Molecular Determinants of Apoptosis Induced by the Cytotoxic Ribonuclease Onconase: Evidence for Cytotoxic Mechanisms Different from Inhibition of Protein Synthesis

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ABSTRACT

Cytotoxic endoribonucleases (RNases) possess a potential for use in cancer therapy. However, the molecular determinants of RNase-induced cell death are not well understood. In this work, we identify such determinants of the cytotoxicity induced by onconase, an amphibian cytotoxic RNase. Onconase displayed a remarkable specificity for tRNA in vivo, leaving rRNA and mRNA apparently undamaged. Onconase-treated cells displayed apoptosis-associated cell blebbing, nuclear pyknosis and fragmentation (karyorrhexis), DNA fragmentation, and activation of caspase-3-like activity. The cytotoxic action of onconase correlated with inhibition of protein synthesis; however, we present evidence for the existence of a mechanism of onconase-induced apoptosis that is independent of inhibition of protein synthesis. The caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone (zVADfmk), at concentrations that completely prevent apoptosis and caspase activation induced by ligation of the death receptor Fas, had only a partial protective effect on onconase-induced cell death. The proapoptotic activity of the p53 tumor suppressor protein and the Fas ligand/Fas/Fas-associating protein with death domain (FADD)/caspase-8 proapoptotic cascade were not required for onconase-induced apoptosis. Procaspases-9, -3, and -7 were processed in onconase-treated cells, suggesting the involvement of the mitochondrial apoptotic machinery in onconase-induced apoptosis. However, the onconase-induced activation of the caspase-9/caspase-3 cascade correlated with atypically little release of cytochrome c from mitochondria. In turn, the low levels of cytochrome c released from mitochondria correlated with a lack of detectable translocation of proapoptotic Bax from the cytosol onto mitochondria in response to onconase. This suggests the possibility of involvement of a different, potentially Bax- and cytochrome c-independent mechanism of caspase-9 activation in onconase-treated cells. As one possible mechanism, we demonstrate that procaspase-9 is released from mitochondria in onconase-treated cells. A detailed understanding of the molecular determinants of the cytotoxic action of onconase could provide means of positive or negative therapeutic modulation of the activity of this potent anticancer agent.

INTRODUCTION

Programmed cell death or apoptosis is an integral part of embryonic development and of homeostasis in higher eukaryotic organisms (for reviews, see Refs. 1–3). With the exception of the specific case of granzyme B-mediated cell death utilized by cytotoxic T-lymphocytes, two autonomous but interacting pathways of apoptosis induction have been identified in mammalian cells: (a) apoptosis induced in response to engagement by proapoptotic cytokines of specialized cell surface death receptors (exemplified, for instance, by the death receptor Fas/APO1/CD95 and its ligand, FasL); and (b) apoptosis triggered by changes in the homeostasis of mitochondria (for recent reviews, see Refs. 2 and 3). For each of the two apoptotic pathways, a triggering event converges through specific signal transduction pathways onto initiation and execution machinery that is comprised of cascades of proteolytic enzymes known as caspases (for reviews, see Refs. 4–6). Before an apoptosis-triggering event, caspases exist as dormant proenzymes. The most apical “initiation” caspase that participates in the death receptor-dependent pathway is caspase-8 (7, 8). Death receptor-triggered autocalytic processing of procaspase-8 releases the active caspase-8 heterotetrameric enzyme, which, in turn, can directly process and activate the “executioner” caspase-3 (9, 10). Similarly, the most apical initiation caspase that participates in mitochondria-dependent cell death is caspase-9. Its autocalytic processing (11) and activation involve the participation of several coactivator molecules, one of which is cytosolic cytochrome c (12). This protein, which is normally loosely attached to the outer surface of the inner mitochondrial membrane (for a review, see Ref. 13), is released from the mitochondria through mechanisms that are still debated into the cytosol in response to apoptotic stimuli (for a review, see Ref. 3). The proapoptotic protein Bak induces apoptosis by triggering the release of cytochrome c from mitochondria (14–20). Activated caspase-9 can directly process caspase-3 and its closest relative, caspase-7 (11). The activation of caspase-8/caspase-3 or caspase-9/caspase-3 cascades ensures the irreversibility of the apoptotic process. Ultimately, activation of executioner caspases leads to the proteolytic cleavage of numerous cellular substrates, the activation of apoptosis-specific nucleases (21, 22) that lead to internucleosomal chromatin cleavage, and the development of a typical apoptotic morphology characterized by nuclear pyknosis and fragmentation and by the blebbing of the cell plasma membranes. In the final step of apoptosis, the cell corpses are engulfed and destroyed by neighboring phagocytic cells.

Based on results obtained in recent knockout models, the apoptotic program in response to DNA damage appears to involve the mitochondrial apoptotic pathway mediated by caspase-9 (23–25). However, in certain Fas-expressing cells, anticancer drugs and radiation activate the transcriptional induction of the gene for FasL that leads to increased FasL expression. FasL further engages Fas and triggers Fas-dependent, caspase-8-mediated cell death (26–28). Furthermore, the absence of a functional wild-type p53 tumor suppressor protein generally impedes the induction of apoptosis by DNA-damaging agents such as genotoxic drugs and ionizing or UV radiation (for reviews see Refs. 29 and 30), thus establishing an important role for...
p53 in the DNA damage-induced apoptosis. However, the relationships between p53 and either of the apoptotic pathways is poorly understood. Despite the incompletely understood mechanisms of apoptosis induced by DNA-damaging chemicals and ionizing radiation, these agents constitute the predominant part of the available anticancer therapy.

An entirely novel approach to induce cytotoxicity in target cancer cells is based on the ability of the amphibian endoribonuclease (RNase) onconase (P-30 protein) to kill rapidly proliferating cells (with a certain preference for cancer cells) both in tissue culture and in the mouse (31–35). Onconase, originally isolated from oocytes of *Rana pipiens* (36), is a member of a growing family of extracellular cytotoxic RNases (for a review, see Ref. 37). The cytotoxic properties of onconase ultimately depend on its ability to enter target cells and on its RNA-hydrolyzing capacity (33). Recently, onconase, in combination with doxorubicin, has been found to dramatically increase the life span of nude mice bearing human breast carcinoma cells (38). Interestingly, pancreatic RNase A, which is strongly homologous to onconase, is not cytotoxic (39). The cellular protein RI, which is an inhibitor of ribonucleases, appears to possess a much greater affinity for RNase A than for onconase (40), thus providing one possible explanation for the different cytotoxic abilities of the two RNases.

