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1. Introduction

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2. Ribosome inactivating protein-induced apoptosis

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synthesis and consequently necrosis. However, it was found that the morphology of cells treated with RIPs resembled that of cells undergoing death by apoptosis. Soon after the report by Griffiths et al. [14] on ricin- and abrin-induced apoptosis, many other bacterial as well as plant toxins were also found to induce apoptosis in mammalian cells [4–9]. Though many reports are available on toxin-induced apoptosis, very little progress has been made on elucidating the mechanism and pathways of RIP-induced apoptosis. Most of the work that has been published is on ST-induced apoptosis.

One of the earliest reports that RIPs are capable of inducing cell death came from the studies carried out by Griffiths et al. in 1987 [14]. They showed that intramuscular injection of ricin and abrin into rats resulted in death of cells in rapidly dividing tissues, where these toxins were known to concentrate. It was observed that the morphology of cells in para-aortic lymph nodes and Peyer's patches of ricin or abrin injected rats resembled that of cells undergoing apoptosis characterized by the formation of apoptotic bodies. It was also found that abrin and ricin treatment resulted in exhibition of apoptotic morpholgy, in addition to heterochromatin condensation and DNA laddering in bovine pulmonary endothelial cells [15] and also in U937, a human macrophage cell line [6]. In HeLa cells, ricin was found to induce the classical apoptotic morphological changes and also activation of caspase-3. Ricin also brought about an arrest of cell cycle at G2/M phase, but no effect on the G0/G1 phase [16]. In addition to ricin and abrin, several other RIPs like ST, ML, saporin and gelonin are also reported to induce apoptosis [11,17–21].

3. Apoptosis induction by ribotoxic stress response

It has been reported in the literature that many chemical agents that disrupt macromolecular synthesis in cells also induce cell death. The list includes cycloheximide (CHX) and actinomycin D, inhibitors of protein synthesis and RNA transcription, respectively. Treatment of HL-60 cells with CHX and actinomycin D resulted in large scale apoptosis [22]. It has also been shown that CHX alone is a poor inducer of apoptosis, but it potentiates tumor necrosis factor (TNF-α)-induced apoptosis [23]. Anisomycin, a low molecular weight protein synthesis inhibitor, was shown to have better apoptosis inducing capacity in U937 cells than CHX and was comparable to ricin in inducing apoptosis [24]. This suggested that the continuous synthesis of certain regulatory proteins is required to keep the apoptotic machinery in check in some fast dividing cell populations.

Earlier, it was shown by Iordanov et al. [25] that ricin, α-sarcin and anisomycin were able to activate the SAP kinases or JNK1 in Rat-1 cells. Damage to 28S rRNA by RIPs resulted in a novel pathway of kinase activation called ribotoxic stress response, resulting in the activation of SAPK/JNK and its activator, SEK1/MKK4. This study also revealed that activation of SAPK/JNK1 is not just due to protein synthesis inhibition, but also due to signaling from 28S rRNA affected by RIPs. Further, not all inhibitors of protein synthesis were able to elicit the ribotoxic stress response. Hence, it was proposed that ribotoxic stress response is specific for inhibitors that either induce damage to the α-sarcin/ricin loop of 28S rRNA or RIPs that ADP-ribosylate the EF-2/EFG, thus arresting translation at the translocation step. Anisomycin and the aminohexose pyrimidine nucleotide antibiotic blasticidin S are known to inhibit the peptidyl transferase activity of ribosomes. These antibiotics also resulted in activation of SAPK/JNK1 further confirming the above mentioned hypothesis. Antibiotics that inhibit protein synthesis at stages other than translocation did not induce phosphorylation of SAPK/JNK1 upon treatment with anisomycin [25].

