3,4-Dichloroisocoumarin Serine Protease Inhibitor Induces DNA Fragmentation and Apoptosis in Susceptible Target Cells (44325)

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Abstract. **3,4-Dichloroisocoumarin (DCI) inhibition of serine proteases generates reactive intermediates that have been theorized to affect apoptosis. To examine this possibility various target cells were treated with different concentrations of DCI and assessed for intracellular nuclear DNA fragmentation and apoptosis. DCI treatment caused oligonucleosomal DNA fragmentation in cell lines expressing high levels of protease activity (LAK cells, NK-92, CTLL-2, L929, 3T3). This DNA breakdown characteristic of apoptosis occurred in a dose-dependent fashion within 4–6 hr of treatment and was confirmed by electron microscopy. In cell lines expressing low levels of protease activity (unstimulated human peripheral blood mononuclear (PBMN) cells, YAC-1 cells), DCI effectively inhibited protease activity without inducing oligonucleosomal DNA fragmentation. ZN^{2+} ions significantly inhibited DCI-induced DNA degradation. The mixture of DCI and BLT esterase active NK cell lysate triggered DNA fragmentation in isolated YAC-1 nuclei. Degree of DNA fragmentation in YAC-1 nuclei was proportional to the level of BLT esterase activity. Cell lysate protease activity, initially inhibited by DCI acylation, was restored by hydroxylamine deacylation, thus preventing DCI-mediated DNA fragmentation. Our results suggest that DCI treatment of cells expressing high levels of protease activity generates toxic levels of acyl-enzyme intermediates. These intermediates may trigger nuclear DNA breakdown and apoptosis by activating endogenous endonucleases. This effect may compromise the analysis of apoptosis in experimental systems using high concentrations of DCI for extended periods.** [P.S.E.B.M. 1998, Vol 219]

3,4-Dichloroisocoumarin (DCI) is a highly potent serine protease inhibitor whose mechanism of action involves acylation of the protease active site. DCI is important in inhibiting protease-mediated proteolysis, and is an excellent *in vitro* probe for various biological functions. DCI is a mechanism-based serine protease inhibitor. It is nonreactive until its isocoumarin ring is enzymatically opened by an active serine protease. This opening of the isocoumarin ring results in acylation of the protease active site $Serine_{195}$ (using the chymotrypsin numbering system). This acylated protease may slowly deacylate or may remain stably acylated. Only acylated proteases can be restored to enzymatic activity by deacylation (1–4).

Cytotoxic lymphocytes, such as cytolytic T lymphocytes (CTLs) and natural killer (NK) cells, mediate cell death through exocytosis of cytoplasmic granules. Perforin, a cytolytic protein found in these granules, forms transmembrane ion permeable pores (5–8). These granules also contain a subfamily of serine proteases called granzymes, some of which trigger target cell apoptosis. Three cytoplasmic granule serine proteases have been reported to cause DNA fragmentation and apoptosis when combined with perforin (9–13). DCI has been used to investigate the role of cytoplasmic granule serine proteases in cell death (14–15).

The effect of DCI treatment on target cells may depend on levels of protease activity within these cells. In cells with low protease activity, DCI may effectively inhibit intracellular proteases with no adverse effects. In cells with high

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protease activity, however, DCI may trigger apoptosis. We hypothesize that this effect is mediated by reactive intermediates generated by the reaction of DCI with proteases. These intermediates may reach toxic levels, depending on the level of protease activity. In this study, we used various concentrations of DCI to treat cells expressing different activity levels of BLT esterase (16–18) and investigated the effect of such treatment on these cells.

Materials and Methods

Cell Lines. L929, 3T3, and YAC-1 cell lines used in this study were maintained in continuous passage using standard tissue culture technique (5% CO_2, 37°C). Complete medium (CM) used for cell culture consisted of RPMI 1640 supplemented with 10% heat-inactivated FCS, 0.1 m*M* MEM nonessential amino acid solution, 2 m*M* L-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin. The human NK-92 cell line (a kind gift from Dr. Hans-G. Klingemann, Terry Fox Lab, British Columbia, Canada) and mouse CTLL-2 cell line were maintained in the same medium supplemented with 100 U/ml IL-2 (Hoffman La Roche, Nutly, NJ). Human LAK cells were generated as described previously (9).