An onconase-based immunotoxin with increased tumor cell specificity is approved for clinical trials. In the present work, we undertook to elucidate the intracellular mechanisms responsible for the cytotoxic effects of onconase. We used a lipofection-mediated delivery of enzyme into mammalian cells and asked whether it could induce characteristic features of apoptosis in these cells. We demonstrate here that onconase treatment caused degradation of cellular tRNA but left rRNA and mRNA apparently undamaged. Onconase induced a characteristic apoptotic pattern of cell death involving chromatin degradation, nuclear pyknosis and fragmentation, cell membrane blebbing, and activation of caspases-9, -3, and -7. The proapoptotic effects of onconase did not require the presence of wild-type p53 and of the FasL/Fas/FADD/caspase-8 proapoptotic cascade. Although onconase-induced cytotoxicity correlated with onconase-induced inhibition of protein synthesis, we present evidence for the existence of an onconase-triggered apoptotic mechanism that is likely to be independent of the inhibition of protein synthesis. Finally, we demonstrate that onconase-induced cell death and caspase-9 activation are accompanied by unusually little release of cytochrome c from mitochondria and a lack of detectable translocation of Bax from the cytosol onto the mitochondria, suggesting that Bax- and cytochrome c-independent mechanisms of caspase-9 activation might be involved in mediating onconase cytotoxicity.

### MATERIALS AND METHODS

**Chemicals.** The source, storage, methods of application, and modes of action of emetine and cycloheximide have been described (41). The caspase inhibitor zVADfmk was from Calbiochem, and it was stored as a 50 mM stock solution in (H<sub>2</sub>SO<sub>4</sub>) at −20°C. Whenever zVADfmk pretreatment was applied, the corresponding control cell received 1 μM of (H<sub>2</sub>SO<sub>4</sub>) per milliliter of medium. Lipofectin Reagent was from Life Technologies, Inc.

**Cell Culture.** HeLa tk<sup>−</sup> cells were maintained in DMEM supplemented with 10% calf serum (HyClone, Logan, UT). All experiments presented here were performed with logarithmically growing cultures that had not reached more than 50% confluence. For this purpose, cells were plated 18–24 h before treatment at a density of 2.6 × 10<sup>4</sup>, 5 × 10<sup>4</sup>, or 2 × 10<sup>5</sup> cells/plate (or well) in 10-cm plates, 6-well plates, or 12-well plates, respectively. The p53<sup>(−/−)</sup> fibroblast cell line was derived from p53<sup>(−/−)</sup> mouse primary embryonic fibroblasts (a generous gift from Dr. Markus Grompe) through spontaneous immortalization. The absence of p53 in these cells at both allele and protein levels was verified by PCR and Western blot analyses, respectively, and it will be described elsewhere.

**Onconase Preparation.** Native onconase was purified from *Rana pipiens* oocytes (Nasco, Fort Atkinson, WI) following the published protocol (36) as described previously (42).

**Lipofectin-mediated Delivery of Onconase.** Delivery of onconase with Lipofectin was performed in DMEM containing 0.5% calf serum. Lipofectin/onconase mixes were prepared following the procedure outlined for the delivery of diphtheria toxin and e-sarcin in Ref. 41. Before the application of the Lipofectin/onconase mixes, the cells were washed once with serum-free DMEM. Four h after the addition of the Lipofectin/onconase mix, the cells were fed calf serum to a final concentration of 10%.

**Antibodies.** Antibodies against PARP (H-250, sc-7150, and A-20, sc-1562), cytochrome c (H-104, sc-7159), Bax (N-20, sc-493HRP), rabbit IgG (sc-2004), and mouse IgG (sc-2005) were from Santa Cruz Biotechnology. Antibodies against caspase-9 (#6571A), caspase-8 (#6623A), caspase-7 (#66871A), caspase-3 (#65906E), and caspase-1 (#64414A) were from PharMingen. The activating (CH11, #05-201) and blocking (ZB4, #05-338) antibodies against human Fas were from Upstate Biotechnology.

**Measurement of Protein Synthesis via [3H]Leucine Incorporation.** Incorporation of [3H]leucine was performed as described previously (40), except that the cells were not subjected to leucine deprivation before onconase treatment and [3H]leucine pulse labeling. Two μCi of [3H]leucine/ml medium was used for labeling.

**Detection of Apoptosis.** For detection of apoptosis using TUNEL, cells were plated at low density on Theranox slides (Nunc) and treated as indicated in the text, and apoptotic cells were detected using the In Situ Cell Death Detection kit from Boehringer Mannheim following the instructions of the manufacturer. For detection of apoptosis by morphological criteria, cells were plated on 6-well plates and treated with onconase as described in the text or in the figure legends. At the indicated times after the treatment, the cells were washed twice with PBS to remove dead cells. Cells that remained attached to the plates were fixed in a solution containing 95% ethanol and 5% acetic acid for 15 minutes at RT. The cells were then rehydrated with several washes in a sodium phosphate buffer (10 mM, pH 7.9) for 1 h. The cells were stained by applying a 10 μg/ml acridine orange solution in sodium phosphate buffer (pH 7.9) for 1 h, followed by extensive washing with sodium phosphate buffer (pH 7.9). Visualization of the incorporated acridine orange into nuclear DNA was achieved in fluorescent microscopy using an ARC Lamp UV source (Ludl Electronic Products Ltd., Hawthorne, NY) and an IM35 Zeiss microscope. Phase-contrast viewing using the same microscope was done with the in-built visual light source. Photographs of cell were taken with a CONTAX 167MT camera (Kyocera Co., Tokyo, Japan) and Kodak 100 Elite Chrome film (Eastman-Kodak). For DNA fragmentation analysis, cells were treated, harvested, and lysed, and nuclei were sedimented as described below for preparation of nuclear extracts. The nuclear pellets were resuspended in 50 mM Tris-HCl (pH 7.8), 10 mM EDTA, and 0.5% (w/v) sodium N-lauryl sarcosinate. DNase-free RNase A (Sigma) was added (f.c. 0.5 mg/ml) for 20 min at 50°C. Proteinase K (Life Technologies, Inc.) was added (f.c. 0.5 mg/ml) for an additional 50 min at 50°C. The samples were then resolved in 2% nondenaturing agarose gels. DNA was visualized using ethidium bromide and UV transillumination.