Shiga toxin 1 has also recently been shown to trigger a similar ribotoxic stress response resulting in the activation of p38 MAPK and JNK, culminating in apoptosis of HCT cells. The finding that mutant ST (E167D) lacking RNA-N-glycosidase activity was not able to induce phosphorylation of p38 MAPK clearly indicates that the enzymatic activity of the A-chain is essential for apoptosis signaling. Further, blocking of p38 MAPK as well as JNK using the inhibitor SB202190 was able to rescue the cells from ST-induced apoptosis [13]. Langer et al. [19,20,26] and others have found that ML are capable of inducing apoptosis in many cell lines. Site specific mutation of E166Q and R169Q in recombinant ML resulted in the loss of protein synthesis inhibitory activity as well as apoptosis inducing capacity of the toxin [20]. Additionally, in the fungal ribotoxin α-sarcin, mutation of His137 to Gin also led to loss of apoptosis induction [27]. This suggests that the glycosidase activity of RIPs is essential for their toxicity. It is known that JNK is involved in stress-induced apoptosis triggered via the mitochondria [28].

In the case of ricin, it has recently been shown to induce apoptotic cell death in the macrophage cell line RAW 264.7 [29]. It was observed that in this case, treatment of RAW 264.7 cells with ricin led to increased secretion of TNF-α by these cells and consequently apoptosis. Ricin B-chain alone did not induce apoptosis and pretreatment with the p38 MAPK inhibitor, SB202190, resulted in appreciable drop in the secreted TNF-α levels and also cell death. Similar results were obtained with another RIP modeccin and the antibiotic anisomycin [29]. However, studies carried out in our laboratory on abrin-induced cell death in Jurkat cells revealed that there was no significant rescue from apoptosis even upon pretreatment of cells with p38 MAPK or JNK inhibitors (Narayanan et al., unpublished data). It is possible that abrin induces multiple signaling pathways resulting in cell death and hence different cell lines may respond differently towards translational inhibition with abrin.

With the evidences available at hand, it is safe to surmise that one of the pathways induced by RIPs could be the ribotoxic stress response through their RNA-N-glycosidase activity, resulting in the activation of SAPK/JNK and p38 MAPK leading to apoptosis [30]. However, the exact mechanism or the pathway by which signals are transmitted from damaged 28S rRNA to the MAPK pathway needs to be investigated further.

4. Apoptosis induction via the stress-induced mitochondrial pathway

Studies on the apoptosis induced by RIPs resulted in the finding that mitochondria are involved in many cases, though the proposed modes of induction of this pathway vary. Most of the studies reported a loss in mitochondrial membrane potential, MMP (Δψm), rapid release of cytochrome c, activation of caspase-9 and a marked increase in the production of reac-
tive oxygen species (ROS) in cells. It is known that any or all of these events can lead to cell death.

In our laboratory, abrin was found to induce apoptosis in Jurkat cells and that it occurs via the mitochondrial pathway of apoptosis. We also determined the kinetics of various apoptotic events induced by abrin and found that the loss of MMP was the earliest event to occur after protein synthesis inhibition. This was followed by caspase-3 activation and DNA fragmentation. Unlike some of the lectins that are known to activate death receptors (DR), abrin induced cell death was independent of caspase-8 and hence DR. Overexpression of Bcl-2 was able to inhibit apoptosis induced by abrin, but these cells were found to die by necrosis albeit after a prolonged exposure [4]. This suggested that the abrin-induced apoptosis follows the intrinsic mitochondrial pathway of cell death.

In the case of ST, it was observed that treatment of HeLa cells with ST resulted in activation of several caspases, including caspase-3, -6, -8 and -9 and apoptosis as measured by DNA fragmentation. It was also reported that enzymatically active ST alone could bring about cell death. Further, the activation of caspase-9 suggested the involvement of mitochondria, and indeed there was a loss of MMP (ΔΨm) in HeLa cells upon treatment with ST [17]. Though caspase-8 activity was found to increase, it could be due to activation by caspase-9 at late stages of apoptosis. Further, the fact that brefeldin A treatment resulted in inhibition of ST induced apoptosis suggests that the DR pathways might not be involved. It is also of importance to note that ST-induced apoptosis was inhibited by overexpression of Bcl-2, an anti-apoptotic protein acting at the mitochondrial level [18].