Preparation of Cell Lysates. DCI-treated and DCI-untreated cells (2×10^6 cells/0.5 ml) were incubated for 20 min in microcentrifuge tubes at room temperature. The cells were then resuspended in ice-cold lysis buffer containing 50 m*M* Tris, 0.3 NP-40, pH 7.5. The debris was pelleted by centrifugation at 14,000*g* for 15 min. The supernatant was used immediately to measure the BLT esterase activity or stored at −70°C until further use. Protein concentrations in cell lysates were determined by the BCA method (Pierce Chemical Co., Rockford, IL) using crystalline bovine serum albumin for calibration.

Preparation of YAC-1 Cell Nuclei. YAC-1 cells were harvested by centrifugation, washed twice with phosphate buffered saline (PBS), and resuspended in buffer containing 10 m*M* PIPES, pH 7.4, 10 m*M* KCl, 2 m*M* $MgCl_2$, and 1 m*M* dithiothreitol. They were allowed to swell on ice for 20 min and then were gently lysed with Dounce homogenizer. The resulting homogenate was layered over 30% sucrose in the above buffer. The nuclei were pelleted by centrifugation at 800*g* for 10 min, washed once with the same buffer, resuspended at a concentration of 5×10^7 nuclei/ml, and immediately assayed for DNA fragmentation.

Inactivation of BLT Esterase (S) by DCI. DCI-treated (0.1–1.0 m*M*) or untreated whole cell lysates (2×10^5 cells/0.5 ml) of each cell line were assessed for esterase activity using colorimetric BLT thioesterase assay described previously with the following modifications (16). Stock reagents included 10 m*M* BLT (Sigma Chemical Co.) in H_2O, and 10 m*M* 5,5-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma Chemical Co.) in dimethyl formamide. These were diluted in Hank's balanced salt solution (HBSS)-HEPES (10 m*M*) to 1 m*M* BLT and 0.5 m*M* DTNB. In each well of a 96-well microtiter plate, we combined dilute DTNB and 50 μl of dilute BLT solution or 50 μl of HBSS-HEPES, with 10 μl of cell lysates diluted in HBSS-HEPES. The contents were mixed on a plate, shaken for 60 sec, and incubated for 5 min at room temperature. We used an extinction coefficient of 13,100 m^{-1} cm^{-1} at 405 nm for the 3-carboxy-4-nitrophenoxide ion produced from the reaction between Ellman's reagent (DTNB) and the freed benzyl mercaptan.

At times thereafter, samples were removed and assayed for residual BLT esterase activity. Control incubations included cell lysate with BLT esterase activity in the presence or absence of solvent. In other simultaneous experiments, the absorbance of the reaction mixture was measured at 325 nm to monitor the DCI isocoumarin ring opening due to DCI reaction.

DNA Gel Electrophoresis. 2×10^6 cells/0.5 ml were incubated with DCI in HBSS containing 2 m*M* $CaCl_2$, 10 m*M* HEPES, and 0.1% DMSO in 5% CO_2 at 37°C. The cells were added to 5 m*M* ethylenediaminetetraacetic acid (EDTA) and 0.5% sodium dodecyl sulphate (SDS) and digested with 0.5 mg/ml proteinase K for 2 hr at 50°C, after which the nucleic acids were precipitated with ethanol. Extracted DNA was resuspended in Tris-EDTA buffer and digested with 15 μg/ml RNase A (Sigma, St. Louis, MO) for 1 hr at 37°C. The samples were separated on a 1.0% agarose gel and stained with ethidium bromide. The DNA was visualized and photographed on a UV light box.

DCI reactive intermediates were generated *in vitro* by mixing DCI and NK-92 cell lysates. Our samples included DCI plus active NK cell lysate, DCI plus heat-inactivated NK cell lysate, and DCI plus buffer only (spontaneous hydrolysis). The isolated nuclei were then added to the reaction mixture at a concentration of 2×10^6/0.5 ml, and DNA fragmentation was assayed as described above.

Electron Microscopy. 2×10^6 YT cells, which also express a high level of BLT esterase activity, were incubated with 2 m*M* DCI for 6 hr. Cells were pelleted, resuspended, and fixed overnight at 4°C in 2% gluteraldehyde. Following fixation, the cells were washed in modified Tyrodes buffer, postfixed, and stained in osmium tetraoxide. Samples were then dehydrated through graded ethanol and rinsed in propylene oxide prior to embedding in PolyBed 812 (Polysciences, Warrington, PA). Sections of 90–100 nm were cut, mounted on uncoated copper grids, and stained with uranyl acetate and lead citrate. Sections were examined and photographed using a Philips model 201 electron microscope.

