

Review

Ricin and Ricin-Containing Immunotoxins: Insights into Intracellular Transport and Mechanism of action *in Vitro*

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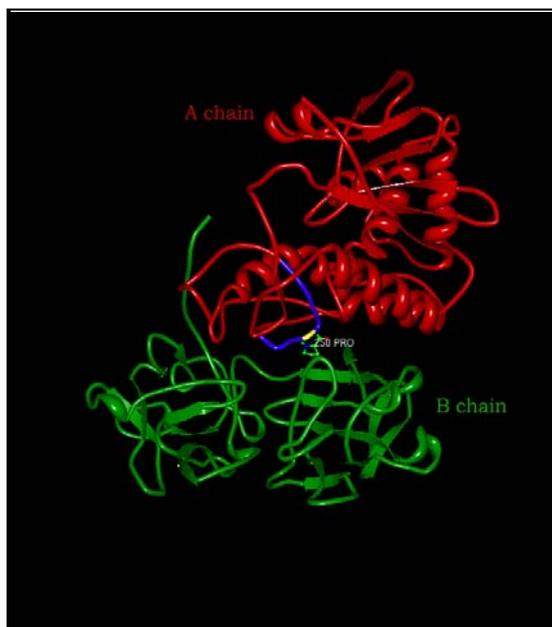
Abstract: Ricin is a type II ribosome inactivating protein (RIP) isolated from castor beans. Its high toxicity classifies it as a possible biological weapon. On the other hand, ricin linked to specific monoclonal antibodies or used in other conjugates has powerful medical applications. Ricin consists of an A-chain (RTA) that damages ribosomes and inhibits protein synthesis, and a B-chain that plays a role in binding and cellular uptake. A number of recent studies have demonstrated that ricin-induced inhibition of protein synthesis is not the only mechanism responsible for cell death. It turns out that ricin is able to induce apoptosis in different cell lines and multiple organs in animals. However, the molecular link between protein synthesis inhibition and ricin-dependent triggering of apoptotic cell death is unclear. This review describes the intracellular transport of ricin and ricin-based immunotoxins and their mechanism of action in different non-malignant and cancer cell lines. Moreover, various ricin-containing immunotoxins, their composition, medical applications and side-effects will be described and discussed. Understanding the mechanism of action of ricin-based immunotoxins will facilitate construction of effectively acting immunotoxins that can be used in the clinic for cancer treatment.

Keywords: ricin; ricin-based immunotoxins; intracellular transport; apoptosis; vascular leak syndrome

1. Introduction

Ricin is a natural, extremely potent protein toxin isolated from castor beans, the seeds of the castor plant, *Ricinus communis*. The name ricin was suggested in 1888 by Stillman who discovered that the active ingredient isolated from the castor seeds was a protein [1]. In the 1890s Paul Ehrlich found that feeding small amounts of castor seeds to mice induced immunity and provided evidence that under such conditions specific neutralizing antibodies were produced [2,3]. The structure and general mode of ricin action were elucidated by Olsnes and Pihl who established the subunit composition of ricin and found that the toxicity is a result of its catalytic action on ribosomes resulting in inhibition of protein synthesis [4]. For this reason, ricin turned out to be the very first identified RIPs (ribosome-inactivating proteins), classified as the class II of this group of protein toxins. Ricin holotoxin is a 64 kDa heterodimeric protein that consists of two polypeptide chains (A and B) joined by a disulfide bond (Figure 1). The A-chain (RTA) inhibits protein synthesis by irreversibly inactivating eukaryotic ribosomes [5,6]. The B chain (RTB) is a lectin, which binds to β -1,4-linked galactose residues [7]. Such residues are widely present on mammalian cell surface glycoproteins and glycolipids, therefore most cell types bind significant amounts of ricin.

Figure 1. Crystallographic structure of ricin. The enzymatically active subunit (A-chain) is in red, whereas the binding domain (B-chain) is in green. The hydrophobic region of ricin A-chain (Val245 to Val256) is indicated in blue, proline P250 is in yellow. The structure has been obtained from the PDB protein data bank (code 2AA1).



Due to its high toxicity and ready availability, ricin has been listed as a Category B Select Agent by the National Institutes of Health and the Centers for Disease Control and Prevention [8]. For this reason a specific vaccine against ricin, RiVax produced by recombinant technology has been developed [9]. Results of the phase I human trial established that the immunogen was safe and induced antibodies anticipated to protect humans from ricin exposure [10]. A phase IB clinical trials showed that alhydrogel-adsorbed RiVax (RiVax/alum) is safe and well tolerated, and induces higher ricin

neutralizing antibody levels in humans than adjuvant-free RiVax [11]. Although ricin can be considered as a serious threat to human health, it has a large potential to be used as a tool in cell biology studies and in medicine. This toxin can serve as a membrane marker and as a probe in investigations of endocytosis and various intracellular pathways [12,13]. Among these intracellular pathways is retrotranslocation of proteins from the endoplasmic reticulum (ER) to the cytosol [14–16], a process that has been intensively studied in recent years as an important part of the glycoprotein folding quality control system operating in the ER [17]. In medicine ricin can be used to bring into cells different biologically active constituents as well as epitopes for vaccination purposes. As an example, adjuvant-carrier properties of ricin B-chain fused to HIV-1 antigen, p24 have been tested [18]. In these experiments, the high levels of antibodies obtained after immunization of mice demonstrated the good adjuvant-carrier properties of RTB when conjugated to a HIV structural protein. Finally, ricin can selectively kill certain cell types, such as cancer cells. It is possible that ricin and other RIPs are more toxic to malignant cells which often have a high rate of protein synthesis during proliferation. It was also suggested that increased sensitivity of tumor cells to toxins may be due to the changes in receptor concentration on their surfaces or altered intracellular transport of toxins [19]. Importantly, the potency of ricin has been combined with the specificity of various targeting moieties to yield immunotoxins (ITs).

In this review we will focus on intracellular transport of ricin and various ricin-based immunotoxins as well as on their mechanisms of action into cells.

2. Ricin-Derived Immunotoxins

Paul Ehrlich proposed that it should be possible to develop drugs that would act as “magic bullets” and kill tumor cells with high specificity [20]. A proposition for production of specific therapeutic agents was based on the idea to couple toxins to antibodies directed against cancer cells, or by conjugating toxins to growth factors (such as EGF or transferrin), hormones and lectins that bind preferentially to some cell types. The first toxin conjugates were produced by chemical coupling between the whole toxin or parts of the toxin and a binding component; more recently genetically coupled conjugates have also been produced [21]. Chemical construction of ITs utilizes reagents that crosslink antibody and toxin [22], genetic construction involves preparation of hybrid genes to produce toxin fusion proteins in *Escherichia coli* [23]. Generally disulfide [24] and thioether bonds [25] are used to form chemical coupled ITs. Disulfide bonds can be reduced inside the target cells, thereby releasing the toxin exerting its inhibitory activity. This type of bond has been used to construct ITs containing ricin A-chain [26]. Thioether bonds are less useful, since mammalian enzymes cannot hydrolyze them and thioether-linked ITs are not cytotoxic to target cells [27]. However, the intact toxin ricin can be linked to antibody through such a bond. When the antibody is bound to the toxin through the RTB, the toxic A-chain can be released in the target cell by reduction of the interchain disulfide bond [28]. The antibodies that have been most widely used in immunotoxins are murine monoclonal antibodies (MAb) belonging to the IgG isotype [29]. In the 1990s instead of using single Fv fragment of antibodies, a new type of disulfide-stabilized Fv (dsFv) was designed in which the light and heavy chains were held together by a disulfide bond. Compared with the single-chain toxins, dsFv-based immunotoxins do not aggregate and have remarkably higher stability at 37 °C, for several days or longer [30]. This is especially important in the treatment of solid tumors.

