The serine threonine kinase RIP3: lost and found

Michael J. Morgan1,* & You-Sun Kim2,3,*

1Department of Pharmacology, University of Colorado School of Medicine, Aurora, Colorado, USA, 2Department of Biochemistry, Ajou University School of Medicine, 3Department of Biomedical Sciences, Graduate School, Ajou University, Suwon 443-749, Korea

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 pleotropic 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-
biology 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 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 “necosome”.

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 necosome 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-clAP1 double knockout and clAP1-clAP2 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-dependant 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 necroosomal 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 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 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 tested, 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 / design/ation | RIP3 status | Reference |
|-----------|-----------------------|-------------|-----------|
| 1-87      | lung adenocarcinoma    | ++          | F         |
| 293T      | transformed embryonic kidney | - | H |
| A172      | glioblastoma          | Limited K   |           |
| A546      | 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 |
| G361      | melanoma              | - M        |           |
| H2009     | lung adenocarcinoma   | +/−         | K/H conflict |
| H322C     | bronchoalveolar carcinoma | - YES K |           |
| H358      | bronchoalveolar carcinoma | - NO K |           |
| H4006     | lung adenocarcinoma   | - K        |           |
| HaCaT     | immortal keratinocyte | ++ K       |           |
| HCC1937S  | breast ductal carcinoma | - NO K |           |
| HCC277    | lung adenocarcinoma   | + K        |           |
| HeLa      | cervical adenocarcinoma | - YES K, K, H |           |
| Hep3B     | hepatocellular carcinoma | - YES K |           |
| HepG2     | hepatocellular carcinoma | - YES K |           |
| HCT-116   | colorectal carcinoma  | - YES K    |           |
| HL70/2    | normal liver immortal cell line | ++ K |           |
| HMLE      | normal breast-immortalized melanoma | - | M |
| HMV-II    | vaginal malignant 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        |           |
| 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 | - F |           |
| 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        |           |
| SNJ35     | hepatocellular carcinoma | - K |           |
| SNJ387    | hepatocellular carcinoma | - K |           |
| SNU423    | hepatocellular carcinoma | - K |           |
| T24       | bladder transitional cell carcinoma | - K |           |
| T47D      | breast ductal carcinoma | - K |           |
| T98G      | glioblastoma           | - YES K, H |           |
| U251      | glioblastoma           | - NO K     |           |
| UO25     | osteosarcoma           | - H        |           |
| U87-MG    | glioblastoma           | ++ K        |           |
| U937      | myelomonocytic blastoid lymphoma | +/− K, H |           |
| UMUC3     | bladder transitional cell carcinoma | - K |           |
| WM856-4   | breast ductal carcinoma | - YES K |           |
| ZR75.1    | breast ductal carcinoma | ++ K |           |

![Fig. 1](http://bmbreports.org) Expression of RIP3 in cancer or other cell lines (solid tumors or comparable tissues). The table shows how the RIP3 expression in various cancer (solid tumors) and other cell lines as determined by western blotting and or reverse transcription-PCR. The estimated relative expression range of the cell is indicated (− 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. (10); K, Koo et al. (38); 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-mediated 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 cell 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 hematopoietic 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 myeloblastic 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 indicated (- 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.
characterized are also shown (right panel of Fig. 2).

**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 to 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 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. Alternatively, 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),
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 CpG sites 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-aza-cytidine, 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 passage 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 effects 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 "ripotosome", or "necosome" 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 activation. This phosho-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 2VAD 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 2VAD. (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 hypomethylating 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 at 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 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.

ACKNOWLEDGEMENTS

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by grant (No. 2011-0030043 and 2014R1A2A1A11052951) to Y.-S. Kim from the Ministry of Education, Science, and Technology. MJM is supported by a Cancer League of Colorado Cancer Research Grant to MJ Morgan from the Cancer League of Colorado, Inc. in association with the University of Colorado Cancer Center.