Results

Various intracellular serine proteases exhibit trypsin-like activity. We used BLT esterase activity as a marker of trypsin-like protease activity in the different cell lines. The esterase activity was assayed spectrophotometrically using a

BLT substrate in the presence of DTNB as a chromagen. The hydrolysis of BLT yields benzyl mercaptan, which reacts with the chromagen to produce the thiophenoxide chromophore. Table I shows the levels of esterase activity in cell lysates of different cell lines after 5 min of incubation in the presence or absence of DCI. In the absence of DCI, NK-92, LAK cells, CTLL-2, L929, and 3T3 cell lines expressed 4–7 times greater levels of BLT esterase activity than unstimulated PBMN cells and YAC-1 cells. In the presence of DCI, BLT esterase activity was inhibited in cell lysates of all cell lines. 100 μ*M* DCI was the minimum concentration required to inhibit BLT esterase activity in 2×10^6/0.5 ml YAC-1 or PBMN cells. In NK-92, LAK cells, CTLL-2, L929, and 3T3 cells, however, 1.0 m*M* DCI was the minimum concentration required to inhibit esterase activity completely. This inhibition by DCI suggests that the BLT activity was due to trypsin-like serine proteases. The long wavelength absorption peak at 325 nm is associated with the isocoumarin ring system. We monitored this peak to evaluate whether DCI was acting as an inhibitor of BLT esterase activity through acylation/ring opening by the same mechanism as for inhibition of serine proteases. Upon ring opening during protease inactivation, the absorption at 325 nm was reduced to negligible levels and was accompanied by a corresponding loss of BLT esterase activity at each time point (Table II).

We examined the effect of DCI on DNA fragmentation in cytotoxic lymphocytes and other tumor cell lines expressing high (LAK, NK-92, CTLL-2, L929, 3T3) and low (unstimulated PBMN cells and YAC-1 cells) levels of BLT esterase activity. We found that a minimum concentration of 1 m*M* DCI was required to induce significant DNA fragmentation in all susceptible target cells. The kinetics and dose response patterns of DNA breakdown were consistent in all cell lines expressing high levels of BLT esterase activity. Figure 1 illustrates the kinetics of DNA fragmentation, which was detectable at 4 hr and became maximal at 6 hr, in NK-92 cells (Fig. 1A), LAK cells, and CTLL-2 cells (not shown). This pattern was also observed in noncytotoxic tumor cell lines including L929 (Fig. 2A) and 3T3 (Fig. 2B). Figure 1 also shows the dose response patterns of DCI-treated cells. DNA fragmentation occurred in a dose-dependent fashion in LAK cells (Figure 1B), CTLL-2 cells (Figure 1C), and NK-92 cells (not shown). However, DCI treatment did not cause DNA breakdown in unstimulated PBMN cells (not shown) or YAC-1 cells (Figure 2C).

Because different reaction environments would be expected to affect DCI's mechanism-based enzyme activity, we also examined the effect of pH on DCI-mediated apoptosis. We found that DCI induced DNA fragmentation more effectively at pH 7.5 than at pH 6.0 (not shown). Spontaneous hydrolysis of unreacted DCI produces transient intermediates. We generated DCI byproducts by hydrolyzing DCI in buffer. We examined the effect of these intermediates by incubating all cell lines for the same periods as DCI treatment. No nuclear DNA degradation was identified in any cell line (not shown).

Hydroxylamine is a chemical that removes acyl inhibitors bound to protease active sites, thus restoring enzyme activity. DCI inhibits serine proteases through acylation of the active site; we hypothesized that hydroxylamine deacylation would block DCI-mediated DNA fragmentation in susceptible cells. We tested this hypothesis by incubating NK-92 cells with 1 m*M* DCI for 5 min, then adding hydroxylamine. In our experimental sample, 0.2 m*M* hydroxylamine plus 1 m*M* DCI did not cause nuclear DNA breakdown (Figure 2D, Lane 1), whereas 1 m*M* DCI alone induced DNA fragmentation (Figure 2D, Lanes 2 and 3). Because Zn^{2+} is a potent inhibitor of endonucleases, we examined its effect on DCI-treated cells. Figure 2D, Lane 3 illustrates DNA fragmentation in NK-92 cells treated with DCI. Lane 4 shows that Zn^{2+} treatment of DCI-treated NK-92 cells blocked DNA fragmentation completely, suggesting that DCI mediates DNA fragmentation through activation of endonucleases.

