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Non-caspase-mediated apoptosis contributes to the potent cytotoxicity of the enediyne antibiotic lidamycin toward human tumor cells

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Abstract

Enediyne antibiotics have been reported to be the most potent cytotoxic antitumor agents. The pathway by which these compounds cleave DNA and induce apoptosis of tumor cells may be different from the caspase-mediated pathways that initiate typical apoptosis. In this report, we studied the apoptosis induced by lidamycin (LDM), a member of the enediyne antibiotic family, and compared the characteristics of LDM-induced apoptosis with those of typical apoptosis induced by mitomycin C or etoposide. Chromatin condensation occurred very rapidly and appeared as speckles in human hepatoma BEL-7402 and breast carcinoma MCF-7 cells after treatment with 1 μM LDM. In addition, co-staining the cells with the mitochondria-specific dye Mitosensor™ and the DNA-specific dye Hoechst 33342 enabled the visualization of mitochondria in normal control and LDM-treated cells but not in mitomycin C-treated cells. Neither the caspase inhibitor VAD-fmk nor the caspase-3 inhibitor DEVD-fmk was able to inhibit the DNA ladder patterns caused by LDM in BEL-7042 or MCF-7 cells. Smaller fragments of histone H1 cleaved by LDM were detected by SDS–PAGE, indicating that the site of LDM action is the internucleosomal structure. Although caspase-9, caspase-3/7, and caspase-6 activities were increased in BEL-7402 cells, and caspase-7 activity was increased in MCF-7 cells after treatment with 1 μM LDM, this occurred much later, indicating that chromatin condensation reached the maximal level rapidly while caspase activities still remained low. Taken together, these results demonstrate that LDM induced rapid DNA cleavage and chromatin condensation independently of caspase activities; this may contribute to its highly potent cytotoxicity toward tumor cells.

Keywords: Lidamycin; DNA cleavage; Apoptosis; Caspases; Hepatoma BEL-7402 cells; Breast carcinoma MCF-7 cells

1. Introduction

Enediyne antibiotics have been shown to have potent antitumor activity due to their unique ability to damage the DNA of tumor cells. LDM (original name C1027), a member of the enediyne antibiotic family, was isolated from a *Streptomyces globisporus* C-1027 strain in China [1,2]. Previous studies showed that LDM is highly toxic to tumor cells and can also effectively inhibit the replication of viruses [3–7]. LDM consists of an apoprotein and a chromophore, with the former serving as a protecting protein and the latter manifesting the ability to damage DNA strands [8,9]. The DNA damage caused by LDM includes double-strand breaks, single-strand breaks, and breaks in non-base regions due to hydrogen atom abstraction from the deoxyribonucleotide backbone [10–12]. The DNA cleavage activity of LDM in a cell is increased by 285-fold over that in a cell-free environment, and, using the episome-containing 935.1 cell line as a model, reveals a preference for intracellular DNA species in the following order: episome > mitochondrial DNA > genomic DNA [13]. Our previous studies showed that LDM retarded hepatoma BEL-7402 cells in the $G_2 + M$ phases and induced apoptosis in human promyelocytic leukemia HL-60 cells [14,15].

Many antitumor agents effect tumor killing through the induction of apoptosis, a conserved cellular suicide

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Abbreviations: Eto, etoposide; LDM, lidamycin; MMC, mitomycin C; and PI, propidium iodide.

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program characterized by chromatin condensation, DNA fragmentation, and cellular shrinkage [16,17]. Two major apoptotic pathways have been defined thus far. One is a caspase-dependent pathway, which is mediated by the activation of death receptors such as Fas or TNFR1, or by a cellular stress signal, and which induces the release of cytochrome *c*, followed by the consecutive activation of Apaf-1, caspase-9, -3, -7, and -6. The final release of a death fragmentation factor then leads to the cleavage of genomic DNA [18–20]. The second pathway is caspase-independent and relies on the release of pro-apoptotic molecules acting as nucleases, nuclease activators and serine proteases, such as HtrA2/Omi, from the mitochondria to the cytosol [21]. Omi can induce cell death and can also inhibit IAPs (inhibitor-of-apoptosis proteins) to augment caspase-dependent apoptosis [19].

Enediyne antibiotics have been shown to damage DNA in isolated nuclei of HeLa cells at the internucleosomal level, and the DNA ladder patterns were not induced by endogenous nucleases or topoisomerase [22]. This suggests that the cleavage of DNA by enediyne antibiotics and the cleavage by caspase-activated factors in the intracellular environment generated by typical apoptosis-inducing agents are different biochemical events. However, further information is needed to compare the differences and to depict the morphological changes in whole living tumor cells versus the biochemical changes observed in a cell-free system. In this study, the apoptotic features induced by LDM in human hepatoma BEL-7402 and breast carcinoma MCF-7 cells were compared with those of typical apoptosis initiated by conventional anti-tumor agents such as MMC and Eto. We showed that LDM can induce a unique type of apoptosis characterized by distinct chromatin condensation and rapid DNA fragmentation. The early phase of this apoptosis is independent of caspase activities and, therefore, contributes significantly to the potent toxicity of LDM toward tumor cells.

2. Materials and methods

2.1. Chemicals

LDM was provided by Professor Lian-fang Jin from our Institute and prepared as a 100 μM stock in 0.9% NaCl solution. MMC (Serva) was also prepared in a 0.9% NaCl solution, while Eto (Sigma) was dissolved in DMSO (Sigma); DMSO was used at 0.1% and showed no toxicity toward the tumor cells. All of the reagents were stored at −20° until used.

