# Ribosome Inactivating Proteins and Apoptosis

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**Abstract** Ribosome inactivating proteins (RIPs) are RNA *N*-glycosidases which potently inhibit translation by inactivating ribosomes. RIPs have also been shown to possess the ability to induce apoptosis. A number of RIPs from different sources have been used to study the mechanism of apoptosis induction. However, it is being observed that these toxins trigger apoptosis in different cell types via different mechanisms; although in most cases mitochondria have been involved, no single common pathway that is followed by the RIPs for apoptosis induction has emerged. There appears to be a consensus that the protein synthesis inhibition and induction of apoptosis by RIPs are independent of each other. In this chapter, we bring together the available studies on apoptosis induction by RIPs.

## 1 Introduction

Ribosome-inactivating proteins (RIPs) inactivate ribosomes which results in potent inhibition of protein synthesis. They are widely distributed in nature and are almost ubiquitously present in plants. RIPs are not only found in many exotic plants but also in some edible crop plants including wheat, maize, barley, tomato, spinach, etc. (Prestle et al. 1992; Ishizaki et al. 2002; Barbieri et al. 2006). In addition to plants, RIPs are also found in bacteria, fungi, and alga (Liu et al. 2002), and have been shown to be phylogenetically related (Girbes et al. 2004). RIPs have been shown to possess RNA *N*-glycosidase activity which is responsible for their RNA depurination ability (Endo et al. 1987). They manifest toxicity by irreversibly damaging the ribosome. The interest in RIPs gained a new momentum with the growing

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evidence of their action on nonribosomal substrates (Barbieri et al. 1997, 2000; Hudak et al. 2000).

RIPs are classically categorized into type 1 and type 2. The type 1 RIPs, like saporin and pokeweed antiviral protein (PAP), include a number of basic monomeric enzymes of approximately 30 kDa. Type 1 RIPs share a number of highly conserved active site cleft residues and secondary structure within the active site region; however, they are distinctly different in overall sequence and posttranslational modifications (Monzingo and Robertus 1992; Mlsna et al. 1993; Barbieri et al. 1993; Husain et al. 1994). There are some smaller type 1 RIPs, with a molecular mass below 30 kDa which are characterized by an N-terminal sequence abundant in arginine and glutamate residues (Ng and Parkash 2002; Ng et al. 2002, 2003; Parkash et al. 2002a, b). Besides the classical type 1 RIPs, there are few type 1 RIPs, like maize b 32, which are synthesized as inactive precursors termed proRIPs. These RIPs are much less prevalent than classical type 1 RIPs and have been characterized only from maize and barley (Bass et al. 1992; Reinbothe et al. 1994; Walsh et al. 1991). Type 1 RIPs play a defensive role in plants and inhibit both plant and animal viruses (Robertus 1996). They penetrate virus-infected cells, inactivate ribosomes and kill the infected cells, thus, terminating viral proliferation (Rappuoli 1997).

Type 2 RIPs, like ricin, abrin and modeccin are heterodimeric proteins consisting of two chains, an A-chain of approximately 30 kDa with enzymic activity, and a B-chain of approximately 35 kDa with lectin properties. The A-chain is linked to the B-chain through a disulfide bond (Olsnes and Pihl 1973, 1981; Stirpe et al. 1977). The B-chain can bind to galactosyl moieties of glycoproteins and/or glycolipids found on the surface of eukaryotic cells (Sandvig et al. 1976; Olsnes and Sandvig 1988; Swimmer et al. 1992; Lehar et al. 1994; Steeves et al. 1999) and mediate retrograde transport of the A-chain to the cytosol where it has access to the translational machinery (Olsnes and Pihl 1981; van Deurs et al. 1986; Beaumelle et al. 1993; Sandvig and van Deurs 1996). The type 2 RIPs vary in their toxicity by about three orders of magnitude (Battelli 2004; Stirpe 2004). On the basis of the considerable differences in their cytotoxicity, and consequently in their toxicity to animals, the type 2 RIPs have been broadly divided into two groups, toxic and nontoxic. The reasons for the difference in toxicities are not completely understood, however binding and entry of the toxin into the cells and/or their degradation and exocytosis appear to be the major contributors (Stirpe and Battelli 2006).

## 2 Mechanism of Action of RIPs

RIPs exhibit RNA *N*-glycosidase activity, which was first demonstrated by Endo et al. (1987). They discovered that the RIPs catalyze removal of a single adenine residue, A4324 in rat liver rRNA, from a GAGA sequence in a universally conserved loop at the top of a stem in 28S ribosomal RNA, which was subsequently termed sarcin/ricin domain or loop (Endo et al. 1987; Endo and Tsurugi 1988).

The catalytic depurination disrupts the binding of elongation factors to the ribosomes, thus arresting protein synthesis at the translocation step (Endo et al. 1987). Later, when the study was extended to more RIPs and they were all found to have similar activity, RIPs were classified as rRNA *N*-glycosidases (EC 3.2.2.22). Although the catalytic mechanism of all RIPs is identical, their activity on ribosomes from sources other than eukaryotes is markedly different (Barbieri et al. 1993; Stirpe et al. 1988). The differences in the toxicity of RIPs toward various cell lines, requirements for different cofactors and variations in the minimal structure of the adenine-containing loop that they can attack, point to their substantial diversity (Carnicelli et al. 1992; Marchant and Hartley 1995). Differential inhibition pattern by molecules that bind and inactivate RIPs has also suggested that local sequence and structure variability exists among RIPs (Brigotti et al. 2000).