We verified that DCI-mediated nuclear DNA fragmentation results in changes in nuclear morphology characteristic of apoptosis. Glutaraldehyde-fixed NK-92 cells treated with DCI were examined by thin section electron microscopy. These cells exhibited the nuclear features of cells undergoing apoptosis. Negative control NK-92 cells incubated in buffer containing 2 m*M* $CaCl_2$, 10 m*M* HEPES, and

Table I. Protease Activity[a]

Cell Lines	Protein (mg)	0.0 μ*M* DCI	100 μ*M* DCI	1 m*M* DCI	Specific Activity Units/mg[b]
NK-92	0.050 ± 0.030	14.0 ± 1.3	11.2 ± 1.4	<0.8	280.0
LAK cells	0.045 ± 0.020	13.5 ± 2.3	10.5 ± 1.6	<0.9	300.0
CTLL-2	0.052 ± 0.018	12.9 ± 2.1	9.7 ± 2.2	<0.7	248.0
L929	0.049 ± 0.030	10.9 ± 1.5	8.3 ± 1.7	<0.8	222.0
3T3	0.050 ± 0.026	9.9 ± 0.3	7.8 ± 1.2	<0.7	198.0
PBMN cells	0.047 ± 0.021	2.0 ± 0.5	<0.8	<0.3	42.5
YAC-1	0.049 ± 0.018	2.1 ± 0.4	<0.6	<0.2	42.8

[a] Expressed as the Average and Standard Error of the Mean values of BLT esterase activity of seven cell lines preparations, 2×10^6/0.5 ml whole cell lysate of each. One unit of BLT esterase activity was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate per minute.
[b] Specific activity in the absence of DCI.

Table II. Kinetics of BLT Esterase Inhibition and 3,4-DCI Ring Opening[a]

Time (min)	In the presence of 1 m*M* 3,4-DCI		
	BLT esterase activity (% of initial value)[b] NK cell lysate	Absorbance at 325 nm[c] NK cell lysate	NO NK cell lysate[d]
0	0	3.5 ± 2	3.5 ± 2
0.5	20 ± 3	2.9 ± 4	3.5 ± 2
1.0	35 ± 4	2.2 ± 5	3.5 ± 2
2.0	54 ± 6	1.6 ± 3	3.3 ± 3
3.0	86 ± 5	1.1 ± 3	3.3 ± 3
4.0	94 ± 4	0.4 ± 2	3.1 ± 2
5.0	96 ± 5	0.10	3.1 ± 2
10.0	96 ± 2	0.10	3.0 ± 2

[a] % Inhibition of BLT esterase activity and 3,4-DCI ring opening was determined as described previously (4, 19).
[b] Inactivation of BLT esterase activity by 3,4-DCI. NK cell lysate (0.045 mg) was incubated in 0.1 *M* HEPES, 0.5 *M* NaCl, pH 7.5, and 1% DMSO. At indicated time, samples were removed and assayed for residual BLT esterase activity. Data are presented from duplicate experiments. Control incubations included NK cell lysate in the presence and absence of solvent. Data expressed as the Average and Standard Error of the Mean values.
[c] BLT esterase-catalyzed opening of 3,4-DCI ring. In parallel experiments 3,4-DCI (1 m*M*) was rapidly added to a solution containing NK cell lysate (0.045 mg protein) in 0.1 *M* HEPES, 0.5 *M* NaCl, pH 7.5, and 1% DMSO. Decreasing absorbance at 325 nm indicates increasing concentration of open 3,4-DCI rings (measured in the presence and absence of BLT esterase). Data expressed as the Average and Standard Error of the Mean values.
[d] Decreasing absorbance indicates nonspecific spontaneous hydrolysis in the buffer.