An alternate pathway of apoptosis induction by abrin has been proposed by Shih et al. [31]. They found that the apoptosis signaling by abrin is independent of its glycosidase activity and is due to its direct interaction with a mitochondrial protein called anti-oxidant protein-1 (AOP-1). They were able to show that abrin colocalized with the AOP-1 on the mitochondria, in addition to showing that AOP-1 interacts with an active site mutant A-chain using yeast two-hybrid system. Also, it was found that abrin was able to bring about a decrease in the anti-oxidant function of AOP-1 and consequently an increase in ROS production, and cytochrome c release into the cytosol. This study suggested that abrin is able to induce apoptosis by production of ROS, loss of MMP and release of cytochrome c into the cytosol. Shih et al. propose that the apoptogenic activity of abrin A-chain could be independent of its RNA-N-glycosidase activity. In addition to inducing loss of MMP, abrin was also found to induce elevation in ROS levels due to mitochondrial damage [4].

A third mode of action of these toxins is supposedly by ROS production in response to stress and by increase in intracellular calcium levels. Ricin induced a rapid elevation of cellular calcium level in hepatoma cells [32]. Trichosanthin, a type I RIP, has been shown to induce; apoptosis in many cells. Trichosanthin induces high levels of ROS production in human choriocarcinoma cells [33]. This event was shown to be dependent on extracellular as well as intracellular calcium levels and ROS production paralleled calcium elevation, suggesting that ROS production might be a consequence of calcium signaling. The anti-oxidant Trolox and ROS scavengers catalase and mannitol were able to rescue the cells from trichosanthin-induced cell death [33]. Recently, many studies have established a role for cellular calcium signaling in the mitochondrial pathway of apoptosis [34]. Also, the connection between calcium mobilization from ER to mitochondria is one of the hot topics of research in cell death. That the toxin-induced intrinsic or mitochondrial pathway of cell death is a complex phenomenon with multiple levels of regulation is reflected by the above mentioned reports. However, the bottom line of these data is that RIPs are capable of inducing stress-induced apoptosis via the mitochondrial pathway.

5. Apoptosis induction by downregulation of anti-apoptotic factors

The fate of a cell, to survive or to die, is decided by the delicate balance between the levels of anti-apoptotic and pro-apoptotic proteins in the cell. Many pro-apoptotic factors are activated during extrinsic as well as intrinsic signaling of apoptosis. During ST-induced cell death in endothelial cells, it was demonstrated that both ST-1 and ST-2 were able to bring about a decrease in the cellular level of the Bcl-2 family protein, Mcl-1, while the levels of Bcl-2 and Bcl-X were unaltered [35]. Mcl-1 is structurally similar to Bcl-2 except that it harbors two PEST sequences that target the protein for degradation by proteasome. The involvement of caspases in the degradation of Mcl-1 was ruled out as z-VAD-FMK, a pancaspase inhibitor was not able to block Mcl-1 degradation. However, specific inhibitors of proteasome, lactacystin and its derivative β-lactone, were able to block the degradation of Mcl-1, thus rescuing cells from ST-induced apoptosis. It is possible that Mcl-1 is continuously synthesized and degraded by proteasome and the continuous presence of Mcl-1 is probably required to prevent the cells from undergoing death. Protein synthesis inhibition by ST leads to a decreased level of Mcl-1, consequently resulting in apoptosis. Another study by Jones et al. [18] reveals that treatment of epithelial cells with ST led to an increase in the levels of the pro-apoptotic protein, Bax. This mechanism of action of RIP toxins might be cell line dependent as it is known that different cell lines express differing levels of pro- and anti-apoptotic factors and needs to be studied further.

