Invited Mini Review

The serine threonine kinase RIP3: lost and found

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Receptor-interacting protein kinase-3 (RIP3, or RIPK3) is an essential protein in the "programmed", or "regulated" necrosis cell death pathway that is activated in response to death receptor ligands and other types of cellular stress. Programmed necrotic cell death is distinguished from its apoptotic counterpart in that it is not characterized by the activation of caspases; unlike apoptosis, programmed necrosis results in plasma membrane rupture, thus spilling the contents of the cell and triggering the activation of the immune system and inflammation. Here we discuss findings, including our own recent data, which show that RIP3 protein expression is absent in many cancer cell lines. The recent data suggests that the lack of RIP3 expression in a majority of these deficient cell lines is due to methylation-dependent silencing, which limits the responses of these cells to pro-necrotic stimuli. Importantly, RIP3 expression may be restored in many cancer cells through the use of hypomethylating agents, such as decitabine. The potential implications of loss of RIP3 expression in cancer are explored, along with possible consequences for chemotherapeutic response. [BMB Reports 2015; 48(6): 303-312]

INTRODUCTION

The way in which a cell dies in response to physiological cues, pathological stressors, or even intentional therapeutic cellular damage can have a profound effect on the remaining non-dying cells of an organism. Diverse biochemical consequences, including inflammation, and increased or decreased cellular proliferation can occur as the result of cell death mechanism. Thus, the study of cell death mechanisms is important in understanding the ultimate outcome of a physiological or pathological situation, whether cell death is induced by pathogen infection, cancer therapeutic, or whatever other stressor may be involved. Of course, identification of a cell death mechanism is also important if one seeks to intervene therapeutically to inhibit or enhance a given cell death process.

The programmed, or "regulated" necrotic cell death process is initiated downstream of many cellular stressors, including the signaling events activated by death receptor ligands, such as TNFα, FasL, or TRAIL (1, 2). Unlike apoptosis — in which caspase proteases activated and are largely responsible for the cellular demolition program, including proteolytic cleavage events, cellular shrinkage, chromatin condensation, nuclear fragmentation, and culminating in the formation of membrane-bounded bodies that are taken up by surrounding cells and by professional phagocytes, such as macrophages — caspase activation is not needed for cell death, and, unlike apoptosis, regulated necrosis results in the rupture of the plasma membrane, thus spilling the contents of the cell and efficiently triggering the immune system and inflammation (2, 3). Thus apoptosis, which has been thought to occur primarily without triggering inflammation (this is, in fact, an oversimplification), is largely perceived as having different consequences than regulated necrosis, which is highly pro-inflammatory (4, 5).

Programmed, or "regulated" necrosis is distinguished from classical necrosis, which is a somewhat random and largely passive injury-initiated process that occurs in response to direct cellular damage. The main difference, (other than their initiation factors) is that specific gene products are required for programmed or regulated necrosis, but not for classical necrosis. Though there is undoubtedly much that we still do not know about programmed necrosis, studies within the past decade have taken us from a state of the field where almost nothing was known about the mechanism of programmed necrosis to having a fairly well characterized pathway, at least in some contexts.

Mixed forms of cell death in which both apoptosis and necrosis play a role, as well as pleotrophic effects of both molecules (e.g. RIP1, which can play a role in both necrosis and caspase activation) and pharmacological inhibitors have made it difficult to make firm conclusions as to the role of programmed necrosis in diseases. However, it is likely that programmed necrosis plays roles in many pathological processes (2, 3, 6), including a facilitative role in tissue damage, such as in ischemia-reperfusion injury (7), and in host defense of viral infections (8), among many other roles. For instance, a very recent publication has implicated regulated necrosis in the path-

ology associated with Multiple Sclerosis (9). In addition, as we will discuss more fully, there is some hint now that repression of necrotic cell death molecules may some a role in tumor cell growth in cancers (58).

THE NECROTIC CELL DEATH PATHWAY

The serine-threonine kinase RIP1 (or RIPK1) was the first protein discovered as part of the cellular necrotic machinery (10, 11). Although it has a substantial role in apoptosis and necrosis, RIP1 plays other important roles in many death receptor and toll-like receptor signaling pathways, including the TNFα pathway, where it is essential for efficient activation of NF-κB and the MAP kinases ERK, JNK, and p38 (12). Although a kinase, most signaling pathways that it is involved in do not actually involve its kinase activity (13), but its function as a scaffolding protein. The exception to this rule is that the serine-threonine kinase activity of RIP1 is required for necrotic cell death signaling by this protein (11, 14, 15). Necrostatin-1, a compound identified in a small molecule screen for inhibitors of programmed necrotic cell death, was later shown to be an inhibitor of RIP1 kinase activity (14, 16). The kinase activity of RIP1 leads to the stabilization of a necrotic complex with RIP3 (17) and other proteins, and the compound necrostatin-1 inhibits the formation of this complex (17, 18), which is often referred to as the "necrosome".