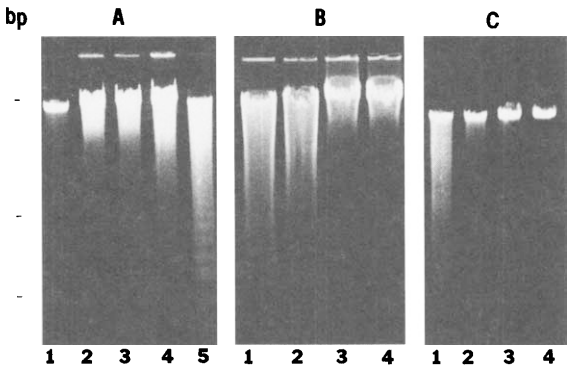

Figure 1. The kinetics and dose responses of DNA degradation by DCI. 2 × 10^6 cells were resuspended in 0.5 ml of buffer (HBSS containing 2 m*M* $CaCl_2$, 10 m*M* HEPES, 1.0% DMSO, and 1.0 m*M* DCI). Cells were incubated at 37°C and 5% CO_2 for various time periods. The extracted DNA was separated by 1% agarose gel electrophoresis (A). Kinetics of DNA fragmentation in NK-92 cells incubated for various times (Lane 1, 6-hr Control with 1.0% DMSO only; Lane 2, 1-hr; Lane 3, 2-hr; Lane 4, 4-hr; Lane 5, 6-hr). (B, C) Dose responses of DNA fragmentation in human LAK cells (B) and mouse CTLL-2 cells (C) incubated for 6 hr as described above (Lane 1, 1.0 m*M* DCI; Lane 2, 0.5 m*M* DCI; Lane 3, 0.1 m*M* DCI; Lane 4, Control with same buffer plus 1.0% DMSO only). DNA standards (bp) indicated by dash (–) on left side in descending order: 9.4, 2.0, and 0.5.

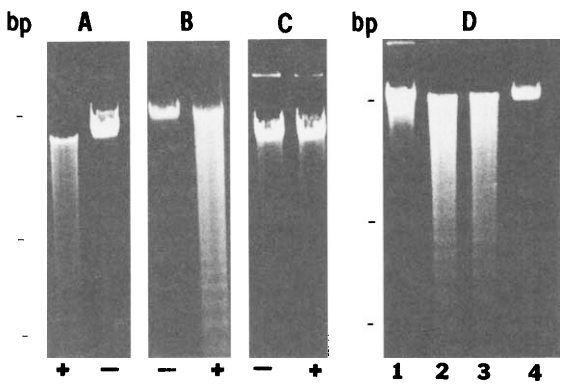

Figure 2. DCI-induced DNA fragmentation in noncytotoxic tumor cell lines, the effect of hydroxylamine deacylation and Zn^{2+}. 2 × 10^6 cells were resuspended in 0.5 ml buffer (see above) and incubated at 37°C and 5% CO_2 for 6 hr. The extracted DNA was separated by 1% agarose gel electrophoresis. (+) indicates incubation with 1.0 m*M* DCI plus 1.0% DMSO and (–) indicates control with buffer containing 2 m*M* $CaCl_2$, 10 m*M* HEPES, and 1.0% DMSO alone. (A) DNA fragmentation in L929. (B) DNA fragmentation in 3T3. (C) No DNA fragmentation in YAC-1 cells. (D) The effect on DCI-mediated DNA fragmentation by hydroxylamine and Zn^{2+} in NK-92 cell line (Lane 1, 1 m*M* DCI plus 0.2 *M* hydroxylamine; Lanes 2 and 3, 1 m*M* DCI; Lane 4, 1 m*M* DCI plus 1 m*M* Zn^{2+}). DNA standards (bp) indicated by dash (–) on left side in descending order: 9.4, 2.0, and 0.5.

1.0% DMSO for 6 hr demonstrated no apoptosis (Fig. 3A). Cells incubated with DCI and 1.0% DMSO demonstrated hypercondensed chromatin domains at the nuclear periphery at 4 hr (Fig. 3B), which subsequently became sharply defined, spherical apoptotic bodies (Fig. 4C and 4D).

