Induction of an apoptotic program in cell-free extracts by 2-chloro-2'-deoxyadenosine 5'-triphosphate and cytochrome c

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ABSTRACT Adenine deoxynucleosides, such as 2-chloro-2'-deoxyadenosine (2CdA) induce apoptosis in quiescent lymphocytes, and are thus useful drugs for the treatment of indolent lymphoproliferative diseases. However, it has remained puzzling why deoxyadenosine and its analogs are toxic to a cell that is not undergoing replicative DNA synthesis. The present experiments demonstrate that the 5'-triphosphate metabolite of 2CdA (2CdA-5'-triphosphate), similar to dATP, can cooperate with cytochrome c and Apaf-1 to activate caspase-3 in a cell free system. Chronic lymphocytic leukemia cells and normal peripheral blood lymphocytes expressed both caspase-3 and apoptosis promoting factor 1. Incubation of the lymphocytes with 2CdA induced caspase-3 activation prior to DNA degradation and cell death. Stimulation of the caspase proteolytic cascade by 2CdA-5'-triphosphate, in the context of DNA strand break formation, may provide an explanation for the potent cytotoxic effects of 2CdA toward nondividing lymphocytes.

The effectiveness of cancer chemotherapy often depends upon the induction of apoptosis in malignant cells. Among antitumor agents, the 2'-deoxyadenosine congeners 2-chloro-2'-deoxyadenosine (2CdA, cladribine) and 9-β-D-arabinofuranosyl-2-fluoroadenine (fludarabine) have the ability to induce apoptosis in nondividing lymphocytes, at concentrations that spare other cell types (1, 2). Fosmidomycin and 2-cytosine deoxynucleosine analogs also have achieved an important place in the treatment of indolent lymphoid malignancies, including hairy cell leukemia, chronic lymphocytic leukemia (CLL), and low grade lymphomas (2, 3).

The cytotoxicity of 2CdA depends mainly upon the selective and progressive accumulation of its 5'-triphosphate metabolite (2CdATP) in lymphocytes that have a high ratio of deoxycytidine kinase (EC 2.7.1.74) to cytosolic 5'-deoxyadenosine-5'-triphosphate, similar to dATP, can cooperate with cytochrome c and Apaf-1 to activate caspase-3 in a cell free system. Chronic lymphocytic leukemia cells and normal peripheral blood lymphocytes expressed both caspase-3 and apoptosis promoting factor 1. Incubation of the lymphocytes with 2CdA induced caspase-3 activation prior to DNA degradation and cell death. Stimulation of the caspase proteolytic cascade by 2CdA-5'-triphosphate, in the context of DNA strand break formation, may provide an explanation for the potent cytotoxic effects of 2CdA toward nondividing lymphocytes.

Various stimuli of apoptosis lead to the activation in the cytoplasm of cysteine proteases with specificity for aspartic acid residues, referred to as caspases. The activated caspases can cleave structural proteins and enzymes necessary for the survival of both proliferating and resting cells (reviewed in refs. 5–7). In addition, caspases have been shown to activate the endonuclease responsible for the internucleosomal cleavage of genomic DNA, a hallmark of apoptosis (8, 9).

One important component of the caspase cascade is caspase-3, which is activated by two sequential proteolytic events that cleave the 32-kDa precursor at aspartic acid residues to generate an active heterodimer of 20- and 12-kDa subunits (10). The activation can either be autocatalytic, or occur via a caspase cascade, similar to the serine protease cascade in the blood clotting process (7). In susceptible cells, caspase activation might amplify preexisting but sublethal apoptotic signals, leading to rapid and irreversible proteolysis.

Recently, Wang and coworkers established a cell free system in which caspase-3 activation in the cytosol is induced by the addition of dATP and cytochrome c (11–13). Three protein factors, designated apoptotic protease activating factors (Apafs), are necessary and sufficient to reconstitute dATP-dependent caspase-3 activation. Apaf-2 has been identified as cytochrome c, and Apaf-3 as caspase-9. Caspase-3 activation begins when caspase-9 (Apaf-3) binds to Apaf-1 in a reaction stimulated by cytochrome c and dATP (11). Because of the structural similarity between dATP and 2CdATP, and the important role of 2CdA in the treatment of indolent lymphoid malignancies, we designed experiments to address the possibility that 2CdATP directly induces caspase-3 proteolysis in a cell free system, and to verify that caspase-3 proteolytic activation occurs in viable lymphocytes and CLL cells exposed to 2CdA in vitro.