RIP3 is an essential downstream partner for RIP1 in most forms of programmed necrosis (17-19), and interacts with RIP1 through a homotypic interaction motif (RHIM) (20). The resulting interaction can create a β-amyloid-like filamentous structure with the result that necrosis is slightly inhibited by amyloid dyes (21). The kinase activity of RIP3 is required for downstream signaling events in necrotic cell death (17-19). Recent data suggests that RIP1 both positively regulates the activity of the necrosome complex after necrotic stimuli, but also negatively regulates promiscuous basal RIP3 induction of necrotic cell death (22-25).

Mixed Lineage Kinase Domain-like protein (MLKL) is the essential target of RIP3 kinase activity and interacts with RIP3 upon induction of programmed necrotic cell death (26, 27). Knockout of MLKL in mice indicates that this protein is essential for necrosis to proceed after necrosome formation (28, 29). RIP3-dependent plasma membrane localization of MLKL is necessary for programmed necrotic cell death to occur (30-32). The translocation of MLKL to the membrane has been alternately reported to leads to plasma membrane disruption by through its activation of ion channels (31, 32) or by directly permeabilizing the plasma membrane (30, 33), perhaps through binding to phosphatidylinositol phosphates (33), although this mechanism has been challenged (34).

In addition to MLKL, Zhang *et al.* identified several metabolic enzymes in screening for interactions with RIP3, including glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL), glutamate dehydrogenase 1 (GLUD1), as well as fructose-

1,6-bisphosphatase 2 (FBP2), fumarate hydratase (FH), glycosyltransferase 25 domain containing 1 (GLT25D1), and isocitrate dehydrogenase 1 (IDH1) (19). PYGL, GLUL, and GLUD1 were verified in their interaction with RIP3 in overexpression systems (19). RIP3 may thus have additional alternate roles in regulating metabolic enzymes associated with glycolysis and the mitochondria.

Important regulation of programmed necrosis occurs when the apoptotic proteins (i.e. FADD and caspase-8) are more prevalently activated in the necrosome complex, which may at least be partially controlled by the prevalence of apoptotic molecules versus necrotic molecules (35). The importance of such regulation of necrosis by the apoptotic molecules in the complex is supported by the fact that developmental defects and lethality of some gene deletions, including FADD, caspase-8, cFLIP-FADD double knockout (but not cFLIP knockout alone), XIAP-cIAP1 double knockout and cIAP1-cIAP2 double knockout that are rescued completely or to some degree by RIP1/RIP3 deficiency (36-43). While caspase inhibitors prevent apoptosis, caspase inhibition often potentiates necrotic cell death (44), due to their inhibition of caspase- dependent cleavage of RIP1 (45), RIP3 (46), and the CYLD deubiquitinase (47) (the last of which potentiates necrotic signaling by removal of K63 linked ubiquitin from RIP1, allowing it to interact with necrosomal components), thus stabilizing RIP1-RIP3-MLKL signaling. Repression of the programmed necrotic pathway by apoptotic proteins, such as FADD and caspase-8, prevents spontaneous cell death and inflammation in lymphocytes (42, 43, 48), keratinocytes (36), and intestinal epithelial cells (49, 50).

EXPRESSION OF RIP3 IS LOST IN CANCER CELL LINES

In a large part, the major models systems for studying programmed necrosis have involved cell death induction the death receptor agonists. While FasL, TRAIL and TNFα ligands often stimulate apoptosis in many cell types in other contexts they kill by programmed necrosis (11, 44, 51-53). Previously to the discovery that RIP3 was an important molecule in the necrotic cell death machinery, some researchers wondered why so few cell lines/types were responsive to such necrotic stimuli. Among the cell lines that have been used study of regulated necrosis are the mouse fibrosarcoma cell line L929, the T-cell leukemia cell line Jurkat (primary T-cells have also been used), the colon cancer cell line HT-29, the myeloid lymphoma cell line U937, and mouse embryonic fibroblast cell lines. Many of these are not very representative of cancer cell lines as a whole in terms of their response to these prototypical necrotic stimuli. L929 cells, for instance, appear to be a very special cell type, and undergo necrotic cell death as the default mode of cell death induced by TNFα treatment, while in other cell types, such as U937, apoptotic cell death is the default mode of cell death when treated with TNFα, and caspase inhibitors such the pancaspase inhibitor zVAD, are used to switch the mode of death to programmed necrosis (16). MEF

cell lines that are deficient in NF-κB activity, such as p65/RelA, TRAF2, and TRAF5 knockout cell lines, also die by a necrotic cell death mode when treated with by TNFα in the presence of zVAD, while in other cell types, the addition of SMAC mimetics or transcriptional or protein synthesis inhibitor in combination with caspase inhibitors is necessary to initiate programmed necrosis (10, 18, 54).