REFERENCES

1. Vandenabeele P, Galluzzi L, Vanden Berghe T and Kroemer G (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 11, 700-714
2. Vanlangenakker N, Vanden Berghe T and Vandenabeele P (2012) Many stimuli pull the necrotic trigger, an overview. Cell Death Differ 19, 75-86
3. Morgan M and Liu Z (2013) Programmed cell death with a necrotic-like phenotype. Biomol Concepts 4, 259-275
4. Kroemer G, El-Deiry WS, Golstein P et al (2005) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. Cell Death Differ 12 Suppl 2, 1463-1467
5. Fiers W, Beyaert R, Declercq W and Vandenabeele P (1999) More than one way to die: apoptosis, necrosis and reactive oxygen damage. Oncogene 18, 7719-7730
6. Moriwaki K and Chan FKM (2013) RIP3: a molecular switch for necrosis and inflammation. Genes Dev 27, 1640-1649
7. Linkermann A, Brasen JH, Darding M et al (2013) Two independent pathways of regulated necrosis mediate ischemia-reperfusion injury. Proc Nat Acad Sci U S A 110, 12024-12029
8. Kaiser Wj, Upton JW and Mocarski ES (2013) Viral modulation of programmed necrosis. Curr Opin Virol 3, 296-306
9. Ofengand D, Ito Y, Najafov A et al (2015) Activation of Necroptosis in Multiple Sclerosis. Cell Rep 10, 1836-1849
10. Lin Y, Choksi S, Shen HM et al (2004) Tumor necrosis factor-induced nonapoptotic cell death requires receptor-interacting protein-mediated cellular reactive oxygen species accumulation. J Biol Chem 279, 10822-10828
11. Holler N, Zaru R, Micheau O et al (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 1, 489-495
12. Morgan MJ and Liu ZG (2010) Reactive oxygen species in TNFalpha-induced signaling and cell death. Mol Cells 30, 1-12
13. Devin A, Lin Y and Liu ZG (2003) The role of the death-domain kinase RIP in tumour-necrosis-factor-induced activation of mitogen-activated protein kinases. EMBO Rep 4, 623-627
14. Degterev A, Hitomi I, Gemscheid M et al (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 4, 313-321
15. Chan FK, Shisler J, Bixby JG et al (2003) A role for tumor necrosis factor receptor-2 and receptor-interacting protein in programmed necrosis and antiviral responses. J Biol Chem 278, 51613-51621
16. Degterev A, Huang Z, Boyce M et al (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 1, 112-119
17. Cho YS, Challa S, Moquin D et al (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137, 1112-1123
18. He S, Wang L, Miao L et al (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 137, 1100-1111
19. Zhang DW, Shao J, Lin J et al (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325, 332-336
20. Sun X, Yin J, Starovasnik MA, Fairbrother VJ and Dixit VM (2002) Identification of a novel homotypic interaction motif required for the phosphorylation of receptor-interacting protein (RIP) by RIP3. J Biol Chem 277, 9505-9511
21. Li J, McQuade T, Siemer AB et al (2012) The RIP1/RIP3 Necrosome Forms a Functional Amyloid Signaling Complex Required for Programmed Necrosis. Cell 150, 339-350
22. Rickard JA, O'Donnell JA, Evans JM et al (2014) RIPK1 regulates RIPK3-MLKL-driven systemic inflammation and emergency hematopoiesis. Cell 157, 1175-1188
23. Dillon CP, Weinlich R, Rodriguez DA et al (2014) RIPK1 blocks early postnatal lethality mediated by caspase-8 and RIPK3. Cell 157, 1189-1202
24. Orozco S, Yatim N, Werner MR et al (2014) RIPK1 both positively and negatively regulates RIPK3 oligomerization and necroptosis. Cell Death Differ 21, 1511-1521
25. Dannappel M, Vlantis K, Kumari S et al (2014) RIPK1 maintains epithelial homeostasis by inhibiting apoptosis and necroptosis. Nature 513, 90-94
26. Zhao J, Jitkaew S, Cai Z et al (2012) Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proc Natl Acad Sci U S A 109, 5322-5327
27. Sun L, Wang H, Wang Z et al (2012) Mixed lineage kinase domain-like protein mediates necrosis signaling down-