Besides having the functional *N*-glycosidase activity there are evidences of RIPs showing activities on nonribosomal substrates (Barbieri et al. 1997, 2000; Hudak et al. 2000). Most of the novel enzymatic activities are related to a presumed RNase or DNase activity (Li et al. 1991; Mock et al. 1996; Nicolas et al. 1997, 1998, 2000; Roncuzzi and Gasperi-Campani 1996). Other enzymatic activities reported for individual RIPs include phospholipase, chitinase and superoxide dismutase activity (Li et al. 1997; Helmy et al. 1999; Sharma et al. 2004; Xu et al. 2008). PAP has been shown to cleave the double-stranded supercoiled DNA using the same active site required to depurinate rRNA, whereas momordin has been shown to have intrinsic RNase activity (Wang and Tumer 1999; Fong et al. 2000).

## 3 Apoptosis

Apoptosis or programmed cell death is a well orchestrated collapse of a cell whereby the specific signaling is activated which ultimately leads to controlled cellular death. The term apoptosis was first used to describe a morphologically distinct form of cell death (Kerr et al. 1972). The mechanism of apoptosis is highly complex and involves a cascade of energy dependent molecular events. There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. In addition, there is now evidence that the two pathways are linked and molecules in one pathway can influence the other (Igney and Krammer 2002).

The extrinsic pathway is initiated by the interaction of the transmembrane receptor with a ligand. FasL/FasR and TNF-α/TNFR1 are the best models that characterize the sequence of events that define the extrinsic pathway of apoptosis. Briefly, the receptors cluster when they bind with the homologous trimeric ligand. Consequently, upon ligand binding, cytoplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. The binding of Fas ligand to Fas receptor results in the binding of the adapter protein FADD (Fas-Associated protein with Death Domain), and the binding of TNF to TNF receptor results in the binding of the adapter protein TRADD (TNFR1-associated

death domain protein) with recruitment of FADD (Hsu et al. 1995; Kelliher et al. 1998; Wajant 2002). FADD then associates with procaspase-8 via dimerization of the death effector domain. At this point, a death-inducing signaling complex (DISC) is formed, resulting in the autocatalytic activation of procaspase-8 (Kischkel et al. 1995). Once caspase-8 is activated, the execution phase of apoptosis is triggered. Death receptor mediated apoptosis can be inhibited by a protein called c-FLIP (FLICE inhibitory protein) which binds to FADD and caspase-8, rendering them ineffective (Kataoka et al. 1998; Scaffidi et al. 1999).

The intrinsic pathway of apoptosis, as its name suggests, is initiated from within the cell. This pathway involves a diverse array of nonreceptor-mediated stimuli that produce intracellular signals which act directly on targets within the cell and are mostly mitochondrial-initiated events. The stimulus that initiates the intrinsic pathway may either be a positive or a negative factor. In other words, there has to be a balance between the pro- and anti-apoptotic factors for a continued cell growth. The presence of negative signals, which could be through the absence of growth factors, hormones and cytokines, can lead to failure of suppression of death programs, thereby triggering apoptosis. The positive signals could be due to a specific factor(s) like radiation, toxins, hypoxia, hyperthermia, viral infections, free radicals, etc. Any of these stimuli can cause changes in the inner mitochondrial membrane that results in opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release of two main groups of proapoptotic proteins from the intermembrane space into the cytosol (Saelens et al. 2004). The first group consists of cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi, which activate the caspase dependent mitochondrial pathway (Du et al. 2000; van Loo et al. 2002a; Garrido et al. 2006). Cytochrome c binds and activates Apaf-1 (apoptotic protease activating factor-1) as well as procaspase-9, forming an "apoptosome" (Chinnaiyan 1999; Hill et al. 2004). The clustering of procaspase-9 in this manner leads to caspase-9 activation. Smac/DIABLO and HtrA2/Omi are reported to promote apoptosis by inhibiting the activity of IAP (inhibitor of apoptosis proteins) (Schimmer 2004; van Loo et al. 2002b). The second group of proapoptotic proteins, AIF (apoptosis-activating factor), endonuclease G and CAD (caspase-activated DNase), are released from the mitochondria during apoptosis, but this is a late event and occurs after the cell has committed to die.

Both, the extrinsic and intrinsic pathways converge at the point of the execution phase which is the final stage of apoptosis. The activation of the effector caspases is the most important step that begins the execution phase of apoptosis. The activated execution caspases in turn activate cytoplasmic endonuclease, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins. Caspase-3, caspase-6, and caspase-7 function as effector or "executioner" caspases, which cleave various substrates (Slee et al. 2001). Caspase-3 is the most important of the executioner caspases and is activated by a number of the initiator caspases like caspase-8, caspase-9, or caspase-10. Caspase-3 specifically activates the endonuclease, CAD (Caspase-Activated DNase). In proliferating cells, CAD is present with its inhibitor, ICAD (inhibitor of caspase-activated DNase) but in the apoptotic cells, activated caspase-3 cleaves ICAD to release CAD (Sakahira et al. 1998).