We hypothesized that toxic levels of acyl-enzyme intermediates mediated DCI's apoptotic effect and that, in these samples, a critical level of BLT esterase activity was required to generate concentrations of intermediates sufficient to induce apoptosis. To test this hypothesis we generated DCI reactive intermediates *in vitro* by mixing NK-92 cell lysates of different BLT esterase activities with 1 m*M* DCI. Then we added isolated YAC-1 nuclei and analyzed the ability of the reaction mixture to induce DNA fragmentation. For negative controls, YAC-1 nuclei were treated with a mixture of DCI plus heat-inactivated NK-92 cell lysates with no BLT esterase activity or with DCI in buffer only. We found that DCI treatment of lysates containing BLT esterase activity caused DNA fragmentation of YAC-1 nuclei (Fig. 4B). YAC-1 nuclei failed to exhibit DNA fragmentation; however, when incubated with heat-inactivated NK-92 cell lysates treated with DCI (Fig. 4C), or when incubated with stable byproducts of spontaneous DCI hy-

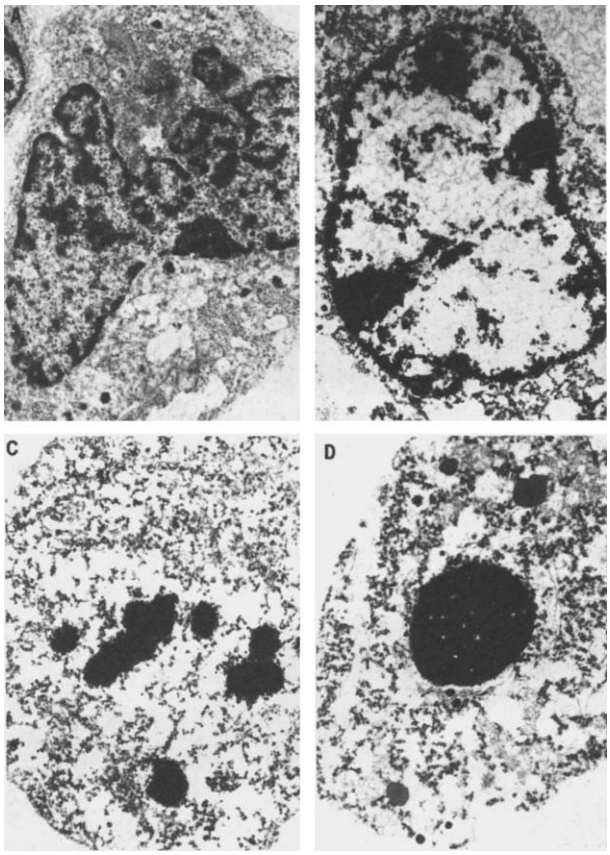

Figure 3. Morphology of NK-92 cells undergoing apoptosis after incubation with DCI for 3–6 hr. (A) Cell incubated in buffer containing 2 m*M* $CaCl_2$, 10 m*M* HEPES, 1.0% DMSO for 6 hr. (B) Cell incubated with 1.0 m*M* DCI for 4 hr, demonstrating perinuclear chromatin condensation characteristic of apoptosis. (C, D) Different cells incubated with 1.0 m*M* DCI for 6 hr demonstrating collapse and fragmentation of nuclear structures and formation of apoptotic bodies. Magnification, 7000× (A and B) and 3200× (C and D).

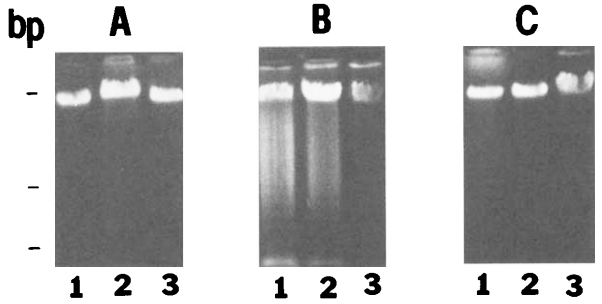

Figure 4. DNA fragmentation in isolated YAC-1 nuclei by DCI. NK-92 cell lysates with different levels of protease activity were added to buffer (see above) and incubated for 10 min. 2×10^6 YAC-1 nuclei were then added and incubated at 37°C, 5% CO_2 for 6 hr. The extracted DNA was separated on 1% agarose gel electrophoresis. (A) YAC-1 nuclei incubated with 1.0 m*M* DCI in buffer. Prior to the addition of the nuclei: Lane 1, hydrolysis of DCI in the buffer for 10 min; Lane 2, hydrolysis of DCI in the buffer for 30 min; Lane 3, hydrolysis of DCI in the buffer for 60 min). (B) YAC-1 nuclei incubated with DCI plus active NK-92 cell lysates (Lane 1, cell lysate with specific activity of 260; Lane 2, cell lysate with specific activity of 140; Lane 3, cell lysate with specific activity of 90. (C) YAC-1 nuclei incubated with DCI plus heat-inactivated NK-92 cell lysates (Cell lysate in lanes correspond with 4B.)