MATERIALS AND METHODS

Synthesis of 2CdATP. 2-Chloro-2'-deoxyadenosine-5'-triphosphate (2CdATP) was prepared by a modification of the general procedure for nucleoside triphosphate synthesis (14). Briefly, unprotected (2-CdA) was phosphorylated with POCl3 in trimethyl phosphate, followed by treatment of the 5'-phosphorodichloridate intermediate with tri-n-butylammonium pyrophosphate in dimethyl formamide. The reaction mixture was neutralized with cold 1.0 M of triethylammonium bicarbonate (pH 8.5) and was chromatographed on a DEAE-Sephadex A-25 column with water and then a linear gradient of triethylammonium bicarbonate. The 2-CdATP was eluted at about 0.8 M of triethylammonium bicarbonate, dried, and stored at −20°C.

Cell Isolation and Analysis. Heparinized peripheral blood samples from normal subjects, or patients with CLL containing at least 80% malignant cells, were fractionated by Ficoll/Hypaque sedimentation. Nonadherent mononuclear cells were resuspended in complete medium (RPMI 1640 medium supplemented with 10% fetal bovine serum) at a density of 1 to 2 × 10⁶ per ml. Cells were incubated at 37°C in an atmosphere of 5% CO₂ with 1 μM of 2CdA or 10 μM of dexamethasone for up to 24 hr, as indicated.

Preparation of HeLa and CLL Extracts. Human HeLa S3 cells (American Type Culture Collection, Manassas, VA; CCL 22) were grown in complete medium (DMEM supplemented with 10% fetal bovine serum) at 37°C in an atmosphere of 5% CO₂/95% air. At 80% confluence, the culture flasks were placed on ice, washed twice with ice-cold isotonic PBS (pH 7.4), and harvested using a cell scraper. Cells were isolated as described.

Abbreviations: 2CdA, 2-chloro-2'-deoxyadenosine; 2CdATP, 2-chloro-2'-deoxyadenosine 5'-triphosphate; CLL, chronic lymphocytic leukemia; Apaf, apoptotic protease activating factor; Δψm, mitochondrial transmembrane potential; PI, propidium iodide; pNA, p-nitroanilide; DOCC₅₀, 3',3'-dihexyloxacarbocyanine iodide; DEVD, Asp-Glu-Val-Asp.

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scribed above. Cells were then washed at 4°C and resuspended in a hypotonic extraction buffer (HEB; containing 50 mM Pipes/50 mM KCl/5 mM EGTA/2 mM MgCl2/1 mM DTT/0.1 mM phenylmethanesulfonyl fluoride). The cells were centrifuged at 1,000 × g to form a tight pellet, and the volume of the cell pellet was approximated. The supernatant was discarded and HEB buffer was added to a volume between 0.5 and 1 × the pellet volume. The cells were allowed to swell for 20–30 min on ice and then lysed in a Dounce homogenizer with 100 strokes of a B-type pestle. The extent of lysis was monitored under the microscope by trypan blue staining. The cell lysate was centrifuged for 30 min at 100,000 × g. The clarified supernatant was used immediately or stored in aliquots at −80°C. The cytoplasmic fraction did not contain microscopically visible whole cells, nuclei, or mitochondria.

**Immunoblot assay for Nucleotide-Induced Caspase-3 Activation.** HeLa and CLL extracts were prepared as described above. Ten microliter aliquots (100 µg of protein) were incubated with the indicated nucleotides, and 10 µM of cytochrome c from bovine heart, at 30°C for 1 h in 15 µl with HEB buffer. At the end of the incubation, 5 µl of 4× SDS sample buffer were added to each reaction. After boiling for 5 min, each sample was subjected to 14% Tris-glycine SDS/PAGE. Caspase-3 was revealed by immunoblotting as described below.