CAD then degrades chromosomal DNA within the nuclei and causes chromatin condensation. Caspase-3 also induces cytoskeletal reorganization and disintegration of the cell into apoptotic bodies. Gelsolin, an actin binding protein, has been identified as one of the key substrates of activated caspase-3.

## 4 Ribosome Inactivating Proteins and Apoptosis

Initially, the cytotoxicity of RIPs was ascribed solely to the inhibition of protein synthesis; however Griffiths et al. (1987) observed for the first time that the morphology of the cells treated with ricin and abrin was similar to that of the cells undergoing apoptosis. They observed a large number of apoptotic bodies in paraaortic lymph nodes, Peyer's patches and ileal crypts of rats intramuscularly injected with ricin and abrin. Abrin, compared with ricin was found to cause more pronounced changes in these tissues. Later, abrin and ricin treatment of bovine pulmonary endothelial cells was also shown to produce apoptotic morphology, in addition to heterochromatin condensation and DNA laddering (Hughes et al. 1996). Soon after the first report on ricin and abrin induced apoptosis, many other plant and bacterial toxins were also found to induce apoptosis in mammalian cells (Chang et al. 1989; Morimoto and Bonavida 1992; Kochi and Collier 1993; Allam et al. 1997; Brinkmann et al. 1997; Narayanan et al. 2004).

As more and more studies were conducted on RIPs, it was clearly established that RIPs induced apoptosis since the toxin treated cells demonstrated the morphological and biochemical events associated with apoptosis. Ricin was observed to induce apoptosis in macrophages independent of the inhibition of protein synthesis (Khan and Waring 1993). Also, it was observed that the ricin-induced apoptosis did not involve the activation of $Ca^{2+}$ dependent endonuclease(s) as there was no immediate increase in $Ca^{2+}$ concentration when macrophages were treated with ricin (Khan and Waring 1993). The cell death induced by ricin, modeccin, *Pseudomonas* toxin, and diphtheria toxin in MDCK cells was found to be strongly inhibited by 1,9-Dideoxyforskolin (DDF) suggesting these protein toxins to invoke a DDF-sensitive common cell death pathway (Oda et al. 1997). However, despite the strong inhibitory effect, DDF did not block toxin-induced DNA fragmentation which suggested that apoptosis and cell death may be triggered through separate pathways by these toxins (Oda et al. 1997).

RIPs have been explored to be developed as therapeutic proteins by coupling with antibodies recognizing cell surface proteins. The conjugates containing RIPs and antibodies, termed immunotoxins have also been studied for their apoptosis inducing properties. Saporin and a saporin containing immunotoxin were found to induce apoptosis in human peripheral blood B lymphocytes and neutrophils, in the B-cell line, Daudi, and in the haemopoietic cell lines, HL-60 and TF-1 (Bergamaschi et al. 1996). The saporin containing immunotoxin was 2–3 logs more effective than the native saporin in inducing apoptosis (Bergamaschi et al. 1996). Momordin, pokeweed antiviral protein from seeds (PAP-S) and saporin, and

their immunotoxins with Ber-H2, a monoclonal antibody directed against the CD30 antigen of human lymphocytes induced apoptosis in the CD30+ L540 cell line (Bolognesi et al. 1996). The immunotoxins made with RIPs were much more potent in inducing apoptosis compared to their free toxin counterparts because of better cell binding and internalization (Bergamaschi et al. 1996, Bolognesi et al. 1996). A replication-defective adenovirus enhanced the apoptotic and cytotoxic activity of a basic fibroblast growth factor-saporin fusion protein by more than ten fold, and caused *in vivo* tumor cell killing at nontoxic concentrations due to enhanced internalization of the ligand–receptor complex and release of the active toxin from the endosomes (Satyamoorthy et al. 1997).

Despite a large number of studies on RIP-induced apoptosis, the exact mechanism by which these toxins induce apoptosis is not very clear. Several reports on various RIPs like abrin, ricin, saporin, gelonin, mistletoe lectins (MLs), Shiga toxins (Stx), etc. describe the induction of apoptosis involving different apoptotic pathways and so far no single general mechanism has emerged for the induction of apoptosis by RIPs. We will address below in this chapter the various mechanisms put forth for the induction of apoptosis by RIPs.

## *4.1 Activation of Intrinsic Pathway of Apoptosis by General Stress*

Mitochondria play a key role in stress induced cell death. Damage to mitochondria leads to loss of mitochondrial membrane potential (MMP) and has been shown to be the key point when the cell commits to die. A cell on exposure to different stress signals, which include toxins, heat, infection by viruses, loss of ATP, etc. responds either to overcome stress by activating various stress genes or can decide to undergo apoptosis. Most studies relating to induction of apoptosis by RIPs suggest that apoptosis is caused by the intrinsic pathway where the MMP changes, followed by rapid release of cytochrome c and activation of caspase-9.

One of the early reports depicted the direct role of mitochondria in RIP-induced apoptosis (Shih et al. 2001). The study showed that abrin could induce apoptosis by directly interacting and activating a thiol-specific 30-kDa antioxidant protein-1 (AOP-1), which resulted in an increase in the levels of intracellular reactive oxygen species (ROS) and release of cytochrome c from the mitochondria to the cytosol, and subsequently activation of caspase-9 and caspase-3 (Shih et al. 2001). Furthermore, ROS scavengers, *N*-acetylcysteine and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl delayed the onset of apoptosis indicating ROS to be an important mediator of abrin-induced apoptosis (Shih et al. 2001).