http://bmbreports.org
stream of RIP3 kinase. Cell 148, 213-227
28. Wu JF, Huang Z, Ren JM et al (2013) Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis. Cell Res 23, 994-1006
29. Murphy JM, Czabotar PE, Hildebrand JM et al (2013) The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. Immunity 39, 443-453
30. Wang HY, Sun LM, Su LJ et al (2014) Mixed Lineage Kinase Domain-Like Protein MLKL Causes Necrotic Membrane Disruption upon Phosphorylation by RIP3. Mol Cell 54, 134-146
31. Cai ZY, Jitkaew S, Zhao J et al (2014) Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. Nat Cell Biol 16, 55-56
32. Chen X, Li WJ, Ren JM et al (2014) Translocation of mixed lineage kinase domain-like protein to plasma membrane leads to necrotic cell death. Cell 154, 105-121
33. Dondelinger Y, Declercq W, Montessuit S et al (2014) MLKL Compromises Plasma Membrane Integrity by Binding to Phosphatidylinositol Phosphates. Cell Rep 7, 971-981
34. Hildebrand JM, Tanzer MC, Lucet IS et al (2014) Activation of the pseudokinase MLKL unleashes the four-helix bundle domain to induce membrane localization and necrotic cell death. Proc Natl Acad Sci U S A 111, 15072-15077
35. Cook WD, Moujalled DM, Ralph TJ et al (2014) RIPK1- and RIPK3-induced cell death mode is determined by target availability. Cell Death Differ 21, 1600-1612
36. Bonnet MC, Preukschat D, Welz PS et al (2011) The adaptor protein FADD protects epidermal keratinocytes from necroptosis in vivo and prevents skin inflammation. Immunity 35, 572-582
37. Ch'en IL, Tsau JS, Molkentin JD, Komatsu M and Hedrick SM (2011) Mechanisms of necroptosis in T cells. J Exp Med 208, 633-641
38. Dillon CP, Oberst A, Weinlich R et al (2012) Survival function of the FADD-CASPASE-8-cFLIP(L) complex. Cell Rep 1, 401-407
39. Kaiser WJ, Upton JW, Long AB et al (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature 471, 368-372
40. Lu JV, Weist BM, van Raam BJ et al (2011) Complementary roles of Fas-associated death domain (FADD) and receptor interacting protein kinase-3 (RIPK3) in T-cell homeostasis and antiviral immunity. Proc Natl Acad Sci U S A 108, 15312-15317
41. Moulin M, Anderton H, Voss AK et al (2012) IAPs limit activation of RIP kinases by TNF receptor 1 during development. EMBO J 31, 1679-1691
42. Oberst A, Dillon CP, Weinlich R et al (2011) Catalytic activity of the caspase-8-cFLIP(L) complex inhibits RIPK3-dependent necrosis. Nature 471, 363-367
43. Zhang HB, Zhou XH, McQuade T, Li JH, Chan FKM and Zhang JK (2011) Functional complementation between FADD and RIP1 in embryos and lymphocytes. Nature 471, 373-376
44. Vercammen D, Beyaert R, Denecker G et al (1998) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. J Exp Med 187, 1477-1485
45. Lin Y, Devin A, Rodriguez Y and Liu ZG (1999) Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. Genes Dev 13, 2514-2526
46. Feng S, Yang Y, Mei Y et al (2007) Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain. Cell Signal 19, 2056-2067
47. O'Donnell MA, Perez-Jimenez E, Oberst A et al (2011) Caspase 8 inhibits programmed necrosis by processing CYLD. Nat Cell Biol 13, 1437-1442
48. Lu JV, Weist BM, van Raam BJ et al (2011) Complementary roles of Fas-associated death domain (FADD) and receptor interacting protein kinase-3 (RIPK3) in T-cell homeostasis and antiviral immunity. Proc Natl Acad Sci U S A 108, 15312-15317
49. Gunther C, Martini E, Wittkopf N et al (2011) Caspase-8 regulates TNF-alpha-induced epithelial necroptosis and terminal ileitis. Nature 477, 335-U108
50. Welz PS, Wullaert A, Vlantis K et al (2011) FADD prevents RIP3-mediated epithelial cell necrosis and chronic intestinal inflammation, Nature 477, 336-U102
51. Denecker G, Vercammen D, Steemans M et al (2001) Death receptor-induced apoptotic and necrotic cell death: differential role of caspases and mitochonrdia. Cell Death Differ 8, 829-840
52. Laster SM, Wood JG and Goodying LR (1988) Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. J Immunol 141, 2629-2634
53. Denecker G, Vercammen D, Steemans M et al (2001) Death receptor-induced apoptotic and necrotic cell death: differential role of caspases and mitochondria. Cell Death Differ 8, 829-840
54. Sakon S, Xue X, Takekawa M et al (2003) NF-kappaB inhibits TNF-induced accumulation of ROS that mediate prolonged MAPK activation and necrotic cell death. EMBO J 22, 3898-3909
55. Yu PW, Huang BCB, Shen M et al (1999) Identification of RIP3, a RIP-like kinase that activates apoptosis and NF kappa B. Curr Biol 9, 539-542
56. Sun XQ, Lee J, Navas T, Baldwin DT, Stewart TA and Dixit VM (1999) RIP3, a novel apoptosis-inducing kinase. J Biol Chem 274, 16871-16875
57. Kasof GM, Prosser JC, Liu DR, Lorenzi MV and Gomes BC (2003) The RIP-like kinase, RIP3, induces apoptosis and NF-kappa B nuclear translocation and localizes to mitochondria. FEMS Lett 473, 285-291
58. Koo G-B, Morgan MJ, Lee D-G et al (2015) Methylation-Dependent Loss of RIP3 Expression in Cancer Represses Programmed Necrosis in Response to Chemotherapeutics. Cell Res 25, 707-725
59. Yang Y, Hu W, Feng S, Ma J and Wu M (2005) RIP3 beta inhibits TNF-induced accumulation of ROS that mediate prolonged MAPK activation and necrotic cell death. EMBO J 22, 3898-3909
60. Newton K, Sun XQ and Dixit VM (2004) Kinase RIP3 is dispensable for normal NF-KBs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and toll-like receptors 2 and 4. Mol Cell Biol 24, 1464-1469
61. Hanahan D and Weinberg RA (2011) Hallmarks of Cancer: The serial threonine kinase RIP3: lost and found Michael J. Morgan and You-Sun Kim
62. Nugues AL, El Bouazzati H, Hétuin D et al (2014) RIP3 is downregulated in human myeloid leukemia cells and modulates apoptosis and caspase-mediated p65/RelA cleavage. Cell Death Dis 5, e1384

