the complex [7]. This cytoplasmic signaling complex, termed “Complex II”, is responsible for induction of apoptosis. Unlike RIP1, which was present in both Complex I and Complex II, RIP3 was only recruited to the caspase-8 associated Complex II, but not Complex I. Interestingly, RIP3 was recruited to caspase-8 associated complex under both apoptotic (TNF alone) and pro-necrotic conditions (TNF + zVAD-fmk). However, when RIP1-associated complexes were examined, it became clear that RIP3 only interacted with RIP1 when cells were stimulated with TNF in the presence of caspase inhibition [6]. Thus, we conclude that the interaction between RIP1 and RIP3 is specifically induced in cells undergoing programmed necrosis.

The RHIM and kinase domains of RIP1 and RIP3 are essential for programmed necrosis

The necrosis-specific interaction between RIP1 and RIP3 requires an intact for both RIP kinases, since tetra-alanine substitutions abolished their interaction. Interestingly, RIP1 and RIP3 underwent necrosis-specific phosphorylation. Inhibition of RIP1 kinase activity with the RIP1-specific inhibitor necrostatin-1 [8] abolished TNF-induced RIP3, but not RIP1 phosphorylation [6]. Necrosis-specific phosphorylation of RIP1 was also absent in RIP3^{-/-} cells [6]. These experiments indicate that both RIP1 and RIP3 are required for their necrosis-specific phosphorylation, possibly through trans-phosphorylation (Fig. 1). Necrosis-specific phosphorylation of RIP1 and RIP3 is important for stable RHIM-mediated RIP1 and RIP3 interaction, since inhibition of RIP1 activity by necrostatin-1 abolished their interaction under pro-necrotic stimulation [6]. Functionally, phosphorylation and assembly of the RIP1-RIP3 pro-necrotic complex is important for activation of Complex II kinase activity, since necrostatin-1 inhibited the induction of necrosis-specific Complex II kinase activity [6]. These results indicate that RIP1 and RIP3 phosphorylation plays a critical role in the early induction of programmed necrosis by promoting formation and activation of the pro-necrotic

signaling complex. Further experiments are required to definitively determine the hierarchy of activation of RIP1 and RIP3.

RIP3 controls ROS production

Reactive oxygen species (ROS) are critical mediators of necrosis in many cell systems [9]. Recently, the Nox-1 [10] and riboflavin kinase [11] were identified as critical upstream regulators for ROS production during TNF-induced programmed necrosis. Both Nox-1 and riboflavin kinase were implicated to signal via the TNFR-1 membrane associated complex, suggesting that ROS is generated early during programmed necrosis to mediate cell death. Interestingly, we found that RIP3 also controls production of ROS during programmed necrosis [6]. As we have mentioned above, RIP3 is recruited to the cytoplasmic Complex II, but not the TNFR-1 associated Complex I. Thus, our results suggest that multiple pathways might control the generation of ROS during programmed necrosis, perhaps in a cell-type specific manner.

The role of RIP3 in T-cell death

In recent years, many of the components of apoptosis pathway have been shown to participate in other signaling pathways. For example, the death receptor adaptor FADD and the initiator caspase caspase-8 were shown to be required for T-cell activation [12–14]. FADD^{-/-} or caspase-8^{-/-} T-cells failed to proliferate in response to T-cell receptor stimulation [15]. Rather, these cells underwent cell death that was inhibited by the RIP1 kinase inhibitor necrostatin-1 or RIP1-specific small interference RNA [16]. Consistently with these observations, we found that RIP3^{-/-} T-cells exhibited enhanced protection against restimulation-induced T-cell death when caspases are inhibited by the broad-specificity caspase inhibitor zVAD-fmk or by vaccinia virus encoded caspase inhibitor B13R/Spi2 [6]. These results indicate that RIP1/RIP3-dependent programmed necrosis may control antigen-specific T-cell expansion when caspases are inhibited, such as that during certain viral infections.

RIP3 participates in anti-viral innate immune responses

The fact that TNF can also induce apoptosis raises questions about the physiological function of programmed necrosis. As we have discussed already, optimal induction of programmed necrosis requires caspase inhibition. Importantly, many viruses encode caspase or apoptosis inhibitors. Thus, infection by viruses that inhibit caspase might skew the cell death response towards programmed necrosis. Indeed, TNF induces cell death with classical necrotic morphology in vaccinia virus infected cells [6, 17]. In contrast, vaccinia virus-infected RIP3^{-/-} mouse embryonic fibroblasts were protected from TNF-induced programmed necrosis [6]. Similarly, vaccinia virus-infected RIP3^{-/-} T-cells were also protected from T-cell receptor induced cell death.

Like most poxvirus infections, vaccinia virus infection causes florid inflammation. The resistance of vaccinia virus infected RIP3^{-/-} cells suggests that RIP3-dependent necrosis might contribute to anti-viral responses. We examined the response of RIP3^{-/-} mice to vaccinia virus infections and found that necrosis in the liver and visceral fat pad was conspicuously absent in RIP3^{-/-} mice. The lack of necrosis in RIP3^{-/-} mice correlated with a lack of inflammation in the infected tissues [6]. Consequently, the RIP3^{-/-} mice failed to control viral replication and succumbed to the infection [6]. These results are consistent with a model in which programmed necrosis controls virus infection by eliminating the viral factory and to promote inflammation through release of “endogenous adjuvants” from the necrotic cells [2] (Fig. 2). In addition to virus-induced inflammation, RIP3 might play a wider role in controlling other inflammatory processes. For instance, RIP3^{-/-} mice were

protected from cerulein-induced pancreatitis [18, 19]. Taken together, our results show that RIP3-dependent programmed necrosis is important for controlling inflammation in certain pathological situations.

In summary, we have shown that a RIP1-RIP3 complex is critical for driving programmed necrotic cell death. It will be of interest in the future to determine whether a common RIP1/RIP3-dependent pathway regulates other physiological and pathological processes that involves necrotic cell injury.

Acknowledgments

This work was supported by NIH grant AI065877. F.K.C. was a recipient of investigator awards from the Smith Family Foundation and the Cancer Research Institute.

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Figure 1.
Trans-phosphorylation of RIP1 and RIP3 stabilizes their interaction during programmed necrosis.

Figure 2.
Programmed necrosis controls the viral factory and promotes inflammatory through the release of endogenous adjuvants.