6. Apoptosis induction due to NAD+ depletion by PARP hyperactivation

There are also reports available on RIP-induced apoptosis that involves alternative pathways of apoptosis. It was reported that treatment of U937 cells with ricin led to a marked decrease in the intracellular levels of NAD+ and ATP before inducing apoptosis [36]. Recent studies by Izyumov et al. [37] showed that decrease in intracellular ATP levels by a combination of inhibitors of glycolysis and oxidative phosphorylation resulted in the mitochondrial pathway of cell death. Poly-(ADP-ribose) polymerase (PARP) was also implicated to play a role in this apoptotic pathway as 3-amino benzamide, a PARP inhibitor, was able to block loss of NAD+ and thus apoptosis to some extent. Indeed, some recent reports conclusively show that hyperactivation of PARP-1 can result in depletion of NAD+ levels and hence can induce mitochondrial damage and apoptosis [38,39].
Barbieri et al. [40] have shown that the RIPs saporin L2, saporin S6, gelonin and momordin have transforming activity that involves auto-ADP-ribosylation of PARP. They also show that RIPs directly depurinate auto-modified PARP releasing adenine from the ADP-ribosyl group. It is known that the auto-modification of PARP is required for the base excision repair mechanism of DNA repair pathway. They propose that depurination of auto-modified PARP could result in the inhibition of DNA repair pathway as well as availability of PARP for further ADP-ribosylation, leading to depletion of intracellular levels of NAD+ thus inducing apoptosis. However, similar studies carried out by our group showed that PARP inhibitor was not able to rescue Jurkat cells from abrin-induced cell death [4]. It is possible that this pathway is relevant only in certain types of cell lines that are sensitive to alterations in cellular NAD+ levels.

7. Apoptosis induction by DNA damage due to nuclease activity

Another mode of apoptosis induction by RIPs is by their non-specific nuclease activity, by which they bring about DNA damage and hence apoptosis. RIPs have been shown to be active in inducing depurination of naked DNA as well as RNA in vitro and are known to have nuclease activity at high concentrations. The nuclease activity has been proposed to be due to the cleavage of DNA backbone at the apurinic/apyrimidinic sites [41]. The RIPs dianthin 30, saporin 6 and gelonin have been shown to have DNase activity, while ricin did not show any such activity under the conditions used for the assay [42]. Contradicting this is the report where ricin and ST have been shown to induce DNA damage by their nuclease activity in human umbilical vein endothelial cells (HUVECs) [12]. Similarly, trichosanthin has been shown to cut supercoiled double stranded DNA into linear and relaxed forms in vitro. This activity has also been observed with other RIPs like gelonin, saporin and dianthin. The nuclease activity of RIPs is still not very clear because the conditions under which the assay was performed and the concentrations of RIPs used vary considerably from one report to another. At least in two cases, contaminating nucleases have been found along with the RIP preparations. Further, it was reported that nuclease free preparations of gelonin, momordin 1, PAP-S and saporin S6 have been found to harbor only the polynucleotide N-glycosidase (PNAG) activity and no DNA nicking or nuclease activity [43]. In spite of all these reports the question whether RIPs are capable of entering nucleus to bring about depurination or nicking of DNA in vivo remains to be answered. With FITC-conjugated abrin, confocal microscopy studies were carried out in our laboratory to find if abrin could be localized in the nuclei of various cell lines. The outcome of the study indicated no nuclear localization of abrin even after it has entered the cytosol. Fig. 1 shows the confocal microscopy of SIHA cells after treating with abrin-FITC for 3 and 6 h. From the figure, it is evident that abrin does not localize to the nuclei of SIHA cells and hence cannot act on the nuclear DNA. Similar results were observed in MCF-7 and HeLa cells (data not shown). This is in contradiction with the earlier studies by Dodson et al. [41], Roncuzzi et al. [42] and Brigotti et al. [12]. It is evident that this aspect needs to be studied in depth to determine whether RIPs can enter the nucleus to induce DNA damage by nuclease activity and trigger apoptosis.