In the case of ricin-based immunotoxins, in the beginning antibodies were conjugated directly to the ricin A-chain, whereas the B-chain was removed [31]. However, ricin may act rather nonspecifically on different cell types. Even without its binding domain, RTA was taken up nonspecifically by macrophages and hepatic nonparenchymal Kupffer cells [32]. It became clear that this uptake was due to the carbohydrate residues present on ricin A-chain, binding to mannose receptors on the liver cells [33]. Chemical deglycosylation resulted in production of deglycosylated ricin A-chain (dgA) immunotoxins, that had significantly prolonged lifetimes in mice, leading to improved therapeutic index [32,34]. These half-lives were improved even further, when the disulfide bond between MAb and the toxin was formed in a hindered fashion using the derivatizing agent 4-succinimidylloxycarbonyl- α -methyl- α -(2-pyridyl)dithio)toluene (SMPT) [26]. However, many ricin immunotoxins in which the binding moiety was conjugated to the whole toxin were more efficient than when it was bound to the A-chain alone [35]. The B-chain may be important for intracellular routing, and it seems to play an important role in the translocation of the A-chain through a cellular membrane to the cytosol [36]. Moreover, it was observed that addition of the ricin B-chain to the immunotoxin resulted in the protection of RTA from proteolytic processing by cathepsins [37]. This protective role of RTB was partially responsible for the higher toxicity of immunotoxins containing ricin holotoxin in comparison to immunotoxins containing only RTA. However, a specificity improvement of immunotoxins containing ricin holotoxin was necessary. The two galactose-binding sites of native ricin were blocked by chemical modification with affinity ligands, creating an altered toxin, called blocked ricin (bR). These sites were blocked with ligands prepared by chemical modification of glycopeptides containing triantennary N-linked oligosaccharides [28]. Blocked ricin had at least a 3500-fold lower binding affinity and was more than 1000-fold less cytotoxic than native ricin for Namalwa cells (a Burkitt's lymphoma line) but maintained the translocation function of the B-chain and the catalytic activity of the A-chain [28]. Compounds known to sensitize cells to some of the toxins may also potentiate the action of immunotoxins [38]. Horssen *et al.* reported that the action of a recombinant ricin A-chain conjugate is potentiated by chloroquine, and proposed that this could also be applied *in vivo* [39].

Today, a large number of laboratories are working with toxin conjugates and immunotoxins. They are being tested clinically on solid tumors and especially on refractory haematological malignancies [40–43]. A regional therapy of recurrent malignant brain tumors with transferrin-CRM107, a conjugate of human transferrin and a genetic mutant of diphtheria toxin (CRM107) that lacks native toxin binding has been tested [42]. It was reported that, at least a 50% reduction in tumor volume on magnetic resonance imaging occurred in 9 of 15 patients who could be evaluated (60%), including two complete responses [42]. In refractory haematological malignancies, malignant cells are often intravascular and more accessible to immunotoxins, including hairy cell leukemia (HCL), Hodgkin's lymphoma, T-cell lymphoma and chronic lymphocytic leukemia (CLL) [44].

Hodgkin's lymphoma (HL) has been demonstrated to be an excellent target for ITs because high concentrations of lymphocyte activation markers such as CD25 and CD30 are expressed on Hodgkin and Reed-Sternberg (H-RS) cells. Several ITs against these antigens have shown potent antitumor effects against H-RS cells *in vitro* and in different HL animal models [45–47]. Ricin immunotoxins against CD25 (RFT5-SMPT-dgA) or CD30 (Ki-4.dgA)-positive Hodgkin's lymphoma have also been exploited in preclinical and clinical studies. The objectives of the phase I trials were to determine the maximum tolerated dose, the dose-limiting toxicities, pharmacokinetics, and antitumor activity. The

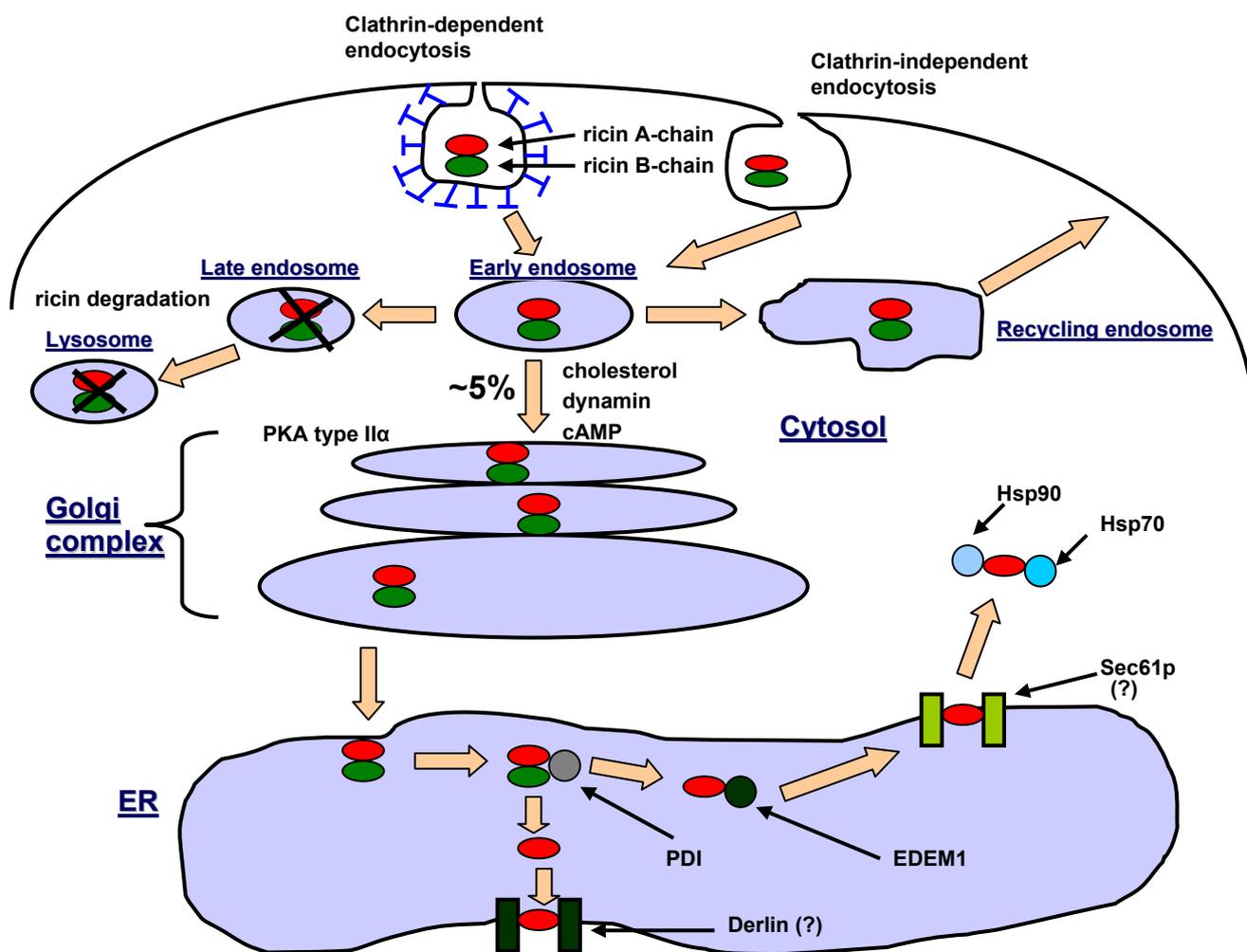
partial remission in clinical studies was 2/15 in the CD25 trial and 1/17 in the CD30 trial [46,48]. Ki-4.dgA was less tolerated than other ITs of this type, possibly due to the low number of CD30⁺ peripheral blood mononuclear cells, and in part due to binding of the IT to soluble CD30 antigen and the resulting circulation of ITs/CD30 complexes. In the next phase I/II clinical studies with these immunotoxins the partial response was 2/27 and 1/15 for RFT5.dgA and Ki-4.dgA respectively [47]. Moreover, different ricin-based immunotoxins against non-Hodgkin lymphomas (NHL) expressing CD5, CD19, and CD22 antigens have been tested in phase I, II and III clinical trials [49,50]. However, the phase III trials with anti-B4-blocked ricin (anti-B4-bR), directed against the CD19 antigen finally fail to support a role for this immunotoxin as consolidative therapy after bone marrow transplant in patients with B-cell lymphoma [50]. On the other hand, it has been demonstrated, that an immunotoxin constructed with RFB4, a murine anti-CD22 monoclonal antibody, and the deglycosylated A-chain of ricin showed activity at safe doses in patients with non-Hodgkin lymphoma and in children with acute lymphoblastic leukemia [51,52]. In clinical testing, RFB4–dgA produced major responses in up to 30% of the patients, although the dose was limited by vascular leak syndrome (see below). The use of a cocktail of two ricin A-chain immunotoxins (RFB4-SMPT-dgA and IgG-HD37-dgA) in NHL patients was also reported. However, the clinical outcome was moderate as only 2 of 22 patients showed a partial response [53]. The potential of HD37-dgA immunotoxin to aggregate suggests one possible basis for problems in these clinical trials. Thus, non-aggregate-forming formulations for these ITs should be pursued prior to future clinical probes. Intact ricin has been suggested to be useful for the regional treatment of cancers, such as ovarian carcinomas [54]. However, in some studies ricin has been linked to the monoclonal antibody L6, which recognizes a determinant that is expressed on carcinomas of the lung, breast, colon, and ovarian and is present only at trace levels in normal tissues [55]. Other data suggested that L6-ricin may be useful for *in vitro* purging of autologous bone marrow from patients with solid tumors and marrow involvement and for *in vivo* regional therapy of L6-positive carcinomas [54]. A conjugate 454A12-rRA consisting of a monoclonal antibody against the human transferrin receptor and recombinant ricin A-chain was designed, and the immunotoxin was tested in a pilot study in eight patients with leptomeningeal spread of systemic neoplasia [56]. In four of the eight patients, a greater than 50% reduction of tumor cell counts in the lumbar CSF (ventricular cerebrospinal fluid) occurred within 5 to 7 days after the intraventricular dose of 454A12-rRA [56]. Goldmacher *et al.* reported a potent anti-CD38-bR immunotoxin capable of killing human myeloma and lymphoma cell lines [57]. The potent specific cytotoxicity of this immunotoxin for tumor cells was combined with its low cytotoxicity for non-malignant cells. N901-bR immunotoxin targets CD56, present on SCLC (small-cell lung cancer), and cells of neuro-ectodermal origin. It has been demonstrated that it can be a potential therapeutic for SCLC [58], what was also confirmed in clinical studies [59]. Recently, a novel approach for ricin-based tumor therapy was tested *in vitro*. According to the design, RTB was expressed by an adenovirus vector targeting cancer tissues while RTA was applied in the form of a purified protein [60]. RTA was concentrated in the cancer tissue where RTB was located due to the strong affinity between RTA and RTB. RTA entered cancer cells with the help of RTB, and exerted its cell-killing function.