**Colorimetric Assay for Nucleotide-Induced Caspase-3 Activation.** HeLa extracts were clarified by 0.2 µm filtration, then 10 µl of extracts (100 µg protein) were incubated in a 96-well plate with the indicated nucleotides, 10 µM of cytochrome c, and 100 µM of DEVD-p-nitroanilide (pNA), at 37°C for 30–60 min, in 50 µl with HEB buffer. The hydrolysis of the substrate was followed spectrophotometrically at 405 nm, in a Molecular Devices MAXline Microplate Spectrophotometer (Menlo Park, CA). Baseline absorbance values from reactions without specificity of the assay was validated by using the caspase-3 inhibitor Ac-DEVD-CHO at 1 μM. DEVD-pNA [sequence; N-acetyl-Asp-Glu-Val-Asp-pNA (15)] and Ac-DEVD-CHO (16) were purchased from Calbiochem.

**Immunoblotting.** Washed cell pellets were lysed in 2× SDS/PAGE sample buffer containing 10 mM DTT, for 5 min at 100°C. Alternatively, washed cells were lysed in RIPA buffer [50 mM Tris-HCl/50 mM NaCl, pH 7.4/1 mM EGTA/0.5% (vol/vol) Nonidet P-40/1 µg/ml aprotinin/1 µg/ml leupeptin/1 mM phenylmethylsulfonyl fluoride]. Lysates were centrifuged at 15,000 × g for 10 min to remove nuclei and the protein content of supernatants was measured by using a modified Coomassie blue assay (Pierce). Proteins were resolved at 125 V on 14% gels and electrophoretically transferred to 0.2 µm of polyvinylidene fluoride membranes (Millipore) for 2 h at 125 V. Membranes were blocked overnight in 1-Block blocking buffer (Tropix, Bedford, MA). Blots were then probed for 1 h with antibodies to caspase-3 (Transduction Laboratories, Lexington, KY), or to Apaf-1 (11). The blots were developed with species-specific anti sera, and visualized by alkaline phosphatase-based enhanced chemiluminescence (Tropix, Bedford, MA), according to the manufacturer’s instructions. The x-ray films were scanned, acquired in Adobe Systems (Mountain View, CA) PHOTOSHOP, and analyzed with National Institutes of Health IMAGE software.

**Measurement of DNA Fragmentation.** DNA fragmentation was assessed by flow cytometry and electrophoresis. Prior to analysis by flow cytometry, cells were fixed in ice-cold 70% ethanol, and incubated with 100 µg/ml of RNase A and 50 µg/ml PI for 1 h at 37°C. Hypodiploid cells were visualized using a Becton Dickinson FACScalibur, and the program MODFIT LT 2.0 (Verity Software House, Topsham, TX).

Prior to electrophoresis, cells were resuspended for 20 min in hypotonic lysis buffer containing 10 mM Tris-HCl, 50 mM NaCl, 10 mM EDTA and 0.2% Triton X-100 (pH 7.5). After centrifugation at 14,000 × g for 10 min, and proteinase K digestion, DNA was precipitated at −20°C in 2 volumes of isopropanol and 0.5 M of NaCl. The pellet was washed and resuspended in 10 mM of Tris-HCl, 10 mM of EDTA, and the samples were then electrophoresed for 2 h at 60 V in 2% agarose with 0.5 µg/ml ethidium bromide. An HaeIII digest of pX174 provided molecular mass standards. After electrophoresis, the gels were photographed on a UV transilluminator.

**Cytosolic Fluorometric Analysis of Mitochondrial Transmembrane Potential (ΔΨm) by 3,3’ Diethyloxacarbocyanine Iodide (DiO[Cl] and Cell Membrane Permeability by PI.** Cells were treated with the indicated amount of 2CdA and 10 µM of the cell-permeable caspase-3/caspase-7-selective inhibitor Ac-DEVD-fmk (sequence; N-acetyl-Asp-Glu-Val-Asp-fluoromethylketone, Enzyme Systems Products, Livermore, CA). Cells were then incubated for 10 min at 37°C in culture medium containing 40 nM of DiOC6 (Molecular Probes, Eugene, OR) and 5 µg/ml PI (Molecular Probes), followed by analysis within 30 min of fluorescence in a Becton Dickinson FACScalibur cytofluorometer. After suitable compensation, fluorescence was recorded at different wavelengths: DiOC6 at 525 nm (FL-1) and PI at 600 nm (FL-3).