63. Liu XY, Lai F, Yan XG et al (2015) RIP1 Kinase Is an Oncogenic Driver in Melanoma. Cancer Res 75, 1736-1748

64. Fukasawa M, Kimura M, Morita S et al (2006) Microarray analysis of promoter methylation in lung cancers. J Hum Genet 51, 368-374

65. Zong WX, Ditsworth D, Bauer DE, Wang ZQ and Thompson CB (2004) Alkylating DNA damage stimulates a regulated form of necrotic cell death. Genes Dev 18, 1272-1282

66. David KK, Andrabi SA, Dawson TM and Dawson VL (2009) Parthanatos, a messenger of death. Front Biosci 14, 1116-1128

67. Sosna J, Voigt S, Mathieu S et al (2014) TNF-induced necroptosis and PARP-1-mediated necrosis represent distinct routes to programmed necrotic cell death. Cell Mol Life Sci 71, 331-348

68. Tenev T, Bianchi K, Darding M et al (2011) The Ripoptosome, a Signaling Platform that Assembles in Response to Genotoxic Stress and Loss of IAPs. Mol Cell 43, 432-448

69. Feoktistova M, Geserick P, Kellert B et al (2011) cIAPs Block Ripoptosome Formation, a RIP1/Caspase-8 Containing Intracellular Cell Death Complex Differentially Regulated by cFLIP Isoforms. Mol Cell 43, 449-463

70. Biton S and Ashkenazi A (2011) NEMO and RIP1 control cell fate in response to extensive DNA damage via TNF-alpha feedforward signaling. Cell 145, 92-103

71. Li JX, Feng JM, Wang Y et al (2014) The B-Raf(V600E) inhibitor dabrafenib selectively inhibits RIP3 and alleviates acetaminophen-induced liver injury. Cell Death Dis 5, e1278

72. Kim SK, Kim WJ, Yoon JH et al (2015) Upregulated RIP3 Expression Potentiates MLKL Phosphorylation-Mediated Programmed Necrosis in Toxic Epidermal Necrolysis. J Invest Dermatol [Epub ahead of print]

73. Motani K, Kushiyama H, Imamura R, Kinoshita T, Nishiuichi T and Suda T (2011) Caspase-1 Protein Induces Apoptosis-associated Speck-like Protein Containing a Caspase Recruitment Domain (ASC)mediated Necrosis Independently of Its Catalytic Activity. J Biol Chem 286, 33963-33972