3. Intracellular Transport of Ricin

Cell-bound ricin is taken in by endocytosis. Since it binds to a variety of cell surface components, all over the mammalian cells, it is able to utilize different endocytic mechanisms (Figure 2). Clathrin-dependent endocytosis was first observed to be used for ricin internalization by Vero cells (African Green Monkey kidney cells) [61]. However, ricin endocytosis can also occur by clathrin- and caveolae-independent mechanisms [13,62], and ricin is endocytosed even when cholesterol was extracted from the membrane [63].

After endocytosis, toxin is initially delivered to early endosomes, from where the majority of the endocytosed toxin recycles back to the cell surface, starts to be degraded and proceeds to late endosomes/lysosomes where further degradation is conducted [64–66] (Figure 2). A minor fraction (~5%) of ricin is transported from early endosomes to the trans-Golgi network (TGN) (Figure 2), as quantified by electron microscopy [67,68]. Strong indications that transport to the Golgi apparatus was important for ricin intoxication came from studies with the Golgi-disturbing drug brefeldin A [69,70], which completely inhibited ricin transport to the Golgi and simultaneously prevented cell intoxication. Importantly, this was observed only in cells where the Golgi was sensitive to brefeldin A [70]. Moreover, an excellent assay for analyzing the intracellular transport of ricin was established by Rapak and coworkers [71]. A short sequence that functions as a tyrosine sulfation site in rat cholecystokinin precursor was added to the C-terminal end of the RTA (ricin-A-sulf-1). Sulfotransferase is a Golgi-specific enzyme catalyzing addition of sulfate to proteins containing the sulfation sites [72]. When mutant ricin sulf-1 is added to cells in the presence of $\text{Na}_2^{35}\text{SO}_4$, the A-chain becomes radioactively labelled due to the sulfotransferase in the TGN, and thus sulfation of ricin A-chain indicates transport to the Golgi apparatus. In cells expressing a mutant of dynamin that blocks clathrin- and caveolae-dependent endocytosis, ricin was still internalised and transported to late endosomes [73]. However, the transport of ricin to the Golgi apparatus was blocked, demonstrating a dynamin-dependent transport route from endosomes to the Golgi apparatus (Figure 2). Furthermore, the cells were protected against ricin intoxication. The latter confirms the importance of transport to the Golgi for ricin toxicity. The Rab9-dependent pathway is a well-characterised route to the TGN, which transports the mannose-6-phosphate receptor (M6PR) from late endosomes. To investigate whether ricin transport to the Golgi apparatus depends on Rab9, cells with inducible synthesis of a dominant-negative mutant Rab9, Rab9S21N were used [74]. Under these conditions transport of M6PR was inhibited, but ricin still intoxicated cells, and ricin sulfation was not changed. Moreover, ricin transport is independent of Rab7 [75], Rab-11 and clathrin [74], but is affected by changes in the cholesterol level. Depletion of cholesterol by addition of methyl- β -cyclodextrin inhibits Golgi transport [76]. Endosome to Golgi transport of ricin is also regulated by signalling in the cell. It has been demonstrated that the presence of the Golgi-localised RII α , the regulatory subunit of protein kinase A (PKA), can contribute to increased endosome to Golgi transport of ricin upon increased levels of cAMP [77] (Figure 2).

Figure 2. Intracellular transport of ricin. Ricin endocytosis can occur both by clathrin-dependent and clathrin- and caveolae-independent mechanisms. From early endosomes the majority of the endocytosed toxin recycles back to the cell surface, starts to be degraded and proceeds to late endosomes/lysosomes where further degradation is conducted. A minor fraction (~5%) of ricin is transported from early endosomes to the trans-Golgi network (TGN), circumventing late endosomes. As indicated, dynamin, cholesterol, cAMP and PKA type II α have been implicated in endosomes to Golgi transport of ricin. Ricin is further transported to the endoplasmic reticulum (ER). In the ER, protein disulfide isomerase (PDI) catalyses reduction of the internal disulfide bond connecting the ricin A- and B-chains. It is likely that enzymatically active A-chain is transported to the cytosol by utilizing Sec61p translocation channel. Probably, ricin can also be transported to the cytosol through Derlin translocation channel. ER degradation enhancing α -mannosidase I-like protein, EDEM1 is directly involved in ricin transport to the cytosol. Hsc70 and Hsc90 cytosolic chaperone machines are involved in RTA folding after retrotranslocation to the cytosol. For references, see the main text.



After arrival in the Golgi complex, ricin is further transported retrogradely to the endoplasmic reticulum (ER) (Figure 2). One mechanism for retrograde transport from the Golgi apparatus to the ER involves movement via coatamer protein I (COPI)-coated vesicles. These vesicles are responsible for

retrograde transport of proteins containing the sequence KDEL or related sequences. Ricin does not naturally have such a sequence, but the addition of KDEL to the C-terminus of RTA increased the cytotoxicity of free RTA [78] and reconstituted holotoxin [13,79]. However, these data do not explain the mechanism of ricin transport to the ER. Interestingly, ricin can bind to calreticulin, a KDEL-tagged protein [80] (Figure 2). It was found that the toxin binds to calreticulin via the B-chain and that terminal galactose residues present on calreticulin are crucial for this interaction. Therefore, it was hypothesized that calreticulin can act as a retrograde carrier for ricin transport from the Golgi to the ER, transport that occurs through binding to KDEL receptors present in COPI-coated vesicles. However, calreticulin does not seem to be a main carrier for ricin, as calreticulin deficient cells remained sensitive to ricin [80]. Although, ricin has never been visualized in the ER by electron or immunofluorescence microscopy [12], it is possible to monitor its transport to this compartment by using mutant ricin with a glycosylation tag [71]. This glycosylation tag consists of three overlapping glycosylation sites, supportive of ER-specific Asn-modification with carbohydrate. In addition, this molecule contains a sulfation tag (described above) and has been called ricin-A-sulf-2 [71]. The second, largely uncharacterized, Golgi-to-ER transport pathway is COPI-independent, controlled by the Rab6 GTPase [81]. It was reported that a dominant negative mutant of Rab6a does not protect against ricin, and furthermore that the cells transfected with a combination of this mutant and a temperature-sensitive mutant of ϵ -COP incubated at the non-permissive temperature, also were not protected against ricin [82]. This lead to the suggestion that ricin enters the ER by a pathway that is independent of both COPI and Rab6a. However, this interpretation was complicated by the finding that there seems to be a compensatory mechanism responsible for ricin transport to the ER in cells with temperature-sensitive ϵ -COP at the non-permissive temperature [83]. Whether a combination of pathways could be responsible for ricin transport at physiological conditions is still an open question.

Translocation of enzymatically active RTA from the ER to the cytosol can occur after reduction of the internal disulfide bond connecting the ricin A-and B-chains (Figure 2). This reaction is catalyzed by the protein disulfide isomerase (PDI) [84], a crucial ER foldase that is responsible for formation, breakage and isomerisation of disulfide bridges.