**Measurement of Cell Death/Survival.** For the 24-h death assay, a modification of the technique of Goillot *et al.* (43) was used. Briefly, HeLa cells were plated in 12-well plates. Eighteen to 24 h later, the cells were treated as described in the text or in the figure legends. Four h after the treatment, the cells were washed twice with PBS to remove dead cells. Cells that remained attached to the plates were simultaneously fixed and stained for 10 min by the addition of a solution containing 0.05% crystal violet, 20% (v/v) ethanol, 0.37% (v/v) formaldehyde, 80% (v/v) H<sub>2</sub>O. The wells were then rinsed extensively with water and dried. Crystal violet was extracted for 1 h

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from the cells by addition of 2 ml methanol/well and vigorous shaking. The absorbance of the extract was measured at 570 nm. After subtracting the background staining from plates containing only growth medium but not cells and processed as described above, the absorbance of control cells was taken to indicate a 100% survival, and the percentage cell survival of the treated cells was calculated accordingly. Percentage cell death was calculated as follows: 100 – percent survival.

Preparation of RNA. Total RNA containing the fraction of tRNA was prepared as follows. Cells were treated as described in the text or in the figure legends. At the indicated times after treatment, the cells were washed twice in ice-cold PBS, scraped in ice-cold PBS, and centrifuged at 16,000 × g for 1 min at 4°C. The cellular pellet was resuspended in 375 μl of a solution containing 10 mM Tris-HCl (pH 7.0), 150 mM NaCl, and 1 mM EDTA; thereafter, 26 μl of 10% (w/v) nonfat dry milk was added to lyse the cells for 2 min on ice. Nuclei were sedimented at 16,000 × g for 30 s at 4°C, and 375 μl of a solution containing 20 mM Tris-HCl (pH 7.8), 350 mM NaCl, 20 mM EDTA, and 1% (v/v) SDS were added to the supernatant. One extraction with 750 μl of phenol/chloroform/isoamyl alcohol (25:24:1) and one extraction with chloroform/isoamyl alcohol (24:1) were performed and RNA from the aqueous phase was precipitated with 2 volumes of ethanol. RNA precipitate was dissolved in RNase-free water, and an equal volume of a 2× RNA-loading solution (50% (v/v) formamide, 6% (v/v) formaldehyde, 20% (v/v) glycerol, 20 mM sodium phosphate buffer (pH 7.0), and 10 μg/ml ethidium bromide without bromphenol blue) was added. The RNA samples were denatured by heating for 10 min at 85°C.

Northern Blot Analysis. RNA samples were resolved electrophoretically in either 1% or 4% denaturing agarose gels (44) and transferred onto Hybond-N membrane (Amersham Life Science). Hybridization with radioactively labeled DNA probes was done using the ExpressHyb hybridization solution (Clontech) following the manufacturer’s instructions. The hybridization probe for tRNA was the oligonucleotide 5′-CTGAGATTAAGTCTCAT-GCTTACCGACTGAGCTAGCC-3′ (Life Technologies, Inc.). The hybridization probe for cyclophilin is described elsewhere (44).

Reverse Transcription of tRNA by Primer Extension. Probing for damage to 28S rRNA by reverse transcriptase-mediated primer extension was done as described previously (40). The following two primers (45) were used (a) 5′-CCACCAGATGGTAGCT-3′; and (b) 5′-CGACATCGAAGGATCA-3′.

Preparation of Nuclear and Cytosolic Extracts for Western Blot Analysis. The following protocol applies to the experiments presented in Figs. 4, 6B, 7A, and 7B. Cells were treated as described in the text and in the figure legends. At the indicated times, both attached and detached cells were collected by scraping directly in the medium, sedimented at 16,000 × g for 1 min at 4°C, washed by resuspending in ice-cold PBS, and resedimented. Cellular pellets were resuspended in 100 μl of a solution containing 10 mM HEPES-KOH (pH 7.9), 60 mM KCl, 1 mM EDTA, 0.5% (v/v) NP40, 1 mM DTT, and Complete Protease Inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN) and lysed for 5 min on ice. Nuclei were sedimented at 735 × g for 5 min and resuspended in a solution containing 250 mM Tris-HCl (pH 7.8), 60 mM KCl, 1 mM DTT, Complete Protease Inhibitor mixture, whereas the post-nuclear supernatants were designated “cytosolic extracts” after removal of cellular debris at 16,000 × g for 10 min at 4°C. For extraction of nuclear proteins, the salt concentration was elevated to 500 mM KCl, and the proteins were extracted at 4°C for 30 min with vigorous agitation, followed by removal of nuclear envelopes at 16,000 × g for 10 min at 4°C.

Western Blot Analysis. Nuclear or cytosolic protein extracts were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Millipore). After the transfer, the nonspecific antibody-binding activities of the membranes were blocked in Tris-buffered saline (TBS, 10 mM Tris-HCl, pH 8.0), 150 mM NaCl containing 0.3% (v/v) Tween-20 and 10% (w/v) nonfat dry milk for 30 min at room temperature. Incubations with appropriate dilutions of primary and secondary antibodies were done for 60 and 45 min, respectively, in TBS containing 0.3% Tween-20 and 5% nonfat dry milk, followed by extensive washes with TBS containing 0.3% Tween-20. Detection of immunolabeled proteins was done using the Renaissance chemiluminescence kit (DuPont New England Nuclear Research Products) and Hyperfilm enhanced chemiluminescence film (Amersham).