Saporin-6, a type I RIP expressed in the seeds of *Saponaria officinalis* plant, induced caspase-dependent apoptosis in human histiocytic lymphoma cell line U937 via the mitochondrial or intrinsic pathway (Sikriwal et al. 2008). Saporin-6, unlike many other RIPs, did not require *N*-glycosidase activity for apoptosis induction, and the apoptosis onset occurred before any significant inhibition of protein synthesis ensued (Bagga et al. 2003; Sikriwal et al. 2008). In another study,

His-tagged saporin was found to be more cytotoxic to U937 cells when combined with *Gypsophila* saponins (Weng et al. 2008). The cytotoxicity was a result of induction of apoptosis triggered by the internalization of saporin facilitated by *Gypsophila* saponins (Weng et al. 2008).

The release of mitochondrial cytochrome c, and the sequential caspase-9 and caspase-3 activations have been shown to be important events in the signal transduction pathway of abrin-induced apoptotic cell death in the HeLa cell line (Qu and Qing 2004). Generation of ROS in response to toxins may cause oxidative stress to cells which might be one of the key factors in inducing apoptosis through the mitochondrial pathway. Abrin was shown to induce apoptosis in Jurkat cells following the intrinsic mitochondrial pathway that involved MMP damage and production of ROS (Narayanan et al. 2004).

In a similar way trichosanthin (TCS), a type-I ribosome-inactivating protein was observed to induce apoptosis in human choriocarcinoma cells, JAR due to generation of ROS (Zhang et al. 2000, 2001). The ROS formation, which preceded the activation of caspase-3, was shown to be dependent on the presence of extracellular $Ca^{2+}$. Furthermore, the antioxidant α-tocopherol prevented TCS-induced ROS formation and thereby rescued the cells from death (Zhang et al. 2000). There are many established roles of calcium in mitochondria induced apoptosis (Hajnoczky et al. 2003). The study by Zhang et al. (2000) indicated the possible role of $Ca^{2+}$ signaling in RIP-induced apoptosis. Though TCS has been shown to induce calcium dependent ROS generation in JAR cells, there is a reported exception in human chronic myeloid leukemia cell line K562 (Li et al. 2007a). In this study, TCS treatment induced a transient elevation in the intracellular calcium concentration followed by a slow increase in ROS production. Calcium chelators and antioxidants did not affect the TCS-induced apoptosis, suggesting that calcium changes and ROS may not be involved in TCS-mediated apoptosis in K562 cells (Li et al. 2007a). TCS was also able to induce effective apoptosis in HIV-1 infected cells which was suggested to account, in part, for its antiviral activity (Wang et al. 2005).

Korean ML treatment resulted in a significant increase in ROS and loss of MMP in human hepatocarcinoma cells (Kim et al. 2004). Furthermore, treatment with the antioxidant *N*-acetyl-L-cysteine reduced ROS induction by ML, preventing apoptosis in Hep3B cells, indicating that oxidative stress is involved in ML-mediated cell death (Kim et al. 2004).

Ricin was shown to induce cell death in human cervical cancer cell line, HeLa which was mediated by the generation of ROS and subsequent activation of caspase-3 cascade followed by downstream events leading to apoptosis (Rao et al. 2005).

*Viscum album* agglutinin-I (VAA-I) was shown to induce apoptosis by ROS-independent mechanism as treatment with catalase, known to degrade $H_2O_2$, failed to reverse VAA-I-induced apoptosis (Lavastre et al. 2002).

Though there are reports on the activation of apoptosis exclusively by the mitochondrial pathway, in some instances apoptosis is induced by RIPs involving caspase-8 through the receptor-independent mitochondria-controlled apoptotic pathway as well. One such example is ML, ML-1 induced apoptosis in leukemic T- and B-cell lines where activation of caspase-8 has been observed along with

caspase-9 and -3 (Bantel et al. 1999). Since caspase-8 is implicated as a regulator of apoptosis mediated by death receptors, it is concluded that apoptosis induced by ML-1 is a receptor-independent mitochondria-controlled apoptotic pathway (Bantel et al. 1999).

Shiga toxin-1 (Stx1) has been shown to induce apoptosis in HeLa cells along with the activation of caspase-8, -6, and -3, loss of MMP, increased release of cytochrome c from mitochondria at 3 to 4 h post-treatment and DNA fragmentation (Fujii et al. 2003). It was concluded that the primary pathway of Stx1-induced apoptosis and DNA fragmentation in HeLa cells was unique and included caspases 8, 6, and 3 but was independent of events in the mitochondrial pathway (Fujii et al. 2003). Similarly, in macrophage-like cells, THP-1, Stx1 activated a broad array of caspases, disrupted the MMP and released cytochrome c into the cytoplasm (Lee et al. 2007). Earlier, it was shown that in THP-1 cells Stx1 and Stx2 activated caspase-3, and the apoptotic signals increased after Stx had reached the Golgi apparatus (Kojio et al. 2000).