In one of the first publications to look at RIP3 protein expression in multiple cell lines in the context of necrotic cell death, He et al. (18) reported that of 14 different cell lines test-

ed, only six cell lines had expression of RIP3 including human T cell leukemia Jurkat and CCRF-CEM cells, human monocytic leukemia U937 cells, mouse fibrosarcoma L929 cells, and WT MEFs, which correlated with their response to necrotic stimulus. They found that RIP3 was necessary for necrotic cell death and they could make other cell lines responsive to necrotic cell death stimuli by ectopic expression of RIP3. Consistent with this, Cho et al. (17) also at this time identified RIP3 to be essential for necrotic cell death using an siRNA screen.

RIP3 expression in cell lines from solid tumors

cell line	origin / designation	RIP3 status	⇑ RIP3 w/ 5-AD?	citation
1-87	lung adenocarcinoma	++		F
293T	transformed embryonic kidney	-		H
A172	glioblastoma	-	Limited	K
A549	lung adenocarcinoma	-	YES	K, F
A549-N	lung adenocarcinoma	-	NO	K
BT549	breast ductal carcinoma	-	YES	K
Calu3	lung adenocarcinoma	-	NO	K
Chang	cervical adenocarcinoma	-	NO	K
COLO205	colorectal adenocarcinoma	+++		M
DLD-1	colorectal adenocarcinoma	-	YES	K
DU145	prostate carcinoma	-	YES	K
FL3	bladder transitional cell carcinoma	-		K
G-361	melanoma	-		M
H2009	lung adenocarcinoma	+ / -		K/H conflict
H322C	bronchioalveolar carcinoma	-	YES	K
H358	bronchioalveolar carcinoma	-	NO	K
H4006	lung adenocarcinoma	+		K
HaCaT	immortal keratinocyte	++		Ki
HCC1937	breast ductal carcinoma	-	NO	K
HCC827	lung adenocarcinoma	+		K
HeLa	cervical adenocarcinoma	-	YES	K, H
Hep3B	hepatocellular carcinoma	-	YES	K
HepG2	hepatocellular carcinoma	-	YES	K
HCT-116	colorectal carcinoma	-	YES	K
HL7702	normal liver immortal cell line	++		K
HMLE	normal breast-immortalized	++		K
HMV-II	vaginal maligant melanoma	-		M
HT-29	colorectal adenocarcinoma	+++		K, H
Huh-7	hepatocellular carcinoma	-	YES	K
KLM-1	pancreatic adenocarcinoma	+		M
LK79	small cell lung carcinoma	-		F
LNCaP	prostate adenocarcinoma	+		K
MCF-10A	breast non-tumor	-		K
MCF-12A	breast non-tumor	-		K
MCF-7	breast adenocarcinoma	-		K, H
MDA-MB-231	breast adenocarcinoma	-	YES	K
MDA-MB-468	breast adenocarcinoma	-		K
NCI-H460	large cell lung carcinoma	-		H
NCI-H1650	bronchoalveolar carcinoma	-	YES	K
NUGC-4	gastric adenocarcinoma	++		M
Panc-1	pancreatic epithelioid carcinoma	-		H
PC3	prostate adenocarcinoma	-	YES	K
RERF-LCMS	lung adenocarcinoma	-		F
S-2	small cell lung carcinoma	-		F
SBC-3	small cell lung carcinoma	-		F
SK-Hep1	hepatic adenocarcinoma	-	Limited	K
SK-BR3	breast adenocarcinoma	+++		K
SKMEL-2	melanoma	-	YES	K
SKMEL-5	melanoma	-	YES	K
SKMEL-28	melanoma	-		M
SNU354	hepatocellular carcinoma	-		K
SNU387	hepatocellular carcinoma	-		K
SNU423	hepatocellular carcinoma	-		K
T24t	bladder transitional cell carcinoma	-		K
T47D	breast ductal carcinoma	++		K
T98G	glioblastoma	-	YES	K, H
U251	glioblastoma	-	NO	K
U2OS	osteosarcoma	-		H
U87-MG	glioblastoma	++		K
U937	myelomonocytic histiocytic lymphoma	++		K, H
UMUC3	bladder transitional cell carcinoma	-		K
WM266-4	melanoma	-	YES	K
ZR75.1	breast ductal carcinoma	++		K