**RESULTS**

**Caspase-3 Activation by 2CdATP.** Liu et al. have reported that the addition of cytochrome c and dATP to cytosolic extracts induces the processing of caspase-3 to active forms capable of inducing apoptotic cell death (12). To determine if 2CdATP was able to replace dATP, a similar cell free system was established using HeLa cells and CLL cells. The addition of cytochrome c (0.2 µg per reaction) and dATP was required for the activation of caspase-3 in both HeLa and CLL extracts (Fig. 1A and B), as demonstrated by the disappearance of the 32-kDa band and the appearance of a 20-kDa band, which represent the cleaved active fragment of caspase-3. dATP alone was not enough to activate caspase-3, probably because during the preparation of the cytosolic extract, the mitochondria were not damaged and did not release sufficient cyto-

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**Fig. 1.** Nucleotide-induced caspase-3 cleavage in cell free extracts. (A) Aliquots (10 µl) of HeLa cell extracts (100 µg) were incubated in the presence (lanes 1, 2, 4, 6, and 8) or absence (lanes 3, 5, and 7) of cytochrome c (10 µM) and various nucleotides (1 mM). Control lane (lane 1), dATP (lane 2), 2CdA (lane 3 and 4), 2CdAMP (lane 5 and 6), and 2CdATP (lane 7 and 8). (B) Aliquots (10 µl) of CLL cell extracts (100 µg) were incubated in absence (Control) or in the presence of cytochrome c (10 µM) and various nucleotides (1 mM), as indicated. Samples were subjected to SDS/PAGE, transferred to a polyvinylidene fluoride membrane, and probed with an anti-caspase-3 antibody.
In the presence of cytochrome c, 2CdATP also induced caspase-3 cleavage, while 2CdA had no effect. The minimal activation of caspase-3 by 2CdAMP may be due to its conversion to 2CdATP by kinases present in the cell-free extracts, thus mimicking the process that occurs in vivo in cells exposed to 2CdA. Other nucleotides were also tested in the CLL extracts (Fig. 1B). 9-β-D-Arabinofuranosyl ATP appeared to be the most potent inducer of caspase-3 activation, followed by dATP and 2CdATP. Under these conditions no caspase-3 cleavage was observed in extracts containing ATP, 2CdA, 9-β-D-arabinofuranosyl-CTP and 2',3'-dideoxy ATP. The quantification of caspase-3 activation by the colorimetric enzyme assay (Fig. 2) showed that dATP is more potent than 2CdATP, especially at concentrations below 100 μM. At concentrations of 1 mM and above, both nucleotides showed similar activities, and were more potent than ATP. The maximal caspase-3 activity induced by ATP was ~60% of the maximal activity induced by dATP and 2CdATP.

Caspase-3 and Apaf-1 in Normal Lymphocytes and CLL. In light of the results obtained in the cell free system, it was important to determine if caspase-3 and Apaf-1 were expressed in CLL cells and normal lymphocytes. Immunoblotting revealed that both components of the caspase cascade were detectable. The expression levels of Apaf-1 were approximately equivalent in the normal and malignant lymphocytes (Fig. 3B). In contrast, the expression level of caspase-3 was higher in CLL cells than in normal cells (Fig. 3A). Densitometric quantitation showed that the mean caspase-3 level in CLL cells was about twice that of normal peripheral blood lymphocytes.

Caspase Activation by 2CdA in Normal Lymphocytes and CLL. Previous work has shown that 2CdA induces internucleosomal DNA cleavage characteristic of apoptosis in sensitive CLL cells (17–19). To determine if the fragmentation was preceded by caspase-3 activation, cells from CLL patients were treated for 24 h with 1 μM 2CdA, and the time courses of caspase-3 and DNA degradation were compared. Leukemic lymphocytes treated with 1 μM 2CdA showed a gradual appearance of cells with a hypodiploid DNA content (Fig. 4). Agarose gel electrophoresis similarly demonstrated increasing amounts of the DNA fragments in an oligonucleosomal pattern (data not shown). Internucleosomal cleavage did not become prominent until 16 h of exposure. Untreated CLL cells also developed some spontaneous apoptosis after 24 h culture.

Immunoblotting experiments showed that freshly isolated CLL cells have already a small amount of cleaved caspase-3 (Fig. 4), as opposed to lymphocytes from normal donors, which did not show any detectable cleavage products (data not shown). 2CdA incubation substantially increased the caspase activation between 4 and 8 h of incubation, after the induction of DNA single strand breaks (20), but before endonuclease activation became prominent. Thus, significant cytoplasmic caspase-3 activation preceded nucleosomal degradation and cell death by 8 h in 2CdA-treated CLL cells.