Preparation of Mitochondria. For the detection of cytochrome c present in Fig. 7C, mitochondria were prepared and analyzed as described in Bossy-Wetzel et al. (46). For the combined analysis of Bax and cytochrome c shown in Fig. 8A, the procedure described in Saikumar et al. (19) was applied, with modifications. Control and appropriately treated cells were harvested by scraping directly in the medium, sedimented, washed once in ice-cold PBS and once in isotonic sucrose buffer [SU buffer-250 mM sucrose, 10 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA (pH 7.1)] and then permeabilized for 1 min at room temperature in SU buffer containing 0.025% digitonin and Complete Protease Inhibitor mixture. The efficiency of permeabilization was nearly 100% as measured by trypan blue exclusion. After sedimenting the cellular pellet, the supernatant was designated “cytosolic fraction.” The pellet of permeabilized cells was resuspended in SU buffer containing 0.5% Triton X-100 and Complete Protease Inhibitor mixture and kept for 10 min on ice, after which the Triton X-100-soluble and insoluble fractions were separated by centrifugation. All three fractions were resolved in 15% SDS-PAGE, transferred onto polyvinylidene difluoride membrane, and probed with appropriate antibodies as shown in Fig. 8A.

RESULTS

(tRNA Is a Specific Target for Onconase in Vivo). Preliminary studies have demonstrated that the cationic lipid vehicle Lipofectin facilitated the delivery of onconase into a variety of cell lines, including HeLa cells (measured by cytotoxicity, see below). To determine the relevant in vivo target(s) for onconase, we treated HeLa cells with Lipofectin and onconase (1 μg/ml) and monitored for 24 h of treatment, see below). Immediately after, 4 h after, or 4 h after the treatment with onconase, the cells were harvested, and total cellular RNA was prepared and resolved electrophoretically in either 1% or 4% denaturing agarose gels. Hydrolysis of 28S or 18S rRNA was undetectable by ethidium bromide visualization in the 1% gel (Fig. 1, Lanes 4–6). We also applied a highly sensitive method (see “Materials and Methods”) based on the lesion-induced arrest of a reverse transcriptase-driven primer extension to detect single-nucleotide damages to domains V and VI of 28S rRNA (41, 45). These domains are directly involved in translational elongation and are the most common targets for fungal antibiotics and polypeptide ribotoxins (see Ref. 41 and references therein). Using two primers that allow the scanning of an approximately 1-kb region within these domains, we failed to detect any sites that were susceptible to cleavage by onconase (data not shown). We therefore concluded that tRNA is an unlikely candidate for an onconase target in vivo.

During the course of the former experiments, we noticed that a band of RNA with a mobility greater than the 28S and 18S rRNAs displayed a graded decrease in ethidium bromide staining after treatment with onconase (Fig. 1, Lanes 4–6). The same band was further resolved in a 4% gel into three distinct RNA species, of which only the species with the highest mobility was significantly affected by onconase treatment (Fig. 1, Lanes 7–9). Based on the apparent mobilities of these three bands, we concluded that they represent the 5.8S rRNA, the 5S rRNA, and the population of tRNAs, respectively. To confirm that the band that decreased in abundance after onconase treatment of cells corresponded to tRNA, we performed Northern blot analysis of the same RNA preparations using a radioactively labeled 40-mer DNA oligonucleotide probe complementary to the human tRNA (5′-CCACCAGATGGTAGCT-3′). Indeed, at 2 and 4 h after exposure of cells to onconase, the relative abundance of tRNA decreased to 68% and 55% of the control cells, respectively (Fig. 1, Lanes 1–3). Furthermore, probing of the same membrane with a probe specific for cyclophilin mRNA revealed a single hybridizing band and an absence of degradation products corresponding to these mRNAs (Fig. 1, Lanes 10–12). The abundance and integrity of three other nonrelated mRNAs (c-jun, c-fos, and survivin mRNAs) was assessed; these mRNAs also displayed a single hybridizing band and an absence of degradation products (data not shown).
Onconase-induced Cytotoxicity. Although onconase is a cytotoxin with intrinsic internalization capacity, its translocation across the cellular membrane is the rate-limiting step of cytotoxic action (33). We avoided this potential complication by Lipofectin-mediated delivery of onconase into all cell types used in this work. To determine the ability of Lipofectin-delivered onconase to kill HeLa cells, the cells were treated with Lipofectin alone or with onconase (0.01, 0.1, or 1 μg/ml) and Lipofectin, and the cell survival was measured 24 h later using crystal violet staining as described in “Materials and Methods.” Fig. 2 demonstrates that onconase caused cell death that correlated strongly with the logarithm of onconase concentration; the half-maximal killing concentration (IC$_{50}$) for onconase in HeLa cells was $\sim$0.05 μg/ml (Fig. 2; Table 1, column C). In contrast, onconase added without Lipofectin to the medium of cells at concentrations of up to 30 μg/ml was not cytotoxic (data not shown).

A potential target for the action of a ribonuclease is the process of protein synthesis. We therefore determined, in parallel to the determination of the cell killing, the effect of onconase on translation. Cells were pulse-labeled with $[^{3}\text{H}]$leucine between 2.5 and 3 h after onconase treatment. As for the killing, onconase caused an inhibition of incorporation of $[^{3}\text{H}]$leucine that correlated with the logarithm of onconase concentration; the IC$_{50}$ for inhibition of protein synthesis was $\sim$0.1 μg/ml (Fig. 2; Table 1, column A). Correlation analysis (percentage of inhibition of protein synthesis versus percentage of killing) revealed that at IC$_{50}$ for inhibition of protein synthesis, onconase kills 65 ± 5% of the cells in 24 h (Table 1, column E).

Can the cytotoxic potential of onconase result entirely from the inhibition of protein synthesis? We reasoned that if this were the case, a similar correlation between cytotoxicity and protein synthesis should be observed for other inhibitors of protein synthesis. To test this hypothesis, we performed the same correlation analysis (translation versus survival) for emetine, a potent and irreversible inhibitor of ribosomal translocation (see Ref. 41 and references therein). We observed reproducibly that at similar levels of inhibition of protein synthesis, onconase is significantly more cytotoxic. For instance, at IC$_{50}$ for inhibition of protein synthesis ($\sim$0.04 μg/ml; Table 1, column A), emetine kills merely 15 ± 5% of the cells in 24 h (Table 1, column E). Although not as pronounced, similar results were obtained using another antibiotic inhibitor of protein synthesis, cycloheximide (Table 1, column E). These results are consistent with the notion that a part of the cytotoxic action of onconase results from onconase-triggered death mechanisms other than inhibition of protein synthesis (see below).