Fig. 1. Expression of RIP3 in cancer or other cell lines (solid tumors or comparable tissues). The table shows showing the RIP3 expression in various cancer (solid tumors) and other cell lines cell lines as determined by western blotting and or reverse transcription-PCR. The estimated relative expression range of the cell is indicted (- to +++, with +++ indicating maximal expression and − indicating no expression detected; tr indicates some very low trace expression). For some cell lines, where indicated, the table shows whether the treatment of the cell line with 5-AD or other hypomethylating agents leads to increased expression of RIP3 mRNA or protein. The following abbreviations for citations are used: F, Fukasawa et al. (64); H, He et al. (18); K, Koo et al. (58); Ki, Kim et al. (72); M, Motani et al. (73); N, Nugues et al. (62); Z, Zhang et al. (19).

At about the same time, Zhang *et al.* (19), reported that NIH 3T3 cells from two different sources had different phenotypes with respect to necrotic cell death, namely that zVAD inhibited TNFα-induced cell death from one source (which they designated A cells), but enhanced cell death in another (which they designated N cells). Very few differences in the gene expression patterns between these 3T3 variants allowed them to determine that the A cells lacked RIP3 expression, while the N cells expressed the RIP3 protein. The significant difference in sensitivity in largely isogenic cell lines helped them identify RIP3 as the differential molecule important for necrotic cell death, but led to another question. Why is RIP3 so differentially expressed in variants of the same cell line?

In retrospect, it is apparent that there were differences observed early on in RIP3 expression between normal cells and tissues, and cancer cell lines. Soon after the discovery of RIP3 (55, 56), and long before its role in necrotic cell death was hypothesized, Kasof *et al.* (57) published a paper that examined RIP3 expression in normal adult human tissues. They were able to detect RIP3 in many tissues using northern blotting; however, RIP3 mRNA was not detected in any of the 23 cancer lines examined in this study (57). In a recent publication in *Cell Research* (58), we carried out a further in-depth analysis of cancer cell lines, and found that RIP3 expression is almost completely silenced in about two-thirds of the 60+ cancer cell lines we have tested. However, cancer cell lines derived from hematopoetic compartments do not seem to have the same frequency of silencing, with only 20% having lost RIP3 expression. When excluding these hematological cancers, about 80% of the remaining cell lines have little detectable RIP3 protein or RNA. However, consistent with the previous data suggesting RIP3 is expressed in normal tissues (55-57, 59, 60), we easily detect expression of RIP3 protein in most normal primary cells and tissues (58). Fig. 1 and Fig. 2 show a summary of data from the literature regarding RIP3 expression (or lack thereof) in cell lines from solid tumors or similar derivative tissues (Fig. 1) or from hematological cancers (Fig. 2). The RIP3 expression status of murine cell lines that have been similarly

RIP3 expression in hematological cell lines

cell line	origin / designation	RIP3 status	⇑ RIP3 w/ 5-AD?	citation
CCRF-CEM	acute lymphoblastic T-cell leukemia	++		H
Daudi	Burkitt's B-cell lymphoma	+		K
EoL-1	acute myeloid (eosinophilic) leukemia	++		K
HEL	erythroleukemia	+		K
HL-60	acute promyelocytic leukemia	tr		K
Jurkat	acute lymphoblastic T-cell leukemia	++		K, H
K562	chronic myeloid leukemia	-	YES	K
KG1	acute myelogenous leukemia	+		K
ML-1	acute myeloblastic leukemia	-		K
MOLM-13	acute monocytic leukemia	++		K
MOLM-14	acute monocytic leukemia	++		K
MV4-11	biphenotypic B myelomonocytic leukemia	++		K
NB4	Acute promyelocytic leukemia	-		K
Raji	Burkitt's B-cell lymphoma	++		K
NOMO-1	acute myeloid leukemia	++		K, M
Ramos	Burkitt's B-cell lymphoma	+++		K
RL7	follicular B cell lymphoma	+		K
THP-1	acute monocytic leukemia	+++		K, M