In its transport to the cytosol ricin A-chain may utilize the Sec61p ER translocation channel (Figure 2) normally involved in transport of newly synthesized proteins into the ER, but also, at least partially, responsible for retrotranslocation of unfolded/misfolded proteins back to the cytosol for degradation. This last process is a crucial step of ERAD (ER-associated degradation) [85]. It had been proposed that ricin may utilize the ERAD pathway in its transport to the cytosol, since misfolded proteins in the ER translocate across the membrane and are degraded by the cytosolic proteasome [86]. However, data demonstrating that ER degradation enhancing α -mannosidase I-like protein, EDEM1, which accelerates ERAD of misfolded glycoproteins [87,88] is directly involved in ricin transport to the cytosol [14] (Figure 2) finally conformed this hypothesis. Close to its C-terminus, the ricin A-chain contains a 12-amino-acid (Val245 to Val256) hydrophobic region (Figure 1), which is hidden in the holotoxin but becomes exposed upon A and B chain dissociation in the ER. It has been demonstrated that substitution of proline 250 into alanine (P250A) in this region results in a dramatic decrease in RTA_{P250A} cytotoxicity in Vero and HEK293 (human embryonic kidney) cells [15,89] without interfering with the ability of the A-chain to reassociate with the B-chain or impairing the enzymatic activity of the A-chain [89]. Decreased cytotoxicity of P250A ricin was explained by increased

endosomal-lysosomal degradation of the toxin as well as reduced transport from the ER to the cytosol [15]. Moreover, transport of modified RTA to the cytosol, in contrast to wild-type RTA, appeared to be EDEM1-independent and the interactions between EDEM1 and RTA_{P250A} were reduced. Since the introduced mutation alters the secondary structure of RTA into a more helical structure, it was concluded that EDEM1 protein recognition might be determined by the structure of the ERAD substrate [15]. It has also been demonstrated that C-terminal hydrophobic sequence within RTA was critical for transport across the ER membrane when yeast was used as a model to study the intracellular trafficking of RTA [90]. Moreover, it has been shown that N-glycosylation that occurs on asparagines 10 and 236 in RTA promotes transport of RTA out of the ER [90]. Ricin interacts with Sec61 α protein, the main component of the Sec61p ER translocation channel. This was demonstrated by co-immunoprecipitation studies [13,14] and found in studies with isolated yeast ER-derived microsomes [91]. Importantly, it was reported that retrograde translocation of ricin A-chain was dependent on a functional Sec61p complex [91]. However, recent results show that ricin transport to the cytosol might be also dependent on another translocon, formed by Derlin proteins [92] (Figure 2). Whether ricin A-chain can use more than one ER translocation channel and whether different translocons can be used at the same time or separately, depending on specific ER resident proteins or uncharacterised membrane carriers remains as an open question. Interestingly, it has been demonstrated that RTA binds directly to the membrane surface in a temperature-independent manner but that the physiologically relevant temperature of 37 °C is required for specific changes in toxin structure and exposure to the membrane nonpolar lipid core. The membrane-bound ricin A-chain loses some α -helical structures undergoing the conformational change that exposes its C-terminal region to the membrane interior [93]. Such insertion into the lipid bilayer might represent an early step in RTA translocation through the ER membrane. It is possible that the P250A ricin mutant described above possessing an elevated level of α -helices is unable to undergo additional conformational changes allowing it to be stably inserted into the ER membrane. This might be another limiting step in P250A RTA retrotranslocation to the cytosol.

An introduction of an internal disulfide bond in the ricin A-chain decreased the cytotoxicity of the resulting mutant holotoxin, suggesting that unfolding is necessary for retrotranslocation [94]. Therefore, in the cytosol RTA must refold into its biologically active conformation to inactivate the ribosomes. An *in vitro* study demonstrated that RTA was able to refold on its own into the correct conformation in the presence of intact ribosomes [95], but dodecaribonucleotide containing the substrate GAGA tetraloop or naked rRNA were unable to reactivate the partially unfolded toxin. It appears therefore, that ribosomal proteins alone or in conjunction with rRNA can facilitate ricin A-chain refolding. Moreover, it has recently been demonstrated that Hsc70 and Hsp90 cytosolic chaperone machines are involved in RTA folding after retrotranslocation to the cytosol [96] (Figure 2). Once the A-chain reaches the cytosol, it must be able to avoid being degraded by the proteasomes. This could partially be due to the fact that that ricin A-chain contains very few lysine residues, the crucial amino acid for attachment of polyubiquitin chains that trigger degradation by the proteasomes [97]. It has been proposed that this mechanism represents an evolutionary strategy to allow ricin to subvert ERAD [98]. However, it is known that, despite low lysine content, ricin is partially degraded by the proteasomes and this degradation can be inhibited by proteasome inhibitor lactacystin [13]. Data

showing that the introduction of additional lysines into RTA reduces its cytotoxicity by increasing the extent of ubiquitin-mediated proteasomal degradation [98] support this observation.

4. Intracellular Trafficking of Ricin-based Immunotoxins

Immunotoxins utilize a variety of cell-specific binding moieties to gain entry into the cell, thus the comparative effects of different trafficking pathways are difficult to evaluate. However, understanding these pathways would be necessary for development of more potent immunotoxins for targeting cancer cells.

Generally immunotoxins enter cells after binding to plasma membrane epitopes. The endocytic pathway involved is likely to be dependent on the epitope and the valency of the conjugate [61]. It was demonstrated that such parameters as epitope topography [99], antibody affinity [100], and rate of intracellular degradation [99] are critical in determining the cytotoxic efficiency of ricin containing immunotoxins. A large number of ITs has been constructed with antibodies that are likely to be internalized by endocytosis from coated pits, however some data indicate that they can be also internalized by clathrin-independent endocytosis, and that toxin taken up by the different pathways is able to intoxicate the cells [101]. This is in agreement with the idea that the different endocytic mechanisms merge in early endosomes. Since ricin is transported to the Golgi complex and from there to the ER, the new binding moiety that replaces the B-chain may have the same ability. Retinoic acid is a transcription activator that defines certain cell fates during development and has a potential in cancer treatment by inducing tumor cell differentiation [102]. It has been demonstrated that it alters the morphology of the Golgi apparatus and protects HeLa (human cervical cancer) cells, Vero and U257 (human leukemic monocyte) cells from ricin toxicity [103,104]. However, it significantly potentiates the efficiency of ricin immunotoxins [104]. This phenomenon may be explained by the fact that native ricin contains a B-chain that is important for routing the toxin through the Golgi and then the ER to reach the cytosol [80]. Immunotoxins that lack a B-chain are much less potent, apparently due to a deficiency in intracellular routing. It is believed that ricin A-chain immunotoxins in the absence of a B-chain may recycle through the trans-Golgi back to the cell surface repeatedly, but that retinoic acid may allow trans-Golgi to *cis*-Golgi movement of ricin A-chain ITs. Thus, by influencing the Golgi, retinoic acid may inhibit the efficient B-chain mechanism of Golgi transport resulting in decreasing native ricin toxicity while potentiating the inefficient ricin A-chain immunotoxin routing. These results indicate a potential role for retinoic acid in cancer therapy, but also put under further discussion the role of ricin B-chain and its modifications in intracellular transport of ricin-based immunotoxins. Timar *et al.* analysed the mode of entry and subsequent routing of an immunotoxin Fib75-SS-ricin A-chain in a human bladder carcinoma cell line with and without the presence of ricin B-chain as a potentiating agent [105]. Fib75 is the monoclonal antibody against human HLA class I-related plasma membrane antigen. It has been demonstrated that ricin B-chain potentiated the toxicity of this immunotoxin by two orders of magnitude and also significantly increased the rate of protein synthesis inhibition [105]. One of the major differences observed between the intracellular trafficking of immunotoxin with and without ricin B-chain was the appearance of ricin chains in the Golgi complex in cells treated with the immunotoxin containing B-chain. The route and rate of internalisation of an immunotoxin is largely determined by the nature of the antigen to which it binds. However, binding of the Fib75-SS-ricin A-B to cell surface galactose-containing receptors was probably dominating,

considering the fact that the binding affinity of the monoclonal antibody was low [105]. A stronger inhibition of protein synthesis induced by Fib75-SS-ricin A-B in comparison to the immunotoxin containing only the A-chain can also be explained by the fact that B-chain protects A-chain immunotoxin from lysosomal delivery and degradation [36,106]. Interestingly, it was even reported that ricin B-chain is able to convert noncytotoxic antibody-ricin A-chain conjugates into a potent and specific cytotoxic agent [107,108]. Moreover, interesting studies with the immunotoxin consisting of ricin A-chain linked to the monoclonal antibody M-T151, recognising the CD4 antigen present on the human T-lymphoblastoid cell line CEM should be also mentioned [109]. The authors showed that the addition of ricin B-chain to CEM cells treated with M-T151-ricin-A-chain enhanced cytotoxicity only eight-fold, indicating that isolated B-chain potentiated the action of the A-chain less effectively than it did as an integral component of an intact ricin immunotoxin [109]. In this system, the galactose-binding sites of the B-chain contributed to cell killing, regardless of whether isolated B-chain was associated with the A-chain immunotoxin or were present in blocked or non-blocked form as part of an intact ricin immunotoxin. Moreover, these findings suggested that the blocked ricin immunotoxin may become unblocked after binding to the target antigen to re-expose the cryptic galactose-binding sites [109].