Onconase Triggers an Apoptotic Cell Death Program. To identify and characterize the mode of cell death that is triggered by...
Table 1 Correlation between onconase-, emetine-, or cycloheximide-induced inhibition of protein synthesis and cell death in the absence or presence of zVADfmk

HeLa cells growing in 12-well plates were treated with 0, 0.01, 0.1, or 1 μg/ml onconase, either in the absence or in the presence of 50 μM zVADfmk added 1 h before onconase. Two h and 30 min after the onconase treatment, half of the plates were labeled with 2 μCi/ml [3H]leucine for another 30 min, after which time [3H]leucine incorporation was determined as described in Fig. 2. Twenty-four h after the treatment, the other half of the plates was used to determine cell survival using crystal violet staining as described in “Materials and Methods.” Both protein synthesis and cell killing were plotted as functions of the concentration of onconase and IC50 values (± SD from experimental points in triplicates) for either effect were calculated using DeltaGraph Professional software from Deltapoint, Inc. (columns A–D). Plotting percentage (of control) cell killing versus m using 0, 0.03, 0.1, or 0.3 μM onconase also induced a characteristic blebbing of the cellular membrane. Membrane blebbing in HeLa cells was detectable as early as 4 h after treatment and continued to be evident at 16 h after the treatment (data not shown for HeLa cells, but see Fig. 3C for p53null mouse fibroblasts). We next investigated whether the mode of onconase-induced cell death involves the activation of an apoptosis-specific DEVDase activity. To this end, we tested for the appearance in the nuclear fractions of HeLa cells of specific cleavage products of the enzyme PARP, a target for caspase-3 (47, 48). Fig. 4A (top) demonstrates that onconase treatment caused a dose-dependent disappearance of the M, 116,000 full-size PARP and a concomitant accumulation of the M, 89,000 cleavage product. In conclusion, the ability of onconase to induce internucleosomal chromatin fragmenta-

Fig. 3. Hallmarks of apoptosis triggered by onconase. A, HeLa cells were treated, where indicated, with 1 μg/ml onconase. Twenty-four h later, genomic DNA was prepared from the nuclei as described in “Materials and Methods.” Equal volumes of DNA samples were resolved in nondenaturing 2% agarose gels, and DNA was visualized by means of ethidium bromide contained in the gel and UV transillumination. Chromatin fragments resulting from internucleosomal cleavage were photographed as described in “Materials and Methods.” A representative case of nuclear pyknosis and fragmentation (karyorrhexis) is shown (inset). Magnification, ×64 (×256 for the inset). C, logarithmically growing p53null cells were treated, where indicated, with 1 μg/ml onconase for 16 h, after which time phase-contrast microscopy of living cells was used to detect membrane blebbing (inset). Magnification, ×64 (×256 for the inset).
Onconase-induced Apoptosis Does Not Require p53. In general, the absence of a functional wild-type p53 protein impedes the induction of apoptosis by certain DNA-damaging agents such as genotoxic drugs and ionizing or UV radiation (for reviews, see Refs. 29 and 30). Although the wild-type p53 gene is present in HeLa cells (49), the levels of p53 protein are kept very low in these cells due to its rapid degradation through the ubiquitin/proteasome pathway (50, 51). To address the possible requirement for a functional p53 protein in onconase-induced apoptosis, we used a mouse fibroblast cell line derived from p53<sup>−/−</sup> embryonic fibroblasts. These p53<sup>−/−</sup> cells are resistant to both UV radiation- and doxorubicin-induced cell death. However, when treated with onconase, the p53<sup>−/−</sup> cells displayed massive cell blebbing (Fig. 3C), nuclear pyknosis, DEVD-specific caspase activity, DNA fragmentation, and massive cell death (Fig. 3C and data not shown). Importantly, we did not observe significant differences between the responsiveness of p53<sup>−/−</sup> and p53<sup>+/+</sup> mouse fibroblasts to onconase (data not shown). These experiments conclusively demonstrated that the presence of a functional p53 protein is not required for onconase-induced apoptosis.

Partial Resistance of Onconase-induced Apoptosis and Caspase Activity to Inhibition by the Caspase Inhibitor zVADfmk. When treated with the anti-Fas agonistic antibody CH11, HeLa cells undergo a massive apoptosis within a 24-h period after treatment (Fig. 6A). This cell death is accompanied by a marked increase in DEVDase activity as measured by the specific cleavage of the p116 PARP, generating the two fragments p89 and p27 (Fig. 6B, compare Lanes 1 and 3). Both cell death and caspase activity induced by CH11 could be completely prevented by pretreatment of cells with the nonspecific caspase inhibitor zVADfmk (Fig. 6, A and B, compare Lanes 3 and 4). Because the CH11 dose used for these experiments (0.5 μg/ml) was approximately 10 times higher than the one required for maximum killing and PARP cleavage (data not shown), a conclusion can be safely reached that zVADfmk (at the concentration used) possesses a complete ability to prevent Fas-mediated apoptosis and caspase activity. However, when applied to pretreat cells before onconase treatment, the effect of zVADfmk was markedly different. zVADfmk was not able to completely prevent cell death but caused a shift to the right in the IC<sub>50</sub> of onconase by more than 1 log (Table 1, compare columns C and D). As expected, this IC<sub>50</sub> shift was not caused by an effect of zVADfmk on the ability of onconase to inhibit protein synthesis (Table 1, compare columns A and B). Rather, the IC<sub>50</sub> shift correlated well with a partial inhibition in onconase-induced DEVDase activity (Fig. 4A, compare top and bottom). Correlation analysis revealed that at IC<sub>50</sub> for inhibition of protein synthesis, onconase kills only 35% of the zVADfmk-pretreated cells, compared with 65% cell killing in the absence of zVADfmk (Table 1, compare columns E and F).

Does zVADfmk interfere with the part of onconase-induced cytotoxicity that results from inhibition of protein synthesis? To address this question, we investigated the effect of zVADfmk on emetine-induced cell death. First, we observed that a concentration of emetine that caused ~70% killing (0.3 μg/ml; data not shown) also caused DEVDase activity, as measured by PARP cleavage (Fig. 4B, top, Lane 4). This DEVDase activity was due to specific caspase activation because zVADfmk almost completely abolished the cleavage of PARP in response to emetine (Fig. 4B, bottom, Lane 4). However, in contrast to the effect of zVADfmk on onconase-induced cytotoxicity, the caspase inhibitor caused only a modest (4.5-fold) increase in the killing IC<sub>50</sub> for emetine (Table 1, columns C and D) and failed to significantly affect the cell killing induced by emetine at IC<sub>50</sub> for translation (Table 1, columns E and F). Taken together, the results presented in Figs. 2 and 4 and Table...
are consistent with the following two notions: (a) cell death caused by classical inhibitors of protein synthesis (emetine and cycloheximide) requires a significant inhibition of the cellular translation machinery, and, despite the appearance of active caspases, this cell death can proceed in the absence of caspase activity as well; and (b) onconase-induced cell death appears to involve a component that is probably independent of inhibition of protein synthesis and that is zVADfmk sensitive (see Fig. 9 and “Discussion”).