drolysis in buffer (Fig. 4A). Furthermore, the degree of DNA fragmentation corresponded to the specific activities of the NK-92 cell lysates (Fig. 4B, Lanes 1–3). DNA fragmentation occurred in isolated YAC-1 nuclei (Fig. 4B, Lanes 1 and 2) only when incubated with DCI-treated cell lysates exhibiting BLT esterase specific activity greater than or equal to 4-fold of the BLT esterase activity expressed in resting PBMN or YAC-1 cells (see Table I). This critical level of BLT esterase specific activity in the cell lysates was comparable to levels found in intact NK-92 cells.

Discussion

Isocoumarin-based synthetic protease inhibitors are potent *in vitro* probes for biological functions. They offer advantages such as serine protease specificity, rapid reactivity, and covalent inhibition. These "suicide," or mechanism-based, serine protease inhibitors remain nonreactive until the isocoumarin ring is enzymatically opened by a serine protease (15). The inhibitor inactivates the protease by acylating the active site serine, remaining tethered to it, and thus interacting only with the protease that activated the isocoumarin. Concurrently, the reagent spontaneously forms highly reactive, unstable acid chloride or ketone intermediates that further react by hydrolyzing to generate acylated serine protease. The acylated form of serine protease may remain stable.

DCI is a protease inhibitor that acts upon intracellular proteases through acylation, and may involve subsequent alkylation. DCI reaction with lymphocyte proteases creates a mixture of alkylated and nonalkylated proteases, thus illustrating that every acylation reaction does not proceed to secondary alkylation (15).

Although DCI is an isocoumarin-based protease inhibitor, its specificity for serine proteases is not absolute. Prolonged exposure of other intracellular proteins and macromolecules to high concentrations of DCI has been shown to result in nonspecific reactions, such as inactivation of glycogen phosphorylase b (19). This phenomenon was exacerbated in this study by the requirement for excess DCI in all reactions to compensate for its instability in aqueous buffer. At low concentration DCI inhibits tryptase (granzymes A or 3) and aspase (granzyme B or 2) activity of CTL or NK cell-specific serine proteases that in turn inhibits CTL or NK cell apoptosis mediated through these serine proteases. Our study explores an interesting alternative mechanism in which intracellular serine proteases trigger apoptosis through interaction with an isocoumarin-based, synthetic, suicide serine protease inhibitor.

The results of this study suggest that the interaction between DCI and intracellular proteases generates reactive intermediates that may reach toxic levels with high concentrations of protease. This is supported by the correlation of DNA degradation with a spectrum of BLT esterase-specific activities in samples of intact cells and isolated nuclei from all cell lines treated with DCI. These results confirm a previous study which found that an excess of DCI (2 m*M*) did

not induce DNA fragmentation in YAC-1 cells with low levels of protease activity (14). This may be due to less acylation of isocoumarin rings and subsequent decreased generation of acyl-enzyme intermediates. It seems likely that a critical threshold concentration of such intermediates is required to cause DNA fragmentation and apoptosis.

The ability of hydroxylamine to prevent DCI-mediated DNA fragmentation was due to deacylation of intracellular proteases. Inhibition of serine proteases by DCI involves an initial rapid and specific acylation reaction, which is then followed by slow, nonspecific alkylation of intracellular proteins. Based on our data, this nonspecific effect does not appear to contribute to DNA fragmentation and apoptosis. The inhibitory effect of Zn^{2+} on DCI-induced DNA breakdown suggests that DCI reactive intermediates mediate such degradation through endonucleases and/or other intracellular proteins. The mechanism by which this is accomplished remains unknown.

Acylation reactions play a role in apoptosis in various experimental systems (20, 21). The active acyl-enzyme complex may enter the nucleus through facilitated diffusion and interact with nuclear DNA as a function of concentration and duration of exposure to the complex. The acylated serine proteases may trigger cell death through mechanisms such as disruption of DNA metabolism. Our study validates additional experiments to investigate molecular mechanisms of DCI/acylated serine proteases–mediated apoptosis. In summary, this study confirms that DCI-mediated DNA fragmentation correlates with high levels of protease activity. The ability of DCI to induce apoptosis may be useful in selectively ablating tumor cells *in vivo* that express high levels of intracellular proteases. These findings encourage future studies investigating potential therapies exploiting this mechanism.

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