In cell killing-based therapies it is important to identify the death pathways induced by the cytotoxic agent. In most cases little is known about the uptake and intracellular transport of the immunotoxins. Further investigation of the toxins themselves as well as of the intracellular fate of the toxin conjugates is likely to broaden our knowledge and pave the way for further improvement of these molecules.

5. Action of ricin and Corresponding Immunotoxins on Ribosomes and Apoptosis

The first experiments describing enzymatic activity of ricin A-chain were performed in cell-free systems by Sjur Olsnes and Alexander Phil at the beginning of 1980s [110]. They showed that the ribosomal 60S subunit was affected by RTA with a turnover number (K_{cat}) of $1,500 \text{ min}^{-1}$. The fact that ribosome inactivation occurred in simple buffers suggested that there are no cofactor requirements for RTA activity. The general mechanism of the enzymatic modification of ribosomes was discovered by Endo and Tsurugi [5,6]. It has been demonstrated that ricin A-chain has N-glycosidase activity; it specifically removes an adenine residue (A4324) from a GAGA sequence in an exposed, strictly conserved loop at the top of a stem in 28S rRNA, the so-called sarcin-ricin domain (SRD). This loop is part of the recognition/binding site for the eukaryotic elongation factor 1 (eEF-1) and the eukaryotic elongation factor 2 (eEF-2) complexes [7,111,112]. The modified ribosomes are, therefore, unable to support protein synthesis [113]. It is likely that the action of RTA alters the dynamic flexibility of the ribosome, especially the flexibility necessary for the transition between the pre- and posttranslocational states of the elongation cycle [114]. RTA is able to depurinate naked 28S rRNA with an identical specificity to that in native ribosomes [115]. However, the presence of ribosomal proteins is required for efficient catalysis, possibly by inducing a conformation in the SRD favourable for the catalysis reaction. Moreover, several studies have now established the importance of the ribosomal stalk structure in facilitating the depurination activity and ribosome specificity of ricin [116–118]. Based on the interactions between RTA and stalk proteins a two-step binding model is proposed for the electrostatic interactions of ricin A-chain with ribosomes [118,119]. Interestingly, not only conformational changes in the rRNA, but also in the RTA are important for efficient inhibition of protein synthesis. It has recently been demonstrated, that the flexibility of the α -helix (residues 99–106) of RTA is responsible for

modulating the depurination activity of ricin A-chain [120]. It was suggested that the flexibility of the α -helix could affect the side chain orientation of Glu-177, which is critical for the depurination activity of ricin [121,122]. Thus, a conserved α -helix might be considered as a potential target for the prevention and treatment of RIP poisoning.

For many years inhibition of protein synthesis induced by ricin A-chain was considered as the main reason for subsequent death of ricin-treated cells. However, many reports demonstrate that ricin toxicity results not only from inhibition of protein synthesis but also from cell apoptosis, and release of cytokine inflammatory mediators [123–127]. One of the earliest reports that ricin is capable of inducing cell death by apoptosis came from studies carried by Griffiths *et al.* in 1987 [124]. They found that ricin was able to cause apoptotic changes in lymphoid tissue and rat intestine and that the morphology of cells in para-aortic lymph nodes and Peyer's patches of rats injected with ricin resembled that of cells undergoing apoptosis characterized by the formation of apoptotic bodies. Ricin-induced programmed cell death or apoptosis has been studied very intensively, since recent studies have demonstrated that incubation of different cell lines with ricin results in cell death associated with typical apoptotic changes such as membrane blebbing, chromatin condensation, and oligonucleosomal DNA fragmentation [128–131].

It has been established that induction of apoptosis can occur via two major pathways, extrinsic, and intrinsic. The extrinsic or receptor-mediated pathway recruits caspase-8 or -10 as the initiator caspase, whereas in the intrinsic or mitochondrial pathway, which is activated by a variety of cellular types of stress and cytotoxic drugs, caspase-9 is the initiator caspase [132]. These caspases can directly or indirectly cleave effector caspases such as caspase-3, leading to apoptosis [133].

It is believed that ricin triggers apoptosis mainly through the cellular stress-induced mitochondrial pathway. Most of the studies report a loss in mitochondrial membrane potential, rapid release of cytochrome *c*, activation of caspase 9 and caspase-3 (but not caspase-8), DNA fragmentation and a marked increase in the production of reactive oxygen species (ROS) in cells [131,134–136]. Activation of caspase-3 leads to cleavage of a number of important cellular regulators, including PARP, poly(ADP-ribose) polymerase, responsible mainly for the DNA repair and cell death regulation. Cleavage of PARP is an important indicator of apoptosis, and ricin-dependent PARP cleavage has been observed in different cell lines including HeLa [135] or U937 cells [137]. The prolonged activation of PARP is known to lead to the poly-(ADP-ribosyl)ation of multiple substrates and depletion of NAD^+ and ATP [138]. The kinetics of DNA fragmentation in ricin-treated U937 cells correlated with the depletion of intracellular NAD^+ and ATP. Moreover, NAD^+ and ATP depletion were reduced, and cells were protected from apoptosis, by treatment with the PARP inhibitor 3-aminobenzamide (3-ABA) [137]. Barberi *et al.* showed that RIPs directly depurinate auto-modified PARP releasing adenine from the ADP-ribosyl group [139]. It is known that the auto-modification of PARP is required for the base excision repair mechanism of a DNA repair pathway. It is possible 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 [139]. Moreover, ricin has been reported to inhibit the repair of DNA single strand breaks induced by treatment of HUVEC-C (human umbilical vein endothelial) or U937 cells with oxidative or alkylating agents [140]. Importantly, the ricin concentration used to inhibit DNA repair was not sufficient to mediate direct DNA damage (see below), or to mediate total protein

synthesis inhibition. In contrast, cycloheximide was shown to effectively inhibit protein synthesis in HUVEC but did not inhibit the repair of DNA caused by hydrogen peroxide. Thus, the protein synthesis inhibitory activity of ricin could be dissociated from inhibition of repair of DNA single strand breaks [140].

For almost two decades it was assumed that RIPs act only on rRNA within ribosomes [5,6]. Studies published in the 1990s suggested however that the intracellular targets of type II RIPs may not be limited only to rRNA [141]. Deproteinized (naked) RNA, synthetic oligoribonucleotides, nuclear and mitochondrial DNA, polyA, tRNA and viral nucleic acids were reported to be depurinated by purified type II RIPs. Brigotti *et al.* [142] working on HUVEC-C cells found that damage to nuclear DNA can be induced by ricin, moreover their data suggested that the early DNA damage, observed in parallel or after the arrest of protein synthesis in HUVEC-C treated with ricin was not a consequence of apoptosis but results from direct action of ricin on DNA [142]. Li and Pestka [143] suggested that although the α -sarcin/ricin loop, ricin and other RIPs may act to mediate rRNA damage through increased expression and activation of host RNases. Toxin-mediated rRNA depurination at multiple sites may be sufficient to activate a ribosome-bound serine/threonine kinase, double-stranded RNA-activated protein kinase (PKR), which may then participate in triggering the ribotoxic stress response (see below) [144].

Ricin-induced caspase-3 activation also results in cleavage of DNA fragmentation factor (DEF), which has been considered as major nuclease responsible for DNA fragmentation [145]. DEF is composed of two subunits of 40 and 45 kDa, termed DEF40 (CAD) and DFF45 (ICAD) respectively. Cleavage of DFF45/ICAD by caspase-3 is required for DNA fragmentation by DFF40/CAD [136]. Both ICAD and CAD were cleaved with kinetics similar to caspase-3 activation [136]. Involvement of CAD in ricin-induced DNA fragmentation in HeLa cells has been confirmed, indicating maximum fragmentation of 70% after 24 h treatment with ricin (1 μ g/mL) [136].