**Onconase-induced Apoptosis Is Not Mediated by Engaging the FasL/Fas Cell Death Cascade.** Recent studies have demonstrated that apoptosis in Fas-expressing cells subjected to DNA-damaging agents is triggered in part through drug- or radiation-induced activation of FasL production and cell surface presentation, followed by autocrine ligation of Fas (26–28). The results obtained using zVADfmk suggested that the mechanisms of onconase-induced cell death are different from those used by Fas ligation. However, the hypothesis could not be ruled out without directly determining whether onconase-induced apoptosis could be mediated in part by onconase-stimulated production of FasL and autocrine ligation of Fas. To test this hypothesis, we used an antagonistic anti-Fas monoclonal antibody, ZB4, that binds to Fas and prevents Fas agonists (such as FasL or CH11) from engaging the receptor and triggering apoptosis (52). Fig. 6C shows that preincubation of HeLa cells with ZB4 abrogated the cell death induced by CH11, whereas Fig. 6D demonstrates that the same pretreatment completely failed to inhibit onconase-induced apoptosis. We therefore concluded that onconase-induced apoptosis does not utilize the FasL/Fas death signaling pathway.

**Procaspases-9, -3, and -7, But Not Procaspase-1 or -8, Are Processed in Onconase-treated Cells.** To determine the caspases that are activated by onconase, we prepared cytosolic extracts from HeLa cells that were treated with Lipofectin alone or with Lipofectin and onconase for 0, 24, or 48 h and studied the processing of procaspase-1, -8, -9, -3, and -7. The use of caspase-1 as a negative control was determined by the fact that this caspase does not seem to be involved in mediating apoptosis (53–55). The (auto)processing of caspase-8 and -9 was detected by the decreased intensity in Western blot analysis of the respective band corresponding to the full-size procaspases. As expected, 24 h after onconase treatment, no significant caspase-1 cleavage was observed (Fig. 7A, compare Lanes 1 and 2 with Lanes 3 and 4). Similarly, both 24 h (data not shown) and 48 h after onconase treatment, the majority of caspase-8 was in the procaspase form (Fig. 7B, Lanes 3 and 4). In contrast, there was a massive processing of caspase-8 in cells treated for 48 h with CH11 (Fig. 7B, Lanes 1 and 2). Procaspase-9, however, displayed onconase-induced processing that was detectable both 24 and 48 h after the
treatment (Fig. 7A, compare Lanes 1–3 with Lane 4 and Fig. 7B, compare Lanes 3 and 4). Procaspase-9 was also processed in response to treatment of cells with CH11 (Fig. 7B, Lanes 1 and 2). Active caspase-9 can directly process two DEVDases, caspase-3 and its closest relative, caspase-7 (11); therefore, if the processing of procaspase-9 observed in onconase-treated cells leads to the generation of active caspase-9, then a subsequent processing of caspase-3 and -7 should be expected. Indeed, cells treated with onconase displayed processing of the procaspase forms and accumulation of the characteristic COOH-terminal $M_r$ 17,000 fragments that result from the specific processing of the $M_r$ 32,000 procaspase-3 and the $M_r$ 35,000 procaspase-7, respectively (Fig. 7A, Lane 4; data not shown for caspase-7). This proteolytic processing of procaspase-3 and -7 is likely to generate the active
Molecular weight markers are shown to the left.

A. processed as described in On, onconase-treated cells.

B. of Bax and cytochrome described in “Materials and Methods.” The abundance and distribution of Bax and cytochrome c were assessed in immunoblot assay. Molecular weight markers are shown to the left. Nonspecific bands are indicated (*). Co, control cells. pIC, polyI·polyC-treated cells.

On, onconase-treated cells. B. cells were treated with onconase and processed as described in A. To avoid overloading Lanes 1 and 2, only 20% of the cytosolic fraction was loaded into these lanes. To allow appropriate comparison of Lanes 1 and 2 with Lanes 5 and 6, the immunoblot signal (procaspase-9 and -8) in Lanes 1 and 2 should therefore be scaled up fivefold. Co, control cells. On, onconase-treated cells.

caspase-3 and -7 because DEVDase activities were detected by means of PARP cleavage in onconase-treated cells (Fig. 4A). Thus, procaspase-9, -3, and -7, but not procaspase-1 and -8, appeared to be activated by onconase.