Effect of 2CdA on Mitochondria. To determine the effect of 2CdA treatment on the Δψm of freshly isolated CLL cells, the fluorochrome DiOC6 was used. The reduction of Δψm has been shown (21) to precede nuclear DNA fragmentation in lymphocyte apoptosis induced by dexamethasone. Either short-term treatment (24 h) of CLL cells with 1 μM 2CdA or long-term (72 h) treatment with 50 nM 2CdA reduced the DiOC6 fluorescence (Fig. 5 C and D). There was a concomitant increase in PI binding to DNA, reflecting a loss of membrane permeability and in the percentage of hypodiploid apoptotic cells. Untreated CLL cells incubated for 24 h had only 1% apoptotic cells, but 13% of low Δψm cells and 8%...
incubated for the times indicated with 50 nM or 1 M of the cell-permeable caspase-3/7-selective inhibitor Ac-DEVD-fmk. Cell membrane permeability and apoptosis. Freshly isolated CLL cells were then assayed by incubating the unfixed cells for 10 min at 37°C in presence of 5 µg/ml PI and 40 nM DiOC6. DNA fragmentation was estimated by flow cytometry after PI staining of fixed cells. In the large graphs, the x-axis is DiOC6 (FL-1) fluorescence, and the y-axis is PI (FL-3) fluorescence. Numbers refer to the percentages of cells in the upper-left quadrant (live cells), the lower-right quadrant (low Δφm), and the lower-left quadrant (normal cells). The small graphs (insets) represent the DNA content of the cells from parallel cultures determined by PI staining of permeabilized cells, and the numbers refer to the percentages of hypodiploid (apoptotic) cells.

PI-positive cells (Fig. 5A). After 72 h of incubation, 20% of the untreated cells were apoptotic, and 19% were PI-positive, and 8% showed a reduced Δφm (Fig. 5B). Thus, a fall in Δφm staining was a sensitive early marker of both drug-induced and spontaneous apoptosis in cultured CLL cells. The incubation of the cells with the cell-permeable caspase-3/caspase-7-selective inhibitor Ac-DEVD-fmk at 10 µM inhibited completely the appearance of apoptotic cells (Fig. 5 E and F). The loss of Δφm was also noticeably reduced, but not blocked completely. Approximately 10% of cells treated with 50 nM 2CdA and with the caspase inhibitor still displayed a low Δφm.

DISCUSSION

The toxicity of 2CdA depends on its intracellular phosphorylation to 2CdATP by the tandem action of deoxyctidine kinase, AMP kinase, and nucleoside diphosphate kinase (22, 23). Because 2CdAMP is dephosphorylated back to the nucleoside by a cytosolic 5′-nucleotidase, both the in vitro sensitivity of cultured leukemia cells (1) and the in vivo response of patients with CLL to 2CdA (4) correlate with the ratio of deoxyctidine kinase to 5′-nucleotidase activities. Because CLL cells divide very slowly, and cell volume remains stable, 2CdATP accumulates in cells with a high kinase to nucleotidase ratio until a new equilibrium is achieved, or death ensues. In patients given 2CdA orally (10 mg/m2), intracellular 2CdATP levels reached 10 µM after 3 h, within cells cultured with 1 µM 2CdA the intracellular 2CdATP levels reached 70 µM (24).

How 2CdATP induces apoptosis in nondividing cells is not yet known. The nucleotide inhibits ribonucleoside diphosphate reductase (25), DNA polymerases α and β (26), DNA ligase, and is incorporated into DNA (27). Together, these actions lead to the progressive accumulation of DNA single-strand breaks (20). The strand break formation by itself would not be expected to kill a noncytosing cell. However, DNA strand breaks induce the activation of poly(ADP ribose)polymerase, with resultant consumption of NAD, and of total adenine nucleotides. The addition of nicotinamide to lymphocyte cultures exposed to 2CdA can delay the onset of cell death, by inhibiting poly(ADP ribose) formation, maintaining adenosine nucleotides, and replenishing NAD (20, 28).