It has been recently demonstrated that human BAT3 (HLA-B-associated transcript 3), a presumed apoptotic regulator present in the nucleus, is specifically involved in ricin-induced cellular changes associated with apoptotic cell death [136]. Co-immunoprecipitation and confocal microscopy techniques supported a direct interaction between BAT3 and ricin A-chain. BAT3 possesses a canonical caspase-3 cleavage site. It has been demonstrated that BAT3 can be a substrate for caspase-3, producing the 131 amino acid C-terminal fragment of BAT3, designed as CTF-131. Importantly, CTF-131, but not BAT3 mediates direct ricin-induced apoptotic morphological changes such as: cell rounding, nuclear condensation, and phosphatidylserine exposure. However, the CTF-131 fragment did not induce DNA fragmentation, suggesting that signalling through additional apoptotic pathways other than BAT3 C-terminal fragment generation may also contribute to apoptosis in ricin treated cells [136]. It is not clear how BAT3 regulates apoptosis, but it has recently been shown that BAT3 can interact with the mitochondrial intramembrane protein AIF (apoptosis-inducing factor) [146]. BAT3 regulates its stability by blocking proteosomal degradation and also induces nuclear translocation of AIF. BAT3 and AIF initially interact in the cytoplasm, where AIF may regulate caspase activation, but it also is involved in chromatin condensation and DNA fragmentation [146]. Several reports suggest an involvement of reactive oxygen species (ROS), upstream of caspase-3 activation in signal transduction pathways leading to apoptosis. These pathways may be regulated by the changes in the oxidation status of the proteins involved in apoptosis signalling [147,148]. It has

been demonstrated that in ricin-treated HeLa cells levels of ROS were increased and levels of free radical scavenger glutathione were decreased [135]. These changes were correlated with maximal caspase-3 activation in the cells. Some data suggests that ROS production might be a consequence of calcium signalling [149], since it has been demonstrated that ricin induces a rapid elevation of cellular calcium levels in hepatoma cells [148].

It has been considered that not only caspases, but multiple proteases with different preferences for apoptotic substrates participate in the cellular stress-induced mitochondrial pathway. Komatsu *et al.* [131] demonstrated that a specific inhibitor of serine proteases, DCI (3,4-dichloroisocoumarin) inhibited cell death, DNA fragmentation and suppressed ricin-induced nuclear morphological changes in U937 cells. Since DCI did not affect the elevation of caspase-3 or caspase-6 activities, its intracellular target may be distinct from those of caspase-related proteases. It was speculated that DCI-inhibitable proteases may act at a site down-stream of caspase-3, or on a separate parallel signal transduction pathway that is independent of caspase-3. Thus, both caspases and serine proteases may be involved in a common pathway of apoptotic cell death induced by ricin [131].

Other important proteins which are believed to play a critical role in determining cell fate are Bcl-2 family proteins [150,151]. They can be classified into two groups according to their opposing functions: anti-apoptotic, that includes Bcl-2, Bcl-xl, Bcl-w and others, and the pro-apoptotic such as Bak, Bax, Bok. It has been demonstrated that over-expressing the Bcl-2 protein could partially prevent cell death induced by ricin in hepatoma cells, BEL7404 [150]. It is possible that overproduction of Bcl-2 can inhibit ricin-induced cell death through titrating the function of its pro-apoptotic homologues, such as Bax. However, it was also shown that ricin does not alter the expression of the Bcl-2 family members in U937 cells [152].

Immunotoxins can eliminate tumor cells not only by inhibition of protein synthesis but also by inducing apoptosis [153,154]. It has been reported that the effect of ricin- immunotoxins on apoptosis, similarly to ricin, can be related to activation of caspase-3 [155]. More data describing this process come from studies performed with anti-p185^{HER-2}-RTA immunotoxin [156]. This immunotoxin was prepared by chemical conjugation of anti-HER-2 monoclonal antibody to RTA. Overexpression of HER-2 receptor has been detected in gastric cancer, and therefore HER-2 could be a useful target for the treatment of this type of cancer. It has been demonstrated that anti-p185^{HER-2}-RTA inhibited cell growth, and the anti-cancer effect of this immunotoxin might be correlated with the activation of caspase-9 and caspase-3, but also with downregulation of COX-2 (cyclooxygenase-2) and NF-κB/p65 [156]. Nuclear factor-κB is a transcription factor for the inducible expression of genes, including COX-2, which may be related to cell proliferation [157]. NF-κB might also protect cancer cells from apoptosis by activating anti-apoptotic genes [158]. COX-2 is frequently overexpressed in gastric cancer [159] and has been associated with tumor angiogenesis, tumor progression, and poor patient survival [160]. COX-2 can upregulate Bcl-2, which may contribute to decreased cytochrome *c* release and further reduced caspase-9 and caspase-3 activation, thus resulting in the inhibition of apoptosis [161]. Therefore, anti-p185^{HER-2}-RTA-induced activation of caspase-9 and caspase-3 may be linked to the downregulation of COX-2 expression. Some cancers that express high levels of Bcl-2 are relatively resistant to apoptosis inducing agents [162]. It is therefore important to determine to what degree the toxicity of ricin and other toxin derived immunotoxins towards cancer cells can be attributed to inhibition of protein synthesis, and to what degree to subsequent induction of apoptosis. Importantly, it

was demonstrated that different cancer cell lines overexpressing Bcl-2 are still very sensitive to immunotoxins [162].

As described above, the majority of the studies support the concept that ricin activates the intrinsic pathway of apoptosis. However, some findings suggest that the extrinsic pathway induction may also contribute to ricin-induced apoptosis in some cell types. Sha *et al.* [163] used TUNEL staining to show that purified ricin A-chain was able to induce apoptosis in mouse embryonic fibroblast (NIH 3T3), by activation of caspase-8 and -3 but not caspase-9. Moreover, studies on U937 cells suggest that ricin B-chain interaction with membrane receptors may be sufficient to trigger apoptotic signalling [164]. Carboxymethylated-(CM-) ricin B-chain was able to cause DNA fragmentation and typical apoptotic nuclear morphological changes, which were indistinguishable from those observed in ricin-treated cells. These results suggest that some apoptotic signals may be triggered during the intracellular processing or trafficking of ricin B-chain by mechanisms other than inhibition of protein synthesis. Thus, protein synthesis inhibition is not necessarily required for all apoptotic systems, and this may depend on cell type and apoptotic stimuli. In a breast cancer cell line (MCF-7) it was found that caspase activation leading to apoptosis and arrest of protein synthesis were distinct pathways after treatment with immunotoxin B3(Fv)-PE38 [165]. MDCK (Madin-Darby canine kidney epithelial) cells have been extensively employed to study the mechanisms of ricin-induced cell death. It has been proposed that induction of apoptosis in MDCK cells could be clearly dissociated from protein synthesis inhibition, as pretreatment with DFP (the serine protease inhibitor, diisopropylfluorophosphate) actually increased ricin-mediated protein synthesis inhibition, yet protected cells from apoptosis [131]. The two events could also be temporally dissociated as indicated by the finding that DFP inhibited ricin-induced DNA fragmentation even when the protease was administered after initiation of significant protein synthesis inhibition [131]. Moreover, it has been demonstrated that co-treatment of cells with cycloheximide and ricin inhibited DNA fragmentation in U937 cells by approximately 20%, and in Vero cells by approximately 50%, suggesting that *de novo* protein synthesis may be required for optimal apoptosis induction by ricin [164]. Finally, important indications that the protein synthesis inhibitory activity of ricin may be dissociated from apoptosis induction came from studies involving the saturated random mutagenesis of cDNA encoding the proricin A-chain [166]. Moreover, a recent study comparing the activity of RTA variants in mammalian cells showed that ribosome depurination did not correlate with induction of apoptosis [167]. It was proposed that a threshold level of depurination is required to trigger stress and apoptotic signaling. Interestingly, Jetz *et al.* [167] proposed that the lack of correlation between depurination levels and the and the levels of protein synthesis inhibition imply that depurination of the ribosome by ricin may not be solely responsible for the translation inhibition. It should be noted however, that the biological significance of protein synthesis inhibition in the cells undergoing apoptosis is still controversial and unclear; it is difficult to relate this process unequivocally to the extrinsic or intrinsic mechanisms of apoptosis induction pathways.