Lack of Massive Release of Cytochrome c from Mitochondria into the Cytosol and of Translocation of Bax from the Cytosol onto Mitochondria in Response to Onconase. Release of cytochrome c from mitochondria is a key event for initiating a caspase-9/caspase-3 cascade and for amplifying the effectiveness of Fas ligation through a caspase-8-dependent activation of caspase-9 (56–58). Most notably, cytochrome c is a cofactor for caspase-9 activation (12). We therefore investigated whether cytochrome c (normally entirely sequestered in the mitochondria) is released into the cytosol on onconase treatment. Twenty-four h after treatment of HeLa cells with onconase, there was a detectable amount of cytochrome c in the cytosolic fraction compared to the absence of detectable cytochrome c in the Lipofectin-treated cells (Fig. 7C, top, compare Lanes 3 and 4). Consistently, however, significantly less cytochrome c was released from mitochondria into the cytosol in response to onconase than in response to Fas ligation (Fig. 7C, top, compare Lanes 2 and 4) and other proapoptotic stimuli including actinomycin D or UV radiation (both not shown; all agents were applied at doses that triggered similar levels of cell death). To find a possible explanation for the low level of cytochrome c release from mitochondria in onconase-treated cells, Recent studies have demonstrated that the transition of Bax from a soluble (cytosolic) form to a mitochondrial membrane-inserted form is sufficient and possibly required for the release of cytochrome c from apoptotic mitochondria, both in in vitro reconstitution systems and in vivo (14–20). In search of a positive control, we investigated the subcellular distribution of Bax in cells induced to undergo apoptosis in response to treatment with polyI·polyC, a mimic of double-stranded RNA. In HeLa cells, polyI·polyC treatment induces classical features of apoptosis (e.g., caspase activation).6 Importantly, polyI·polyC (under treatment conditions described in Fig. 8A), in HeLa cells, causes inhibition of protein synthesis with kinetics and amplitude similar to those induced by onconase and triggers a similar degree of cell death within 24 h after treatment.6 For instance, in the experiment shown in Fig. 8A, polyI·polyC caused ~70% cell death, and onconase caused >65% cell death 24 h after the treatment. Fig. 8 shows that in the control cells, the majority of Bax protein was found in the cytosol, with a minor fraction found attached to sedimentable structures that were soluble in Triton X-100 (Fig. 8A, top, compare Lanes 3 and 4). Six h after polyI·polyC treatment, there was a dramatic redistribution of Bax; the protein levels were almost undetectable in the cytosol and were greatly increased in the Triton X-100-soluble organellar fraction (Fig. 8A, top, compare Lanes 2 and 5). In contrast, in onconase-treated cells, the levels of Bax protein were substantially decreased in both the cytosolic and the organellar fractions, and there were no indications of a subcellular redistribution of Bax (Fig. 8A, top, compare Lanes 3 and 6). The disappearance of Bax from onconase-treated cells was almost complete 24 h after the treatment (data not shown). The differences in the subcellular location of Bax in polyI·polyC- and onconase-treated cells correlated with even more dramatic differences

6 M. S. Iordanov and B. E. Magun, unpublished results.
in the location of cytochrome c. In the control cells, the majority of cytochrome c was found in the Triton X-100-insoluble organellar fraction (Fig. 8A, bottom, compare Lanes 1 and 7). After polyI:C polyC treatment, the Triton X-100-insoluble organellar fraction was substantially depleted of cytochrome c, and the levels of cytochrome c were dramatically increased in the cytosol (Fig. 8A, bottom, compare Lanes 2 and 8). In contrast, in onconase-treated cells, there was no detectable release of cytochrome c 6 h after the treatment (Fig. 8A, bottom, compare Lanes 3 and 9). The results shown in Figs. 7 and 8A raised the possibility that a yet-to-be-identified cytochrome c-independent mechanism for caspase-9 activation is triggered in cells in response to onconase. Recently, it was discovered that a substantial portion of procaspase-9 is sequestered in mitochondria (59, 60). On apoptotic stimulation, mitochondrial procaspase-9 was found to translocate to the cytosol and undergo a subsequent proteolytic activation there (59, 60). Therefore, using the approach described in Fig. 8A, we investigated whether procaspase-9 undergoes translocation to the cytosol from an organellar (presumably mitochondrial) storage site before its proteolytic activation in response to onconase treatment (Fig. 7, A and B). Indeed, the organellar fraction of onconase-treated HeLa cells displayed decreased levels of procaspase-9 6 h after the treatment (Fig. 8B, top panel, compare Lanes 5 and 6), concomitant with a detectable increase in the levels of soluble cytosolic procaspase-9 (Fig. 8B, top panel, compare Lanes 1 and 2). In contrast, procaspase-8 was found to be entirely cytosolic (Fig. 8B, bottom panel, Lanes 1 and 2) and, as described above (Fig. 7, A and B), unchanged in response to onconase treatment.

**DISCUSSION**

Onconase is a prototypic member of a growing family of endoribonucleases that are found in the bodily fluids of vertebrates from amphibians to mammals and that seem to have emerged as part of evolutionary ancient antiviral and antiparasitic defense systems (37). The most interesting property of onconase is its potential for use as a nonmutagenic alternative for (or a supplement to) the conventional DNA-damaging therapy of cancer. Two major directions of research could contribute to the realization of this potential: (a) increasing the target cell specificity and efficiency of delivery of onconase; and (b) understanding the mechanisms of onconase-induced cytotoxicity. In this work, we have begun to address the question of the intracellular mechanisms used by onconase to kill target cells. The most important finding we report here is that on internalization, onconase elicits an apoptotic death signal that seems to be, in part, independent of inhibition of protein synthesis.

**Apoptotic Mode of Cell Death Induced by Onconase.** Adverse toxins are known to trigger cytolysis via nonapoptotic mechanisms. One such example is the drug capsaicin, the pungent ingredient in chili peppers, that has a therapeutic potential for targeted killing of primary afferent neurons (61, 62). However, a nonapoptotic mode of cell death could hamper the potential application of onconase in the therapy of cancer because nonapoptotic cell death could produce severe inflammatory and immune complications in patients. Although previously reported as apoptotic (63, 64), we present, for the first time, a detailed characterization of the mode of cell death induced by onconase, demonstrating that onconase-treated cells display classical hallmarks of apoptosis such as chromatin fragmentation, nuclear pyknosis and karyorrhexis, plasma membrane blebbing, and activation of caspases.

**The Nature of the Onconase-induced Death Signal(s): Possible Involvement of tRNA?** Previous studies have demonstrated that the cytotoxicity of onconase invariably depends on its catalytic capacity as a ribonuclease (33, 40). The data presented in this work prompt a speculation that the major determinant of onconase-induced cytotoxicity is a death signal that is generated in response to induced RNA hydrolysis. This signal is generated even when concentrations of onconase are applied that are otherwise insufficient for severe inhibition of translation. The only RNA population significantly affected by onconase in HeLa and mouse fibroblast cells appeared to be tRNA (Fig. 1; data not shown for fibroblasts). This is in agreement with our previous findings that onconase preferentially degrades tRNA in vitro in reticulocyte lysates and when injected into Xenopus laevis oocytes (34). Therefore, the most likely candidate for an apoptosis-signaling intermediate is onconase-damaged tRNA. Although we cannot exclude the existence of certain onconase-sensitive mRNAs, we consider this possibility unlikely. The coding region of cyclophilin mRNA contains 67 potential sites for hydrolysis by onconase (5′-UpG-3′), yet apparent hydrolysis of this highly abundant mRNA was undetectable in onconase-treated cells (Fig. 1). Curiously, onconase easily cleaves rRNA and diverse mRNAs (including cyclophilin mRNA) in vitro (data not shown). This finding demonstrates that intracellular conditions determine the specificity of onconase in vivo. Among the possible candidates for such specific conditions, we consider the proper subcellular localization of both target RNAs and onconase and/or specific posttranscriptional modification(s) of onconase. These possibilities are currently being explored.