RIP3 expression in murine cell lines

cell line	origin / designation	RIP3 status	⇑ RIP3 w/ 5-AD?	citation
4T1	mouse breast cancer	tr	YES	K
B16	mouse melanoma	-	YES	K
DA1-3b	acute myeloid leukemia	-		N
NIH3T3-"A"	mouse embryonic fibroblast	-		Z
NIH3T3-"N"	mouse embryonic fibroblast	+		Z
L929	mouse fibrosarcoma	+++		K, H
MEF	mouse embryonic fibroblast	++		K, H
Raw 264.7	murine macrophage	+++		*
WEHI-3B	myelomonocytic leukemia	-		N

Fig. 2. Expression of RIP3 in cell lines derived from hematological malignancies and murine cell lines. The tables show showing the RIP3 expression in various cancer and other cell lines cell lines as determined by western blotting and or reverse transcription-PCR. Murine cell lines are shown in the right panel. The estimated relative expression range of the cell is indicted (- to +++, with +++ indicating maximal expression and − indicating no expression detected; tr indicates some very low trace expression). For some cell lines, where indicated, the table shows whether the treatment of the cell line with 5-AD or other hypomethylating agents leads to increased expression of RIP3 mRNA or protein. The following abbreviations for citations are used: H, He *et al.* (18); K, Koo *et al.* (58); M, Motani *et al.* (73); N, Nugues *et al.* (62); Z, Zhang *et al.* (19), *Y.-S. Kim, unpublished observation.

RIP3 IS SILENCED IN PRIMARY CANCERS

Loss of RIP3 in cancer cells is not completely surprising, since tumor formation often selects against the expression of cell death proteins, and resistance to cell death processes is one of the established hallmarks of a cancer cell (61). However, a major question is whether this is merely a tissue culture phenomenon, or whether there are also clear differences in RIP3 expression in primary cancers compared to normal tissue. To address this question, we have examined RIP3 expression in 75 primary breast cancers compared to the normal breast tissue from the same patients. Largely consistent with the percentages obtained in the previous analysis of cell lines from solid-tumors, about 80-85% of cancer tissue samples have reduced RIP3 expression compared the respective normal breast tissue from the same patient (58). In terms of breast cancer subtypes, luminal subtypes were only slightly less likely to be RIP3 deficient (73% for luminal A and 84% for luminal B) compared to the other subtypes (95%, Her2 positive; 90%, triple negative). Thus, RIP3 is largely silenced in the primary breast cancers we have examined, regardless of subtype.

Intriguingly, although a decreased percentage of hematological cancer cell lines have RIP3 deficiency compared to solid tumor-derived cell lines, we still found that a number of AML patient samples had reduced RIP3 protein and mRNA expression.

This is consistent with a recent study by Nugues et al. (62) that showed that RIP3 expression was reduced in most AML samples compared to cells from healthy donors, whereas the expression of RIP1 did not differ significantly. Thus, there are at least two types of primary cancers in which reduced or absent RIP3 expression is observed compared to normal cell types or tissues, and this may indicate that RIP3 deficiency could be associated with cancer development or progression. It is unclear at this point why loss of RIP3 expression may provide some selective advantage to cancer cells, possibly by either by repressing tumorigenesis or by repressing cell growth, and is an obvious avenue for future investigation. However, it is tempting to speculate that there may be a basal amount of necrotic cell death that occurs in a growing cell population such that elimination of RIP3 becomes advantageous. Alternately, other activities of RIP3 may explain such a propensity for RIP3 silencing. For instance, although often involved together in the regulated necrotic pathway, the presence of both RIP3 and RIP1 appear to regulate the activity of the other protein to some extent (22-25, 35), and therefore it is possible that in some circumstances the presence of RIP3 may repress RIP1 activities. Since RIP1 has recently reported to be an oncogenic driver in melanoma (63), such a repressive activity may make RIP3 silencing advantageous in tumor growth.

RIP3 IS SILENCED BY GENOMIC METHYLATION

The cause of differential RIP3 expression in some isogenic cell lines and the lack of RIP3 expression in many cancer cell lines has remained largely speculative until recently.