8. Discussion

Table 1 shows the toxicity with respect to apoptosis induction by various RIPs and the low molecular weight protein synthesis inhibitor, CHX. The table includes unpublished data gathered from studies carried out in our laboratory on various adherent and non-adherent cell lines with abrin and observations from the literature. It can be seen from the table that though there is not much difference in the IC50 for protein synthesis inhibition in Jurkat cells and U937 cells, the extent of apoptosis is different. Also, the cell lines Raji, U937 and U266B1 did not show much apoptosis even up to 72 h, but instead exhibited necrotic cell death (Bora et al., unpublished observation). It is known that the B-cell line Raji over-expresses the anti-apoptotic protein, Bcl-2. Studies have shown that Bcl-2 overexpression in Jurkat cells results in necrosis rather than apoptosis upon treatment with abrin [4]. Though the table indicates that RIPs induce cell death by apoptosis, there are considerable differences in the kinetics as well as extent of apoptosis observed for a given concentration of toxin.

Though all RIPs induce protein synthesis inhibition, it is possible that the pathway of apoptosis induced by RIPs depends upon the cells as well as the RIP used. Studies carried out by Brinkmann et al. [44] in genetic screens for cDNAs that confer resistance to PE-induced apoptosis resulted in the identification of a gene that encoded an antisense fragment of the cellular apoptosis susceptibility (CAS), a gene that is involved in nuclear import and chromosome segregation. While downregulation of CAS gene was able to confer resistance to PE and DT induced apoptosis, it was not, able to inhibit cell death induced by ricin. This suggests that the mechanism of apoptosis induction might depend on the mechanism of the action of the toxin rather than protein synthesis inhibition, the end result of ribosome inactivation. Further, the picture is made more complex by contradictory reports in the literature with respect to the involvement of p38 MAPK and JNK 1/2 in CHX-induced apoptosis [45,46].
Fig. 2 summarizes various signaling cascades and pathways that are triggered by RIPs to induce cell death. It is quite clear from the picture that most of the pathways are funneled into the mitochondria. It is well known that mitochondria play a major role in several stress induced cell death pathways and damage to mitochondria with subsequent loss of mitochondrial membrane potential has been known to be the point of no return in apoptotic cascades. It is logical to assume that RIPs do not activate the cellular apoptosis machinery directly as the primary activity of these toxins is to inactivate ribosomes and stop protein synthesis in cells. Cells are known to respond to several stress conditions like heat shock, infection by viruses or loss of cellular ATP. Cells depending upon the extent of stress they are exposed to, either try to overcome the stress by activating various stress response genes or undergo cell death. But how the cells decide when to activate the stress negating pathway and when to induce apoptosis is still not known. Mitochondrion is a good candidate for sensing the stress induced by RIPs, namely ribotoxic stress in cells, as it is the origin of intrinsic apoptotic signaling cascade.

### 9. Future prospects

Since the potential application of RIP toxins lies in their targeted delivery in cancer immunotherapy, it is imperative that a systematic study be carried out on various cancer cells and cell lines to determine their efficacy in inducing apoptosis. Such a study will throw light on the sensitivity of cancer cells to apoptosis induced by RIPs. It is desirable that toxin mediated therapy of cancers induces apoptotic cell death and not necrosis, since necrosis results in the spillage of cellular content to extracellular milieu, thereby triggering inflammatory response, which can be deleterious. Also, knowledge about the mechanism of RIP-induced cell death pathways will also enable us to tackle the problem of development of resistance to these toxins by cancer cells.