Stress to organelles other than mitochondria can also induce apoptosis. Treatment of HL-60 cells with TCS demonstrated the involvement of mitochondrial pathway as there was reduction of MMP and release of cytochrome c and Smac besides the activation of caspase-9 (Li et al. 2007b). Furthermore, TCS treatment induced upregulation of endoplasmic reticulum chaperone BiP and transcription factor CHOP (CCAAT/enhancer-binding protein (C/EBP)-homologous protein), and also activated caspase-4, which for the first time strongly supported the involvement of the endoplasmic reticulum stress pathway in TCS-induced apoptosis (Li et al. 2007b). Subsequently, Stx1 was also shown to induce apoptosis through endoplasmic reticulum stress response in myelogenous leukemia cell line, THP-1 (Lee et al. 2008). Treatment of THP-1 cells with Stx 1 resulted in the increased activation of the ER stress sensors IRE1, PERK and ATF6, and increased expression of the transcriptional regulator CHOP and the death domain-containing receptor DR5 (Lee et al. 2008).

## *4.2 Activation of the Extrinsic Pathway of Apoptosis*

There are few reports demonstrating the involvement of death receptors in the induction of apoptosis by RIPs. One such study demonstrated that apart from direct induction of apoptosis in response to inhibition of protein synthesis by the enzymic action of ML- A chain, it could also indirectly induce apoptosis in Fas+ tumor cells through activated FasL+ lymphocytes (Büssing et al. 1999).

Korean ML induced apoptosis in a human colon cancer cell line, COLO, and an antagonizing antibody against tumor necrosis factor receptor 1 was able to decrease activation of caspases, particularly caspase-8, in COLO cells treated with ML suggesting the possibility of the extrinsic pathway of apoptosis to be involved (Khil et al. 2007). Shiga toxin- 2 (Stx2) has been shown to induce apoptosis by activation of both the intrinsic and extrinsic pathways of apoptosis (Fujii et al.

2008). Similarly, Polito et al. (2009) provided evidence for the involvement of more than one pathway in the apoptosis induced by ricin and saporin. However, it was suggested that the activation of the extrinsic pathway may not be essential in apoptosis induced by these RIPs. There are few other reports mentioning the activation of caspase-8 by RIPs, however they have ruled out the involvement of the receptor pathways in the cell death (Bantel et al. 1999; Kiyokawa et al. 2001).

### *4.3 Impaired Balance Between and Pro- and Anti-Apoptotic Factors*

In the normal cell there exists a delicate balance between the pro- and anti-apoptotic factors. The antiapoptotic factors, Bcl-XL and Bcl-2 are located in the outer mitochondrial membrane and promote cell survival, whereas the proapoptotic factors, Bax, Bid, Bak and Bad are in the cytosol where they act as sensors of cellular damage or stress. Some RIPs have been shown to alter the balance of pro- and anti-apoptotic factors by either increasing the expression of proapoptotic or decreasing the expression of antiapoptotic proteins.

ML was observed to induce apoptosis by down-regulation of Bcl-2 and up-regulation of Bax, thereby activating caspases in p53-positive, SK-Hep-1 and p53-negative, Hep 3B cell lines (Lyu et al. 2002). Induction of apoptosis by the *N*-acetyl-galactosamine-specific toxic lectin from *V. album*, ML-III in human lymphocytes has been shown to be associated with a decrease of nuclear p53 and Bcl-2 proteins and induction of telomeric associations (Bussing et al. 1998).

Apoptosis induced by Stxs (Stx1 and Stx2) in epithelial cell line HEp-2 was observed to be mediated through the enhanced expression of the proapoptotic protein Bax which could be blocked by the over expression of Bcl-2 by transient transfection (Jones et al. 2000). Subsequently, it was found that Bid, a proapoptotic member of the Bcl-2 family was also induced upon Stx1 treatment of HEp-2 cells followed by the activation of various caspases (Ching et al. 2002). Stx also induced cell death in human renal proximal tubular epithelial cells, HK-2 by stimulating the expression of proapoptotic protein Bak, and silencing of Bak gene gave partial protection against Stx-mediated apoptosis (Wilson et al. 2005). In another study, Stx-1 and Stx-2-induced death in endothelial cells was found to be accompanied by a dose dependent decrease in the expression of Mcl-1, an antiapoptotic Bcl-2 family member, with no change in the expression of Bcl-2 and Bcl-xl (Erwert et al. 2003). Mcl-1 is structurally similar to Bcl-2 except that it harbors two PEST sequences that target the protein for degradation by proteasome. Using proteasome specific inhibitors, the degradation of the Mcl-1 could be prevented which rescued the cells from Stx-induced apoptosis suggesting a role for Mcl-1 in protecting endothelial cells against Stx-1-induced apoptosis (Erwert et al. 2003).

TCS-induced apoptosis in HeLa cells was accompanied by a decreased expression of Bcl-2 and phosphorylation of cyclic AMP response element-binding protein (CREB), which regulates the expression of Bcl-2 (Wang et al. 2007). The study thus

suggested the possibility of CREB playing a critical role in the regulation of Bcl-2 expression in TCS-induced HeLa cell death (Wang et al. 2007).

Agrostin, a type 1 RIP isolated from the seeds of *Agrostemma githago* showed down-regulation of the intracellular level of Bcl-2 protein (Chiu et al. 2001). Ricin-induced apoptosis in hepatoma cells, BEL7404, was accompanied by increased expression of Bak and decreased levels of Bcl-xl (Hu et al. 2001). In a similar way, abrin-derived peptide (ABP) was also observed to induce apoptosis in Dalton's lymphoma which was marked by a reduction in the ratio of Bcl-2 and Bax protein expression, and consequently activation of caspase-3 (Bhutia et al. 2009).