Another important mechanism for induction of apoptosis caused by ricin is ribotoxic stress response (ROS). This signalling cascade was first described by Iordanov *et al.* [168]. They observed that treatment of cells with certain protein synthesis inhibitors including ricin caused activation of jun-N-terminal kinases (JNKs), while other protein synthesis inhibitors, such as cycloheximide or puromycin failed to activate these kinases [168]. JNKs, p38 and extracellular-receptor kinases (ERKs) belong to the MAPK (mitogen-activated protein kinase) family. The “stress activated protein kinase family” or

SAPKs is sometimes used to further describe p38 and JNKs. It has been demonstrated that the activation of SAPKs/JNKs, and also their activator SEK1/MKK4 results from damage to 28S rRNA by ricin. These studies also reveal that activation of SAPK/JNK pathways is not just due to protein synthesis inhibition, but is connected with signalling from 28S rRNA affected by ricin. Since not all inhibitors of protein synthesis were able to induce ROS, it was proposed that this 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/EF-G, thus arresting translation at the translocation step [168]. Jetzt *et al.* [169] showed that both ricin and ricin A-chain induced apoptosis of MAC-T cells, (an immortalized bovine epithelial cell line), via a mechanism involving caspase-3 and -7 activation, PARP cleavage and DNA fragmentation. These results are similar to data describing the mechanisms of how RTA-derived immunotoxins induce apoptosis in different cell lines, already described in this review. However, while both p38 and JNK kinases were shown to be activated by RTA and ricin holotoxin, the JNK pathway appeared to be more important in inducing the apoptotic response by RTA in the MAC-T cells [169]. Therefore, it is likely that apoptosis caused by ricin A-chain immunotoxins can involve ribotoxic stress response. However, it should be noted that the mechanisms of ROS induction involved after addition of holotoxin and RTA-derived ITs may differ, since it was shown that ricin and ricin A-chain activate the ribotoxic stress response with different kinetics and specificity [169].

Genes that are upregulated by MAPKs include proinflammatory cytokines such as IL-8, GRO- α , IL-1 β , and TNF- α [170–172]. Ricin has been shown to induce expression of TNF- α , IL-1, IL-6 and IL-8 in human monocytes/macrophages [144,173–175]. Higuchi *et al.* [176] showed that ricin induced apoptotic cell death in murine macrophage-like RAW 264.7 cells. The apoptotic changes were reflected by nuclear morphological criteria, severe protein synthesis inhibition and increased release of TNF- α . Interestingly, the maximum level of TNF- α secretion was attained at the concentration at which only partial inhibition of protein synthesis and apoptotic nuclear morphological changes were induced [176]. This stimulation was attributed to the presence of ricin A-chain, whereas isolated B-chain completely failed to stimulate TNF- α secretion [176]. It has been proposed that ability of RTA to induce TNF- α secretion may be related to RTA induction of ribotoxic stress response. This hypothesis was confirmed in the experiments in which a specific inhibitor of p38 MAP kinase blocked ricin-induced apoptosis and the secretion of TNF- α [176]. Interestingly, the same effect was observed when RAW264.7 cells were incubated with a broad spectrum caspase family proteins inhibitor. However, this inhibitor had no effect on ricin-induced activation of p38 MAP kinase, suggesting that the activation of p38 kinase occurs upstream of the caspase-activation cascade [176]. Thus, multiple pathways, including cysteine proteases and MAPKs may contribute to ricin-induced TNF- α production and apoptosis. The precise mechanisms of signaling for increased expression of cytokines involved in apoptosis may be specific for each cytokine and cell type. For example, inhibitors of the JNK or p38 MAPK pathways blocked ricin-induced TNF- α expression by RAW 264.7 cells, while IL-1 expression was inhibited by p38 MAPK inhibitors but increased by JNK inhibitors [177]. Thus, these data suggest that MAPK pathways may induce or suppress the expression of specific cytokines. Several studies have shown that ricin activates the transcriptional factor NF- κ B which regulates the expression of genes encoding inflammatory and pro-coagulant mediators [178,179]. However, NF- κ B is also thought to be a major anti-apoptotic regulator which acts by limiting JNK activation [180,181]. How the

balance of survival and apoptotic signals mediated by MAPKs and NF- κ B are altered by ricin in the presence of a robust cytokine response remains to be fully characterized.

Recently published results also show a role for UPR (unfolded protein response) and ER-stress in ricin induced apoptosis. The ER is the intracellular site for correct folding and post-translational processing of secretory as well as membrane proteins. It possesses a specialized set of chaperones and enzymes that ensure proper folding proteins as well as provide effective quality control system of this process [16]. The UPR is signalled by three transmembrane proteins with luminal domains that sense the changes in the ER environment and cytosolically disposed effector domains: RNA-dependent protein kinase like ER kinase (PERK); inositol-requiring ER to nucleus signal kinase-1 (IRE1) and activating transcription factor-6 (ATF6). PERK is a serine/threonine kinase, IRE1 possesses both kinase and endoribonuclease functions, and ATF6 is a transcription factor [182–184]. These sensors associate with the chaperone binding immunoglobulin protein (BiP, also known as GRP78) [185]. In the presence of unfolded proteins, BiP dissociates from the sensor molecules, allowing the sensors to dimerize and become activated by auto-phosphorylation (PERK and IRE1) or translocated to the Golgi and proteolytically cleaved (ATF6). PERK, ATF6 and IRE1 then act on multiple downstream substrates. PERK dimerizes and autophosphorylates its kinase domain upon release from BiP, followed by activation of eukaryotic initiation factor 2 α (eIF2 α), leading to global inhibition of translation and a decreased protein load on the ER. ATF6 translocates to the Golgi apparatus where it is processed into an active transcription factor that regulates expression of genes involved in ER quality control [184]. Ire1 was the first UPR transducer to be discovered in mammalian cells and is the homologue of the sole yeast UPR transducer, Ire1p [186]. Like the mammalian IRE1 isoforms, Ire1p possesses serine/threonine kinase and endoribonuclease functions. In the presence of unfolded proteins, Ire1p oligomers form, leading to proximity induced autophosphorylation and activation of kinase and ribonuclease functions. HAC1 mRNA is the yeast orthologue of mammalian XBP-1 mRNA. Activated Ire1p removes an intron from unspliced HAC1 to produce the mRNA transcript for the basic leucine zipper transcription factor Hac1 which then binds to promoters containing unfolded protein response elements [187]. Parikh *et al.* [188] showed that enzymatically active ricin A-chain suppressed induction of the UPR. HAC1 mRNA transcripts were not spliced in ricin A-chain expressing yeast. It was suggested that ricin A-chain affects signaling at a point upstream of RNA splicing. Ricin may not only bind rRNA, but also may bind to mRNA, and ricin binding to unspliced HAC1 mRNA may mediate adenine depurination with subsequent inhibition of Ire1p endonuclease function [188]. RTA could potentially activate the UPR, which could contribute to its apoptotic effects. However, results of another study found that RTA alone failed to activate either the IRE1/XBP1 or the PERK/eIF2- α sensors of the UPR pathway [189]. This agrees with studies in yeast [188]. In contrast to these data, ricin was recently reported to inhibit eIF2- α phosphorylation and to decrease intact ATF-6 protein in two human cancer cell lines, MDA-MB-231 (human breast adenocarcinoma) and HCT-116 (colorectal carcinoma cells), indicating that these two UPR pathways were induced by toxin treatment [190]. However, these investigators also found no XBP-1 splicing in response to toxin exposure. Therefore, one potential mechanism by which RTA could inhibit phosphorylation would be to reduce general protein synthesis so that BiP fails to dissociate from IRE1 and PERK, thereby preventing their activation. Moreover, it was proposed that inhibiting the UPR allows RTA to increase cell death through a mechanism that is independent of protein synthesis inhibition. This supports

findings in yeast using RTA mutants which show that protein synthesis inhibition and cell death can be uncoupled [191]. As mentioned above, PKR kinase can be an essential mediator in initiation of apoptosis. PKR activates (phosphorylates) the eukaryotic translation initiation factor eIF-2a, which is also activated by the UPR response [192]. Phospho-eIF-2a down-regulates translation initiation, suggesting that ricin may inhibit global protein synthesis by more than one mechanism.

The UPR response is activated by hypoxia in solid tumors, resulting in resistance to chemotherapy [193]. Similarly, increases in spliced XBP1 relative to unspliced XBP1 have been shown to correlate with poor prognosis in breast cancer [194], and XBP1 has been proposed as a therapeutic target for solid tumors [195]. Therefore, the ability of RTA to inhibit UPR may make it more potent in targeted therapy for cancer. This may be particularly useful in cancer cells where UPR is already upregulated by conditions such as hypoxia.