A number of years ago, Fukasawa et al. (64) had reported hypermethylation of the RIPK3 gene, among several other genes, in lung cancer cell lines and in primary small cell lung cancers. However, no follow-up was done to determine whether this hypermethylation represented a general phenomenon and whether it was responsible for lack of RIP3 protein expression in cancer cells or cell lines in general. In our recent study (58),
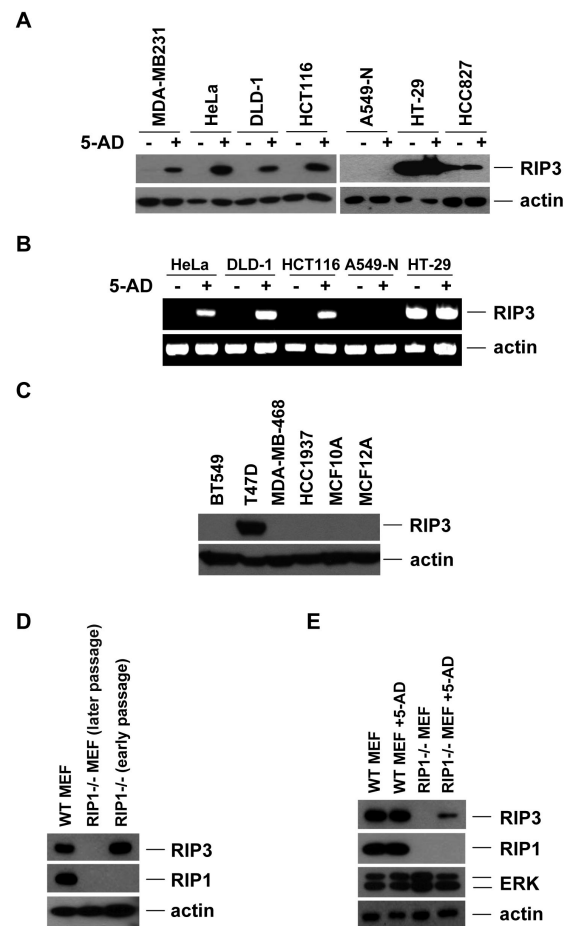

Fig. 3. RIP3 is silenced by methylation in multiple cancer and other cell lines, but can be upregulated in response to hypomethylation agents, such as decitabine (5-AD). (A) Western blotting showing the RIP3 expression in various cancer cell lines in response to 5-AD (2 μM) for 4 d. (B) Reverse transcription-PCR products from cancer cell lines following 4 d treatment with 5-AD (2 μM). (C) Western blotting showing the lack of RIP3 expression in Murine Embryonic Fibroblast (MEF) cell lines, including RIP-/- MEFs characterized by Zhang et al. (19). (D) Western blotting showing the RIP3 expression in MEF cell lines in (B) in response to 5-AD (2 μM) for 4 d.

we examined possible reasons for loss of RIP3 expression in cancer cells, including DNA methylation or histone deacetylation. We reported that, in a majority of cases, methylation of the *RIPK3* genomic sequence, particularly on GpGs near the transcription start site (TSS), was correlated with RIP3 silencing in both cancer cell lines and in primary breast tumors. Further experiments suggested that expression of the maintenance methyltransferase, DNMT1, was inversely correlated with RIP3 expression in some cell lines, and may be the methyltransferase responsible for RIP3 maintaining silencing in cancer cell lines.

Most importantly, while HDAC inhibitors had little effect on RIP3 expression on their own, the use of hypomethylating agents, such as decitibine (5-aza-2'-deoxycytidine, also abbreviated as 5-AD), 5-azacytidine, and RG108, reduced DNA methylation near the *RIPK3* TSS, and consequently restored both RIP3 mRNA and protein expression in a majority cells where RIP3 had previously been silenced (Fig. 3A, B) (58). The fourth column of Fig. 1 and 2 indicate the cell lines in which this has been examined, and whether 5-AD upregulates RIP3 in these cells. This restoration of RIP3 expression also occurred in murine cells, indicating that the gene methylation mechanism of silencing is conserved in mouse cells (58). Thus while RIP3 silencing appears to be regulated epigenetically through methylation of its genomic sequence, an important consequence of silencing through this mechanism is that the RIP3 silencing appears to be reversible in most cases. Since hypomethylating agents are reasonably well-tolerated in patients, this opens the therapeutic possibility to manipulate RIP3 expression in cancer cells to make them sensitive to programmed necrosis.

SELECTION FOR RIP3 SILENCING IN VITRO

There are some indications that RIP3 silencing may affect growth more than it affects tumorigenesis with regard to its selection in cancer. Support for this notion comes from data relating to RIP3 expression in non-cancer cell lines. For instance, though RIP3 protein expression is absent in BT549, MDA-MB-468, and HCC1937 breast cancer cell lines, it is also absent in our passages of the "normal" (i.e. non-tumorigenic) breast cell lines MCF10A and MCF12A (Fig. 3C). Additionally, there are reports of cell lines that have variants lacking RIP3 expression, such as the NIH 3T3 A and N cell lines reported by Zhang *et al.* (19) and H2009 cells as conversely reported by us and He *et al.* (18, 58). While no one has examined these variants to verify if it is indeed methylation of the RIPK3 genomic sequence that is responsible for RIP3 silencing in one of the variants versus the other variant, it is likely that these differences suggest that selection for silencing by methylation also occurs in vitro. As a further example, Zhang *et al.* (19) also reported that when analyzing RIP1 and RIP3 expression in a particular RIP1 knockout MEF cell line (10, 11), they found that it was defective in both RIP1 and RIP3 expression, and therefore had