Protein phosphorylation–dephosphorylation is one of the major signaling mechanisms for modulating the functional properties of proteins involved in gene expression, cell adhesion, cell cycle, cell proliferation, and differentiation. It has been shown that phosphorylation of Bcl-2 proteins regulates their ability to inhibit apoptosis (Adams and Cory 2001). Phosphorylated Bcl-2, Bad, and Bax have an antiapoptotic function and their dephosphorylation is required for proapoptotic activity (Verma et al. 2001). Khwaja et al. (2008) highlighted the potential phosphorylation and glycosylation sites on evolutionarily conserved residues of Bad, Bax and Bcl-2 proteins *in silico*, and suggested that ML-I may induce downstream signaling events that include alternative phosphorylation and O-GlcNAc modification of Bcl-2, Bax, and Bad for tumor cell apoptosis through binding to the cell surface receptors.

## *4.4 Induction of Apoptosis in Response to Ribotoxic Stress*

The term ribotoxic stress was first used to describe the cellular response to toxicants that perturb the functioning of the 3′-end of the large 28S ribosomal RNA (Iordanov et al. 1997). During translation, the 3′-end of the large 28S rRNA functions in aminoacyl-tRNA binding, peptidyltransferase activity, and ribosomal translocation (Uptain et al. 1997). Toxin induced disruption of this activity results in the activation of various kinase pathways like JNK and p38 MAP kinase and/or alterations in ERK1/2 signaling (Iordanov et al. 2002, 1997; Iordanov and Magun 1998). In most cases, active ribosomes appear to be required as mediators of this signaling response and many of the inducers of the ribotoxic stress response at least partially inhibit protein synthesis. However, not all inhibitors of protein synthesis were able to elicit the ribotoxic stress response. Thus, 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 ADP-ribosylate the EF-2/EF-G and arrest translation at the translocation step (Iordanov et al. 1997).

The first evidence which highlighted the role of kinases in apoptosis came from a study with two different protein synthesis inhibitors, ricin and cycloheximide (Geier et al. 1996). Treatment of MDA-231 cells with ricin and cycloheximide induced apoptosis, and the results indicated the possibility of the involvement

of several distinctive pathways with protein kinase C also playing a role (Geier et al. 1996). Later, Iordanov et al. (1997) observed that ricin, α-sarcin and anisomycin were able to activate SAPK or JNK1 in Rat-1 cells. This study also suggested that activation of SAPK/JNK1 was not only due to protein synthesis inhibition, but also due to signaling from 28S rRNA triggered by the toxins. Thus, damage to 28S rRNA by RIPs resulted in ribotoxic stress response. Subsequently, ML was shown to induce apoptosis in cancer cells which was mediated by activation of JNK/SAPK (Kim et al. 2000). Furthermore, three distinct components of mistletoe, including β-galactoside- and *N*-acetyl-D-galactosamine-specific lectin II, polysaccharides, and viscotoxin were found to induce apoptotic cell death in U937 cells (Park et al. 2000). The mistletoe extracts markedly increased the phosphotransferase activity of JNK1/SAPK in these cells. Lectin II was the most potent in inducing apoptosis as well as JNK1 activation in U937 cells (Park et al. 2000). The ML-II-induced apoptosis in U937 cells was preceded by the activation of ERK1/2, p38 MAPK and SAPK/JNK (Pae et al. 2001). The apoptosis was significantly enhanced when ERK1/2 activation was selectively inhibited by PD098059, a MAP kinase inhibitor and was markedly reduced when an activator of ERK, 12-O-tetradecanoylphorbol-13-acetate, was used in U937 cells. Inhibition of p38 MAPK activity with p38-specific inhibitor, SB203580, partially inhibited lectin-II-induced DNA fragmentation. These results suggested that ERK1/2 and p38 MAPK may have opposite effects on cell survival in response to cytotoxic ML-II (Pae et al. 2001).

Two protein kinases, protein kinase A (PKA) and C (PKC), were shown to play a crucial role in apoptosis induced in cancer cells by Korean ML-II (Pae et al. 2000). The study demonstrated that exposure of human leukemia cells, HL-60 to ML-II induced apoptosis but the treatment of these cells with a PKA or PKC activator suppressed apoptosis. PKA and PKC inhibitors reversed the suppression of apoptosis by the activators, suggesting the involvement of PKA or PKC in the ML-II-induced apoptosis in HL-60 cells (Pae et al. 2000). The ML-II has also been shown to induce apoptotic cell death through Akt signaling pathway along with the inhibition of telomerase activity and the activation of caspase-3 (Choi et al. 2004). *Viscum album coloratum* agglutinin (VCA), isolated from Korean mistletoe induced apoptotic killing in hepatocarcinoma Hep3B cells which was preceded by a significant increase in ROS and loss of MMP (Kim et al. 2004). Treatment of Hep3B cells with VCA resulted in JNK phosphorylation which was abolished with the pretreatment of cells with a JNK inhibitor suggesting the necessary role of the phosphorylation in VCA-induced apoptosis. Furthermore, Hep3B cells overexpressing JNK1 or stress-activated protein kinase (SEK1) were more susceptible to cell death induced by VCA- (Kim et al. 2004).

A contradictory report on the role of PKC came to light using a specific inhibitor, participation of calcium-dependent proteases, or when PKC was excluded, in the apoptotic process induced by ricin (Hu et al. 2001).