| Table 1 | Effects of type I and type II RIPs and the low molecular weight inhibitor, CHX, on protein synthesis and cell death induction in various cell lines |
|---|---|---|---|---|---|
| Cell line | IC₅₀ for PSI | Concentration | Time (h) | % apoptosis observed | Reference |
| **Abrin** | | | | | |
| Jurkat | 0.2 ng/ml | 10 ng/ml | 12 | 30 | [4] |
| U937 | 0.75 ng/ml | 1 μg/ml | 72 | 20° | Bora et al. b |
| Raji | 35 ng/ml | 1 μg/ml | 72 | 8° | Bora et al. b |
| U266Bl | 3.0 ng/ml | 1 μg/ml | 48 | 20° | Bora et al. b |
| BeWo | 7.5 ng/ml | 1 ng/ml | 48 | 55 | Bora et al. b |
| KB | 10 ng/ml | 100 ng/ml | 48 | 40 | Bora et al. b |
| MCF-7 | 0.4 ng/ml | 10 ng/ml | 36 | 25 | Surendranath et al. d |
| T47D | 0.4 ng/ml | 10 ng/ml | 36 | 36 | Surendranath et al. d |
| Ovcar-3 | 0.3 ng/ml | 10 ng/ml | 36 | 35 | Surendranath et al. d |
| U937 | NA² | 0.1 nM | 6 | 40 | [6] |
| **Ricin** | | | | | |
| U937 | NA | 1 nM | 6 | 18 | [6] |
| **Shiga toxin** | | | | | |
| HeLa | NA | 10 ng/ml | 4 | 64 | [17] |
| HEp-2 | NA | 12.5 ng/ml | 24 | 20 | [18] |
| THP-1 | NA | 1 ng/ml | 5 | 48.2 | [11] |
| **Mistletoe lectin** | | | | | |
| MOLT-4 | NA | 100 pg/ml | 24 | 22 | [20] |
| Lymphocytes | NA | 50 ng/ml | 72 | 40 | [19] |
| HUVEC | NA | 50 μg/ml | 48 | 25 | [21] |
| **Trichosanthin** | | | | | |
| JAR | NA | 50 ng/ml | 24 | 30 | [33] |
| **Immunotoxin RFB4(dsFv)PE38** | | | | | |
| CA 46 | 0.6 ng/ml | 1 ng/ml | 24 | 16 | [8] |
| Daudi | 20 ng/ml | 1 ng/ml | 24 | 84 | [8] |
| JD38 | 0.3 ng/ml | 1 ng/ml | 24 | 16 | [8] |
| Namalwa | 1.5 ng/ml | 1 ng/ml | 24 | 27 | [8] |
| Raji | 0.4 ng/ml | 1 ng/ml | 24 | 27 | [8] |
| **Cycloheximide** | | | | | |
| MRC-5 | NA | 50 μg/ml | 72 | 45.2 | [22] |
| HL-60 | NA | 50 μg/ml | 48 | 48 | [22] |

*NA: data not available.

°Necrosis was observed.

ᵇUnpublished observation by Namrata Bora.

ᶜExtensive necrosis was observed at 72 h.

ᵈUnpublished observation by Kalpana Surendranath.
At present, the molecular link between protein synthesis inhibition and the triggering of apoptotic cell death is unclear. Is it the direct damage to the 28S rRNA that is induced by the RNA-N-glycosidase activity of RIPs or ADP-ribosylation of EF-2/EF-G in the case of DT and PE that triggers apoptosis? Is it the loss of protein synthesis in the cells that acts as the critical factor in the signaling of apoptosis? Do RIPs interact with mitochondria and affect them directly? These are some of the questions that need to be addressed in order to understand the mechanism of translation inhibition induced cell death.

In addition to plant tissues, proteins that bring about adenine glycosylase activity under certain specific cellular stress conditions have been reported to be found in mammalian tissues also. This activity is very similar to that of many plant RIPs and has been observed in extracts of cells or tissues under stress or infection [47]. It is, therefore, probable that a mechanism of cell death induction that is triggered during cellular stress responses like protein synthesis inhibition exists in cells. RIPs might activate this pathway to trigger cell death upon translational inhibition.

Fig. 2. Various pathways of RIP-induced apoptosis. The possible mechanisms and pathways by which RIPs can induce cell death are shown.

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References