In the present study, 2CdATP and cytochrome c initiated an apoptotic program in cell free cytosolic extracts, from both HeLa and CLL cells, as measured by the activation of caspase-3. Exposure of viable normal or CLL lymphocytes to 2CdA also induced caspase-3 activation. Time course analysis indicated that caspase activation occurred early, at the same time as DNA strand break formation, but before internucleosomal cleavage became prominent. The inhibition of caspase enzymatic activity by the cell-permeable caspase-3/caspase-7-selective inhibitor Ac-DEVD-fmk blocked 2CdA-induced apoptosis and partially prevented the 2CdA-induced reduction of the (Δ[Δm]) of freshly isolated CLL cells.

Recently, Liu and coworkers (8) identified in HeLa cytosol an heterodimeric protein of 45 kDa and 40 kDa, termed DNA fragmentation factor (DFF) that functions downstream of caspase-3 to trigger DNA fragmentation. A similar protein was found in the cytoplasm of mouse lymphocytes by Enari et al. (9). By cleavage of this inhibitor of caspase-activated DNase (ICAD), caspase-3 activates the caspase-activated DNase (CAD), enabling it to migrate to the nucleus and degrade DNA.

In most cell types, DNA strand break formation causes the activation of both poly(ADP ribose)polymerase and p53 dependent pathways (reviewed in refs. 29–32). In quiescent cells poly(ADP ribose)polymerase activation can induce an abrupt drop in NAD and in total adenine nucleotide pools (20). The activation of p53 has been shown to increase the synthesis of enzymes that generate or respond to oxidative stress (33). The combination of reduced adenine nucleotides and increased oxidative stress may impair the function of the F1F0-ATPase in the inner mitochondrial membrane, thereby promoting the release of cytochrome c into the cytoplasm (34, 35). The released cytochrome c could work together with Apaf-1, caspase-9, and increasing concentrations of adenine deoxynucleoside 5′-triphosphates to start a feed forward amplification cascade of caspase activation.

In summary, the data are consistent with the following mechanism of action of adenine deoxynucleosides in resting lymphocytes (Fig. 6). The deoxynucleoside enters cells and is converted progressively to its active 5′-triphosphate form, which causes DNA strand break formation, activating poly(ADP ribose)polymerase and p53. In consequence, NAD and total adenine nucleotides decrease, oxidative stress increases, and mitochondrial integrity wanes. In untreated cells, the concentrations of ATP and cytochrome c in the cytoplasm are insufficient to trigger the caspase cascade. However, the binding of Apaf-1 to caspase-9, and caspase-3, in the presence of high concentrations of adenine deoxynucleoside 5′-triphosphates and small amounts of released cytochrome c, leads to the cleavage of caspase-3, converting it to an active autocatalytic protease, in a process analogous to blood clotting.
The active caspase-3 then stimulates, in turn, the CAD endonuclease that irreversibly degrades DNA. This model suggests that the baseline levels of caspase activation, in addition to the rate of adenine deoxynucleoside 5'-triphosphate formation, may be a factor that influences sensitivity to 2CdA, fludarabine, and deoxycoformycin chemotherapy. Malignant lymphocytes that display greatest sensitivity to 2CdA, fludarabine, and deoxycoformycin chemotherapy, may undergo spontaneous apoptosis, due to a subthreshold level of oxidative stress. In untreated cells, the concentrations of ATP and cytochrome c (c.c.) in the cytoplasm are insufficient to trigger the caspase cascade. However, the binding of Apaf-1 to caspase-9, in the presence of 2CdATP and small amounts of released cytochrome c leads to the cleavage of caspase-3, converting it to an active autocatalytic protease, in a process analogous to the blood clotting. The active caspase-3 then stimulates, in turn, the CAD endonuclease that irreversibly degrades DNA.

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Fig. 6. Activation of the apoptotic pathway by adenine deoxynucleosides. An adenine deoxynucleoside, such as 2CdA, enters cells and is converted progressively to its active 2CdATP. The 2CdATP causes DNA strand break formation, activating poly(ADP ribose) polymerase and p53, with resultant depletion of NAD and adenine nucleotides, and a concomitant increase in oxidative stress. In untreated cells, the concentrations of ATP and cytochrome c (c.c.) in the cytoplasm are insufficient to trigger the caspase cascade. However, the binding of Apaf-1 to caspase-9, in the presence of 2CdATP and small amounts of released cytochrome c leads to the cleavage of caspase-3, converting it to an active autocatalytic protease, in a process analogous to the blood clotting. The active caspase-3 then stimulates, in turn, the CAD endonuclease that irreversibly degrades DNA.