Ricin- induced apoptosis was preceded by the release of TNF-α in a dose dependent manner in mouse macrophage cell line RAW 264.7 (Higuchi et al. 2003). However, galactose-specific ricin B-chain alone did not cause release of

TNF-α and apoptosis suggesting that receptor-binding of ricin through the B-chain is not enough. Inhibition of the release of TNF-α by pretreatment of the RAW 264.7 cells with a specific p38 MAP kinase inhibitor resulted in significant inhibition of ricin-induced apoptosis indicating that a specific attack on 28S rRNA by ricin resulting in ribotoxic stress and the activation of p38 MAP kinase are contributors to ricin-induced apoptosis (Higuchi et al. 2003). In case of ML, only the hololectin was able to induce apoptosis and isolated A- and B-chains were not cytotoxic (Vervecken et al. 2000).

Exposure of primary macrophages to ricin *in vitro* also led to the activation of SAP kinases, increased expression of proinflammatory mRNA transcripts and subsequently increase in the synthesis and secretion of TNF-α, and apoptotic cell death (Korcheva et al. 2007).

Tamura et al. (2003) demonstrated that in Vero cells the apoptosis signaling pathways, triggered by ricin were sensitized in butyric acid-treated cells, while the pathways leading to protein synthesis inhibition by the toxin were relatively unchanged.

Stx1 has also been shown to induce the ribotoxic stress response (Smith et al. 2003). Treatment of intestinal epithelial cell line, HCT-8 with Stx1 induced expression of c-jun and c-fos, and activated JNK and p38 within 1 h which persisted for 24 h. However, using a catalytically defective mutant toxin, in which the active site glutamate was replaced with aspartate, could not activate JNK and p38 indicating that RNA *N*-glycosidase activity is required for the induction of apoptosis. Moreover, blocking Stx1-induced p38 and JNK activation with the inhibitor SB202190 prevented cell death and was able to rescue cells from Stx- induced apoptosis (Smith et al. 2003). Treatment of macrophage-like cells, THP-1 *in vitro* with Stx1 resulted in the simultaneous induction of apoptotic and survival signaling pathways in these cells; and a limited apoptosis and prolonged JNK and p38 MAPK activation was observed (Lee et al. 2007). JNK is known to be involved in stress-induced apoptosis triggered via the mitochondria (Tournier et al. 2000). The absence of JNK causes a defect in the mitochondrial death signaling pathway, including the failure to release cytochrome c, thus indicating that mitochondria are influenced by proapoptotic signal transduction through the JNK pathway (Tournier et al. 2000).

Verotoxins (VT1 and VT2) stimulated a weak, transient increase in JNK activity and a strong activation of both p38 MAP kinase and ERK activity in human monocytes, which was sustained in the case of p38 MAP kinase 3 (Cameron et al. 2003). 293T cells expressing PAP did not show inhibition of translation even when approximately 15% of the ribosomal RNA was depurinated (Chan Tung et al. 2008). PAP expression induced the activation of JNK, and the enzymatically inactive mutant PAPx did not affect kinase activity. However, JNK activation did not result in apoptosis as there was an absence of caspase-3 and poly (ADP-ribose) polymerase cleavage. Thus, unlike other RIPs discussed above, the stress response triggered by PAP expression did not result in cell death (Chan Tung et al. 2008).

## 4.5 The Intrinsic Nuclease Activity of Toxins

As mentioned previously, RIPs possess many different types of activities and among them is their nonspecific DNase activity. RIPs like dianthin 30, saporin-6 and gelonin were identified to exert a specific nuclease activity on supercoiled DNA (Roncuzzi and Gasperi-Campani 1996). In the plasmid, pBR322 four specific sites of cleavage by dianthin 30 and saporin-6, and two specific sites of cleavage by gelonin were identified and mapped (Roncuzzi and Gasperi-Campani 1996). TCS has been shown to cleave the supercoiled double-stranded DNA and relaxed circular DNA to produce linear DNA (Li et al. 1991). In addition, TCS was observed to contain one calcium ion per protein molecule, suggesting a role for calcium in its endonucleolytic activity (Li et al. 1991). Stx1 was also shown to damage the single-stranded DNA by depurination (Brigotti et al. 2001). Ricin and Stx have been suggested to damage nuclear DNA in whole cells by means that are not secondary to ribosome inactivation (Brigotti et al. 2002). The non-specific degradation of DNA by RIPs can, in turn, induce apoptosis. It has been previously shown that most of the saporin-6 was found to be present in the nucleus before the onset of apoptosis (Bagga et al. 2003). Recently, an immunotoxin, StxA1-GM-CSF comprising of the catalytic domain of Stx, as the killing moiety, and GM-CSF as the cell targeting moiety showed the ability to induce apoptosis and DNA double strand breaks in different cell lines (Roudkenar et al. 2008).

## 4.6 Alternate Pathways

Though generally RIPs have been found to induce apoptosis by following the known classical pathways of apoptosis, they have also been shown to induce apoptosis through alternative pathways. In the subsequent section we highlight some studies providing evidences for alternate pathways being activated by RIPs to induce apoptosis.