**The Complex Nature of Onconase-induced Cell Death.** A hypothetical model of the cytotoxic pathways triggered by onconase is presented in Fig. 9 and is discussed below. One of the first effects of onconase on target cells is the degradation of cellular tRNA. An inevitable effect of sustained tRNA degradation is inhibition of protein synthesis. Prolonged inhibition of translation, in turn, will induce cell death that results, in part, from caspase-mediated apoptosis (see, for instance, the example with emetine, Figs. 4B and 5A). However, three lines of evidence suggest that onconase-induced apoptosis does not entirely result from inhibition of protein synthesis. First, whereas
antibiotic inhibitors of translation (as exemplified in Fig. 5A by emetine) induce apoptotic cell death with relatively slow kinetics, onconase triggered a markedly early apoptosis (as measured by the appearance of TUNEL-positive cells; Fig. 5A). Second, emetine and cycloheximide required significantly higher levels of inhibition of translation to induce cell death than onconase (Table 1; data not shown for cycloheximide). Third, zVADfmk, although almost entirely abolishing DEVDase activity induced by emetine (Fig. 4B), could not significantly protect cells from emetine-induced cell death (Table 1, compare columns E and F). It is likely that in the presence of zVADfmk, the emetine-treated cells die from secondary necrosis, a form of cell death that has been recently postulated for cells in which certain aspects of apoptosis are impaired or proceed in an atypical way (65). Onconase-induced DEVDase activity was, overall, similarly susceptible to zVADfmk (Fig. 4), but the inhibitor increased the killing IC50 for onconase by more than 1 log (Table 1, compare columns C and D) and efficiently reduced the cytotoxic potential of onconase at IC50 for translation (Table 1, compare columns E and F).

In contrast to both onconase and antibiotic inhibitors of protein synthesis, cell death and DEVDase activation in response to Fas ligation are completely inhibited by zVADfmk (Fig. 6, A and B). The most obvious mechanistic difference between Fas- and onconase-induced cell death is the inability of Fas ligation to inhibit protein synthesis in HeLa cells (data not shown). Our results are therefore consistent with the notion that in addition to the mode of cell death that is dependent on protein synthesis inhibition (and shared by any inhibitor of translation in HeLa cells), onconase triggers a complementary proapoptotic mechanism that is zVADfmk sensitive. This complementary proapoptotic mechanism activates caspase-9 and -3/7 but is distinct from the classical Fas-induced pathway (FasL/Fas → FADD → caspase-8 → Bid → Bax → cytochrome c → caspase-9 → caspase-3). Furthermore, our results (Figs. 6C and 8) also raised the possibility of a Bax- and cytochrome c-independent mechanism of caspase-9 activation in cells treated with onconase. Alternatively, it is conceivable that feedback self-amplifying caspase “loops” are involved in executing the onconase-induced cell death program. Subsequent to its processing and activation by caspase-9, caspase-3 can process and activate additional molecules of caspase-9, thus leading to the self-amplification of the caspase cascade (11). We have detected caspase-3/7 (DEVDase) activity as early as 1–2 h after onconase application (data not shown). At these time points (and up to 6 h, see Fig. 8A), we were unable to detect a measurable release of cytochrome c. However, it is possible that low levels of cytochrome c release and active caspase-9 (below the sensitivity of the assays used here) lead to the initial increase in DEVDase activity and that these DEVDases, in turn, process more procaspase-9, thereby amplifying the caspase-9/ caspase-3,-7 cascade without further involvement of Bax or cytochrome c.

Onconase in Comparison with Other Cytotoxic Enzymes Used for Designing Anticancer Immunotoxins. Experimental evidence from different laboratories shows that the mechanisms of cytotoxic actions of natural toxins used to design novel therapeutic agents with anticancer properties (such as ricin A chain and Pseudomonas exotoxin A) are more complex than mere of inhibition of protein synthesis, a property which these agents have in common (see Ref. 41 and references therein). For instance, Keppler-Hafkemeyer et al. (66) have investigated the mode of cell death triggered by a genetically engineered immunotoxin containing Pseudomonas exotoxin A as a cytotoxic agent. Pseudomonas exotoxin A causes inhibition of protein synthesis through inactivation of translation elongation factor EF-2 (see Ref. 41 and references therein). Similar to our findings using onconase, Keppler-Hafkemeyer et al. have found that the antibody-Pseudomonas exotoxin A fusion protein also triggered apoptosis and activated caspases (66). However, zVADfmk has been inefficient in preventing Pseudomonas exotoxin A-induced cell death, despite the ablation of apoptotic morphology (66). In this respect, Pseudomonas exotoxin A displayed a behavior more reminiscent of emetine (as described in this work) than of onconase (see Table 1). Therefore, in light of the possible use of both cytotoxic enzymes for immunotoxin design, future parallel studies are required to investigate whether onconase and Pseudomonas exotoxin A use the same or different cytotoxic mechanisms.

Lack of p53 Dependence of the Onconase-induced Apoptotic Program: Possible Implications in Therapy. We demonstrate in this work that RNA damage might be a physiologically relevant death signal in mammalian cells. Similar to DNA damage, this signal is ultimately communicated to caspases involved in inducing an apoptotic phenotype. However, unlike DNA damage, the ability of RNA damage to induce cell death is not affected by the presence or the absence of a functional p53 protein (Fig. 3C and text). The vast majority of human tumors have inactivated the function of p53, either through acquired or inherited mutation in one allele, followed by loss of heterozygosity or by altering the expression or function of critical p53 regulators, such as the human homologue of mdm-2. Increased resistance of some tumors with inactivated p53 to conventional DNA-damaging therapy has been reported (67, 68). Therefore, both the p53 independence and the nonmutagenicity of RNA damage make RNA an attractive target for developing novel proapoptotic anticancer strategies.

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REFERENCES

ONCOSE-INDUCED APOPTOSIS


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