to ectopically reconstitute RIP3 expression in this cell line for their experiments. We have verified the lack of expression of RIP3 in this particular MEF cell line, however, we found earlier passages of these same RIP1-/- MEFs express normal amounts of RIP3 (Fig. 3D). Additionally, treatment of the RIP3-deficient RIP1 KO MEFs with 5-AD restores RIP3 protein expression in these cells (Fig. 3D), indicating that the loss of RIP3 expression is not a permanent loss, but is due to silencing by methylation. This suggests that there is some selective pressure against RIP3 expression during cell passaging in culture that selects for RIP3 silencing by methylation. In this case, RIP1 loss may have contributed to additional selective pressure since under some situations RIP1 is thought to negatively regulate RIP3-dependent cell death activity to some extent (22-25). However, this further argues that, even in relatively normal and non-tumorigenic cell lines such as MEFs, RIP3 expression can be selected against through methylation and silencing in routine cell culture, which may suggest that RIP3 silencing affects cell growth as opposed to tumorigenesis with respect to tumor cells.

CONSEQUENCES FOR RIP3 SILENCING IN CANCER

What are the consequences of RIP3 being silenced in cancer cells? One obvious consequence is that RIP3 is deficient in most of the cell lines used to derive much of the cellular and molecular information for a large majority of the published biological literature. Thus, a lot of the main literature derived from experiments *in vitro* does not adequately reflect possible affects of RIP3 expression on various cell death and signaling pathways that may be expected to occur in normal cells. As mentioned, the pathological consequences of RIP3 silencing in a tumor are that it likely confers a selective advantage to the tumor cells, and may facilitate their growth, thus leading to negative consequences in a patient. Thus, one of our analyses of gene expression data indicates there is some benefit of RIP3 expression on metastasis-relapse (MR)-free survival of breast cancer patients (58).

Since RIP3 is involved in programmed necrosis and other forms of cell death, we hypothesized RIP3-deficiency might have additional effects on the response of cells to chemotherapy — effects that may not yet have been observed due to widespread RIP3 deficiency in cell lines. Regulated necrotic cell death has previously been implicated to contribute cell death processes in response to DNA alkylating agents that are sometimes used as chemotherapeutic agents (65). However, in the case of alkylating agents, the DNA repair protein poly (ADP-ribose) polymerase (PARP) was required for cell death (65), and more recent data suggests that that the PARP-1 dependent necrotic process, which involves the PAR polymer and translocation of AIF from the mitochondrial to nucleus, and which is referred to as "parthanatos" (66), and RIP3-dependent necrotic cell death, also sometimes referred to as necroptosis, are largely distinct pathways (7, 67). The contribution of RIP1/RIP3-dependent necrosis to chemotherapeutic cell

death has been largely unexplored, with a couple of notable exceptions. The so-called ripoptosome complex has been implicated in etoposide-mediated cell death through the activation of both apoptosis and necrosis (68). This complex is thought to contain both RIP1 and RIP3, as well as FADD, caspase-8, and possible caspase-10 and cFLIP (68, 69), and assembles upon disruption of IAP proteins (69). In this prior study, RIP3 contributed to caspase activation (68), but necrosis also occurred in some cell lines, though neither study were able to show the presence of RIP3 in the ripoptosome in response to etoposide. In another study, RIP1 kinase activity was shown to be important in etoposide-induced caspase activity and cell death downstream of ATM through autocrine TNFα signaling (70).

In our recent study (58), we sought to determine whether RIP3-dependent necrotic cell death was important in the cell death response to etoposide and to other DNA damaging agents, as well as to other typically-used chemotherapeutic drugs. We were surprised to find that expression of RIP3 sensitized cancer cells not only to etoposide and to doxorubicin, but to a wide diversity of standard chemotherapeutic agents, including many with disparate mechanisms of action, with chemotherapy classes including topoisomerase inhibitors, taxanes, platinum complexes, anthracyclines, and antimetabolites (58). Indeed, we have found that RIP3 expression has sensitized cancer cells to most of the chemotherapeutic agents that we have tried, in multiple cell lines, and in experiments where we have either ectopically expressed RIP3 in deficient cell lines or knocked RIP3 down in cell lines with endogenous RIP3. In addition, in experiments where we induced RIP3 expression using hypomethylating agents, the agents sensitized to chemotherapeutics in a RIP3-dependent fashion (58). We found the hypomethylating agent 5-AD is also highly synergistic with doxorubicin in treating breast cancer tumors in orthotopic xenografts in mice at doses of these compounds that have little anti-tumor activity on their own (58).