### 4.6.1 PARP Activation Resulting in $NAD^+$ Depletion

PARP (poly(ADP-ribose)polymerase) is an abundant nuclear protein involved in a number of cellular processes involving mainly DNA repair and programmed cell death. PARP, in response to DNA damage, undergoes auto-modification by forming poly (ADP-ribose) polymers using $NAD^+$ (Lindahl et al. 1995). A prolonged PARP activation leads to an excessive consumption of $NAD^+$ resulting in the depletion of ATP pool (Sims et al. 1983), which has been proposed as a mechanism for DNA damage-induced cell death in many cell types (Cherney et al. 1987). It has been conclusively shown that the depletion of $NAD^+$ levels as a result of PARP-1 hyperactivation induces mitochondrial damage and apoptosis (Chiarugi

and Moskowitz 2002; Yu et al. 2002). The first evidence for the involvement of PARP activation and NAD depletion came to light in the case of ricin-induced apoptosis (Komatsu et al. 2000). It was observed that U937 cells exposed to ricin showed an increase in PARP activity and depletion of intracellular $NAD^+$ and ATP. A PARP inhibitor, 3-aminobenzamide (3-ABA), prevented the depletion in $NAD^+$ and ATP levels and concomitantly protected U937 cells from the lysis that followed the ricin treatment (Komatsu et al. 2000).

Later, some RIPs, including ricin, saporin-L2, saporin-S6, gelonin and momordin, were observed to depurinate the automodified enzyme poly(ADP-ribosylated) poly(ADP-ribose) polymerase, thereby releasing adenine from the ADP-ribosyl group (Barbieri et al. 2003). It was suggested that depurination of auto-modified PARP could result in the inhibition of DNA repair pathway as well as the availability of PARP for further ADP-ribosylation, leading to depletion of intracellular levels of $NAD^+$ thus inducing apoptosis (Barbieri et al. 2003).

### 4.6.2 Down-Regulation of Telomerase

Telomerase is a cellular reverse transcriptase which adds DNA sequence repeats, TTAGGG to the 3′ end of DNA strands in the telomere regions in all vertebrates, thus providing stability to the chromosomes. The enzyme is usually not active in normal somatic cells and is specifically activated in many malignant cells. Several protooncogenes and tumor suppressor genes either directly or indirectly have been implicated in the regulation of telomerase activity (Liu 1999). Telomerase dysfunction has been found to be a key determinant in governing the sensitivity to anticancer agents (Lee et al. 2001).

Korean ML was shown to induce apoptosis in hepatocarcinoma cells by inhibiting the telomerase activity (Lyu et al. 2002). ML induced apoptosis in both p53-positive, SK-Hep-1 and p53-negative, Hep 3B cells through down-regulation of telomerase activity. Telomerase activity in p53 positive cells was greatly reduced after 24 h of treatment with ML, whereas the telomerase activity decreased gradually in p53 negative cells (Lyu et al. 2002). Subsequently, it was observed that the inhibition of telomerase activity and induction of apoptosis resulted from decreased phosphorylation of Akt survival signaling pathways (Choi et al. 2004).

### 4.6.3 Inhibition of Histone Deacetylase

Histone deacetylases (HDACs) catalyze the removal of acetyl groups from N-terminus of histones, leading to chromatin condensation and transcriptional repression. Recently, a 30-kDa type I RIP, MCP 30 isolated from bitter melon, *Momordica charantia* seeds has been shown to induce apoptosis as a result of inhibition of HDACs (Xiong et al. 2009). Furthermore, it was found that MCP 30 could also promote acetylation of histone-3 and -4 proteins (Xiong et al. 2009).

### 4.6.4 Degradation of Cytoskeleton Proteins

Cytoskeleton proteins, e.g., actin, lamin and tubulin, provide mechanical support to the cells and hardwire the cytoplasm with the surroundings to support signal transduction. *V. album* agglutinin-I (VAA-I) induced apoptosis in eosinophilic AML14.3D10 (3D10) cells was found to be associated with the degradation of lamin B1 and activation of caspase-1, -2, -3, -4, -7, -8, -9, and -10. VAA-I induced gelsolin degradation was reversed by the pan-caspase inhibitor *N*-benzyloxycarbonyl-V-A-D-O-methylfluoromethyl ketone (z-VAD). Also, paxillin, vimentin and lamin B1 were cleaved by caspases in VAA-I-induced 3D10 cells (Lavastre et al. 2005). Moreover, treatment of purified human eosinophils with VAA-I was found to induce apoptosis, degradation of gelsolin and lamin B1, but unlike 3D10 cells, cleavage of lamin B1 and cell apoptosis was not reversed by z-VAD in eosinophils (Lavastre et al. 2005).

### 4.6.5 Nitric Oxide-Mediated Apoptosis Pathway

TCS was found to induce apoptosis by increasing the expression of inducible nitric oxide synthase (iNOS)mRNA expression and protein levels and this phenomenon was significantly inhibited when L-NIL, a specific inhibitor of iNOS, was added to the cells treated with TCS (Li et al. 2005).

## 5 Conclusion

It is now clearly evident that most RIPs induce apoptosis. Generally, the apoptosis induced by RIPs involves the caspase dependent mitochondrial pathway, and is independent of protein synthesis inhibition. The triggers include ROS, ribotoxic stress, activation of kinases and in some instances consequences of the direct enzymatic activities of RIPs. RIPs have now been acknowledged as multifunctional proteins which may account for the absence of a single common pathway for the induction of apoptosis by them.

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