6. Side-effects of Immunotoxins Made with Ricin

Most research on immunotoxins has focused on their possible use in the therapy of cancers. As described above, some promising results have been obtained, especially on tumors transplanted in animals, and also in patients with various forms of cancers, especially of haematological origin. Compared with conventional chemotherapeutic agents, RIP-containing immunotoxins should have some advantages, being very potent, acting on both dividing and nondividing cells, and not inducing resistance. Unfortunately, the toxicity of some of these agents has prevented their further clinical development, and immunotoxins and other toxin conjugates are not commonly used in tumor therapy. One exception is denileukin diftitox (trade name Ontak), combining interleukin-2 and diphtheria toxin [196]. In 1999 Ontak was approved by the U.S. Food and Drug Administration (FDA) for treatment of cutaneous T-cell lymphoma (CTCL).

The most common side-effects of RIPs-containing immunotoxins are: hepatotoxicity, renal insufficiency, fatigue, fevers and nonspecific immunological responses which includes formation of antibodies against MAb and RIP [197]. Treatment with RIPs immunotoxins may cause allergic reactions and even anaphylactic shock. Moreover, the clinical trials show that, despite inherent specificity, essentially all of the RTA-immunotoxins caused a common dose-limiting toxicity called vascular leak syndrome (VLS). VLS seems to be the main side-effect of ricin-derived immunotoxins. VLS has a complex etiology involving damage to vascular endothelial cells, extravasation of fluids and proteins from the blood to the tissues, interstitial edema, and organ failure [198]. It is believed that the immunotoxin's cell-targeting moiety is not responsible for inducing VLS because this toxicity occurs in patients treated with RTA-immunotoxins of various specificities and with whole antibodies or Fab fragments linked to RTA [199,200]. Therefore, RTA itself is thought to cause VLS by damaging vascular endothelial cells. The exact mechanism of this damage is however unclear. There are two general ways that ricin IT-induced VLS may occur. The first hypothesis tells that VLS is a direct consequence of ricin A-chain cytotoxicity and eventual cell death. Evidence for this hypothesis is based upon the inhibition of protein synthesis and further increase in cell permeability in a time and dose dependent manner observed in human HUVEC-C cells [201,202]. Another hypothesis for RTA-induced VLS is based on the observation that RTA binds to endothelial cells and can cause morphologic changes prior to the detectable protein synthesis inhibition [203,204].

Since there are no suitable animal models for RTA-mediated VLS, Lindstrom *et al.* [202] developed an *in vitro* system in which they measured the integrity of the vascular endothelial cell monolayer in terms of its permeability. In their experiments they mostly used dgRTA since this derivative or RTA is the one most frequently used in clinically tested ricin immunotoxins (see above). They found that RTA directly increased the permeability of HUVEC-C monolayers in a time- and dose-dependent manner and that the enzymatic activity of RTA is required for inducing monolayer permeability. Therefore, it has been proposed that RTA directly damages HUVEC-C through the following cascade of events: protein synthesis inhibition by ribosomal inactivation, cell death, and gap formation in the monolayer leading to an increase in permeability [202].

Soler-Rodríguez *et al.* [201] reported that ricin A-chain and RTA-containing immunotoxins directly affected the morphology, permeability, and viability of primary HUVEC monolayers. However, their data differ with respect to the kinetics and temporal sequence of events compared to the results presented by Lindstrom *et al.* [202]. They found that RTA rapidly induced morphologic changes, including cell rounding and disruption of monolayers, before protein synthesis inhibition. To determine whether these changes were related to the disruptions in endothelial cell interactions with the extracellular matrix, the effect of dgRTA on HUVECs was analysed in the presence of fibronectin (Fn), an extracellular matrix protein, which plays role in the maintenance of vascular integrity [205]. They reported that RTA specifically bound Fn. Moreover, it has been demonstrated that the cell rounding and ultimate cell detachment from the monolayer mediated by dgRTA was inhibited by exogenous Fn [205]. Thus, investigators suggested that RTA binding to Fn was sufficient to perturb cell-cell or cell-extracellular matrix interactions and to change the morphology and permeability of endothelial cell monolayers [205]. Importantly, levels of serum Fn was decreased significantly in patients who developed severe VLS, after treatment with RTA-derived immunotoxins [206]. However, the decrease in the levels of serum Fn was accompanied by increase in the serum TNF- α , what may indicate that particular intracellular cascade signalling pathways are triggered in this case and that proinflammatory cytokines may play a critical role in the VLS. Based on these results and also on data indicating that in severe combined immunodeficient (SCID) mice with human skin xenografts, systemically administered dgRTA-ITs induced VLS in the human skin, but not in the adjacent mouse skin [207], it was considered that ricin A chain and other RIPs may contain homologous structural motifs that may effect cell-cell and cell-matrix interactions and thereby damage human endothelial cells. It has been proposed that (x)D(y) motif where $x = L, I, G, \text{ or } V$ and $y = V, L, \text{ or } S$ can be important in the induction of VLS. In the case of ricin the LDV sequence (residues 74–76) was partially exposed on intact toxin [203]. Moreover peptides containing the LDV motif attached to the anti-C22 MAbs (RFB4), specifically damage HUVECs *in vitro*. The LDV sequence in RTA is probably responsible for the initiation of events leading to VLS-like symptoms *in vivo* because injection of RFB4-RTA peptides containing the native (but not mutated or deleted) LDV sequence caused vascular leak in lungs and in human skin xenografts in a manner analogous to that of the RFB4-dgRTA IT. It was also demonstrated that dgRTA uses its LDV sequence, at least in part, to bind to HUVECs [203]. It appeared that the aspartic acid (D75) in the LDV sequence in RTA and the C-terminal flanking threonine play critical roles in both: the binding of RFB4-RTA-LDV to HUVECs and in its ability to damage these cells [204]. Importantly, the LDV motif in RTA can damage endothelial cells by caspase-3 mediated apoptosis and additionally it may compete with Fn for binding to cell surface

integrins leading to disruption between epithelial cells and extracellular matrix [204]. These results suggested that ITs prepared with RTA mutants containing alterations in LDVT may kill tumor cells *in vivo* in the absence of VLS. In other studies, a series of recombinant ricin A-chains with mutations in LDV sequence or in amino acids flanking it in the three-dimensional structure were generated [208]. These mutated ricin A-chains have been evaluated alone and as immunotoxins for their activity, ability to induce pulmonary vascular leak in mice, pharmacokinetics, and activity in tumor-xenografted mice. One mutant (Asn97→Ala; N97A) was comparable to the ricin A-chain used before in all respects, except that it did not cause vascular leak at the same dose. When immunotoxin RFB4–N97A was used, it was more effective and tolerated at higher doses in xenografted SCID mice, thereby leading to improved antitumor activity [208,209]. However, RFB4–N97A has never been tested in humans. Since animal models are rarely quantitatively predictive of clinical results in patients, clinical testing would be necessary to determine its ultimate value [209].

It is considered that to achieve a balance between reduced nonspecific toxicity and anti-tumor activity, the ideal RTA-derived immunotoxin should have reduced direct cytotoxicity or/and reduced ability to cause morphological changes to endothelial cells prior to the protein synthesis inhibition, and, additionally, have poor immunogenicity. The majority of clinical trials with ricin-based ITs showed that VLS was the dose limiting toxicity, being the largest problem for treatment with ITs. Investigators are now considering new experimental approaches using modified RTA to decrease direct damages to endothelial cells [202,210], less cytotoxic but still potent RTA [15,202] or short-lived monoclonal antibodies [51]. When mutant chimeric (mCRFB4) antibodies, which lack the ability to bind to the neonatal Fc receptor were used, this antibody coupled to RTA induced less pulmonary vascular leak in mice [210].

7. Conclusions

The potential application of RIP toxins lies in their targeted delivery in cancer immunotherapy. Increased knowledge about intracellular trafficking of toxins and toxin conjugates and the mechanism of their action in cells may allow the construction of a new generation of immunotoxins that could fulfil the high expectations put to this research field. In addition to a direct effect on protein synthesis, the efficiency of induction of apoptosis will be important to obtain therapeutically useful immunotoxins.

At present, there is no optimal treatment to reduce vascular toxicity while preserving antitumor activity. The mechanism of VLS is complex and can involve damage of epithelial cells and leukocytes, release of cytokines and of inflammatory mediators, alteration in cell-cell and cell-matrix adhesion and in cytoskeleton function [211]. However, the epithelial cell damage observed *in vitro* or in animal models may not be relevant to VLS in humans because of differences between animal and human vasculature and between macrovasculature and microvasculature in humans. During the last several years a huge progress has been made in understanding the mechanisms of IT-mediated VLS. Our increasing knowledge about mechanisms of action of toxin-containing immunotoxins has led to products with decreased VLS. On the other hand, more effort is still required to generate toxin-containing immunotoxins which do not damage epithelial cells but retain antitumor activity.

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