Of course, in these experiments RIP3 could be influencing cell death through other cell death mechanisms, such as by activating caspases and apoptosis, rather than apoptosis. Though we cannot exclude this possibility that this is also occurring, we have found the following combined points of evidence suggest that RIP3-dependent programmed necrosis is occurring in response to chemotherapeutics (or at least in response to the DNA damaging agents doxorubicin and etoposide, since we have not tested all chemotherapeutic agents as rigorously as these) :

1) Treatment with these compounds induces the formation of the "ripoptosome", or "necrosome" complex including the RIP1, RIP3, FADD, and caspase-8 proteins [as well as the TRADD protein, which is interesting because, unlike Biton and Ashkenazi (70), we found no requirement for autocrine TNFα signaling in cell death induced by DNA damaging agents].

2) These compounds induce the RIP3-dependent phosphorylation of MLKL on Serine 358, consistent with MLKL acti-

vation. This phospho-MLKL colocalizes with RIP3, at least at early stages of treatment.

3) MLKL is immunoprecipitated with RIP3 in treated cells only.

4) Knockdown of MLKL inhibits cytotoxicity by these compounds in a similar manner as RIP3 knockdown.

5) The pancaspase inhibitor zVAD has a minimal effect on cell death at the doses of agents we are using in HT-29 cells. The temporal kinetics of caspase-8 cleavage largely remain the same in the presence or absence of RIP3 upon treatment with a chemotherapeutic agent.

6) On the other hand, three pharmacological inhibitors of programmed necrosis— necrostatin-1, necrosulfonamide, and dabrafenib— all inhibited cell death to a greater extent than zVAD. (necrostatin-1 inhibits RIP1 kinase activity (14); dabrafenib inhibits RIP3 kinase activity (71); and necrosulfonamide inhibits MLKL functions downstream of RIP3 phosphorylation (30)). In addition, MLKL phosphorylation induced by these drugs was inhibited by necrostatin-1 and dabrafenib, but not necrosulfonamide, consistent with their mechanisms of action during programmed necrosis.

Thus, we feel the evidence is sufficient to indicate that RIP3-dependent necrosis is activated in response to chemotherapeutics. This suggests the possibility that RIP3 deficiency in cancer patients may contribute to chemotherapy resistance to some extent, or at least that the addition of hypomethylating agents to chemotherapeutic regimens may increase the efficacy of some chemotherapeutics. Significant additional work in *in vivo* cancer models is still necessary to show whether this additional mechanism of cell death by programmed necrosis will likely have a significant effect in the treatment of human patients by chemotherapeutics. Of course, irrespective of the mechanism of cell death, the manipulation of RIP3 expression by hypomethylating agents may be a way to make chemotherapy more effective. Ideally, future clinical trials could screen for RIP3 deficiency in cancers to determine whether hypomethylation agents should be added in combination with the given cytotoxic chemotherapeutic drugs.

CONCLUSION

Here we have discussed data that indicates that the expression of RIP3 is lost in a large number of cancer cells, and that the mechanism for this silencing is largely due to DNA methylation of the *RIPK3* genomic sequence. There is further evidence that RIP3 expression is regulated in this manner in least three types of primary cancers, including breast cancers (58) and AML (58, 62), where both *RIPK3* methylation and RIP3 silencing have been observed, and in small cell lung cancers, where *RIPK3* methylation has been seen to a large extent (64). Given the propensity for RIP3 loss in cell lines of many other cancers, we expect that RIP3 silencing may be prevalent in other primary cancers as well. All of the given data suggest that deficiency of RIP3 is selected for in cell lines and tumor

cells and likely during tumor development and/or growth, although evidence in non-tumorigenic cells may suggest effects on the latter, rather than the former. Nevertheless, RIP3 deficiency is likely to have important biological and therapeutic consequences, including possible effects on the responses of tumor cells to chemotherapy. Fortunately, it appears that RIP3 expression may be restored in a majority of cells with RIP3 deficiency by treating them with hypomethylating agents, thus restoring sensitivity of these cells to programmed necrosis. This fact not only has the potential to positively impact the field of necrotic cell death cell biology by allowing scientists to examine the necrotic pathway in more depth in a much broader range of cell lines than has been previously available, but also potentially opens up new therapeutic possibilities for the treatment of cancer by potentially restoring RIP3 expression in human patients.

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