2.2. Cell lines

The human hepatoma BEL-7402 cell line was obtained from the Institute of Cell Biology, the Chinese Academy of Sciences, and the human breast carcinoma MCF-7 cell line was provided by Dr. Kenneth Cowan from the National Cancer Institute (USA). Both cell lines were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (HyClone) and 2 mM glutamine, and incubated at 37° in a humidified atmosphere containing 5% CO_2.

2.3. Chromatin condensation shown by co-staining of BEL-7402 cells with Hoechst 33342 and PI

BEL-7402 cells were treated with either 1 μM LDM for 6 hr, 100 μM Eto for 18 hr, or 30 μM MMC for 18 hr. They were then stained by both of the DNA-specific fluorescent dyes Hoechst 33342 (2 μg/mL) (Sigma) and PI (40 μg/mL) (Sigma) [23,24] for 10 min at 37°. Next, cells were washed once, incubated in serum-free RPMI 1640 medium, and observed under a fluorescence microscope (OLYMPUS) equipped with a λ_{455nm} filter. In each condition, 200–250 cells were counted.

2.4. Cell death features observed by co-staining with the mitochondria-specific apoptotic dye Mitosensor™ and the DNA-specific dye Hoechst 33342

MMC-treated cells in suspension and LDM-treated adherent cells were co-stained with 5 μg/mL of the mitochondria-specific dye Mitosensor™ (Clontech) and 1 μg/mL of Hoechst 33342 at 37° for 20 min. After being rinsed once, cells were incubated in serum-free RPMI 1640 medium and observed under a fluorescence microscope equipped with a λ_{515nm} filter. In typical apoptotic cells, because of possible changes in the mitochondrial membrane, the mitochondria are invisible and the cell nuclei fluoresce green, whereas in normal control cells mitochondria fluoresce red.

2.5. DNA isolation and agarose gel electrophoresis

Genomic DNA was isolated from both BEL-7402 and MCF-7 cells after they were treated with 1 μM LDM for various times as described previously [24]. In the caspase inhibitor experiment, the ApoAlert® caspase inhibitor VAD-fmk (10 μM) or the ApoAlert® caspase-3 inhibitor DEVD-fmk (10 μM) (Clontech) was added together with LDM. Briefly, cells were scraped down and lysed in lysis buffer [EDTA (10 mM/L), Tris (50 mM/L), sodium lauryl sarcosine (0.5%, w/v)] for 30 min. Cell lysates were then treated with 100 μg/mL of RNase A (Sigma) and 100 μg/mL of proteinase K (Merck) at 50° for another 3 hr. The DNA samples were extracted three times with phenol:chloroform (1:1, v/v). DNA samples (8–15 μg) were run on 2% agarose gels and the gels were stained by 2 μg/mL of ethidium bromide before being photographed with UVP ImageStore 7500 film (Life Sciences).

2.6. Cleavage of histone H1 by LDM in vitro

The cleavage of histone H1 by LDM *in vitro* was detected using the method of Zein *et al.* [25,26]. LDM and calf histone H1 or bovine albumin fraction V (Gibco) were prepared in double-distilled water. The reaction solution contained 1 μL (10 μg) histone H1 or 1 μL (10 μg) bovine albumin fraction V and 1 μL LDM (at the indicated concentrations) with or without 1 μL leupeptin (Sigma) (20 μg/mL in 50 mM Tris·HCl, pH 7.4) in a total volume of 10 μL. The reactions were carried out at 37° for 12 hr. The resultant protein bands were separated by SDS–PAGE (17% for histone H1 and 10% for bovine albumin fraction V).

2.7. Measurement of caspase activities

The caspase-9 assay kit (Clontech), the caspase-3/7 assay kit (Promega) and the caspase-6 assay kit (BioVision) were used to measure their respective activities, according to the instructions of the manufacturers. The results were presented as the absorbance of (the treated group/absorbance of the untreated group) × 100% (the value of the untreated control group was assumed to be 100%). The same cell populations were used for determination of the relationship between the percentage of cells with chromatin condensation and those with caspase activity. Cells (1×10^5) collected at different times were fixed in 70% ethanol and stored at 4°. They were then stained with Hoechst 33342, and the percentage of cells with chromatin condensation was counted under a fluorescence microscope (200 total - cells/sample).

3. Results

3.1. Effect of LDM on rapid chromatin condensation in hepatoma BEL-7402 and breast carcinoma MCF-7 cells

Cell populations with no more than 5% necrotic cells [represented as PI-positive and necrotic (PIN)] were selected for the experiments. Typical apoptotic chromatin condensation occurred in BEL-7402 cells after they were treated with MMC or Eto, the features of which were consistent with those described previously [17]. Typical apoptotic cells (designated as APO) were detached from the monolayer and apoptotic bodies formed (Fig. 1C and D). Interestingly, a unique and atypical chromatin condensation (represented as C) was observed in PI-negative BEL-7402 cells treated with LDM, and this became more evident when using higher LDM concentrations such as 1 μM (Fig. 1B and Table 1). This type of chromatin condensation was unique and distinct from those of typical chromatin condensation. First, these cells did not detach from the monolayer during the chromatin condensation process. Second, many smaller "dots" representing segregated condensed chromatin appeared as early as 0.5 hr of LDM treatment, and these "dots" became more evident and clearer after 3 hr of LDM incubation (data not shown and see Fig. 5 below). Finally, there was no formation of apoptotic bodies observed at the late stage of cell death. We also observed similar apoptotic features in LDM-treated MCF-7 cells (data not shown).

The percentages of various types of chromatin condensation were calculated and compared in BEL-7402 cells treated with 30 μM MMC, 100 μM Eto, or 0.01, 0.1, and 1 μM LDM for 6, 12, 18, and 24 hr (Table 1). The "C"-type

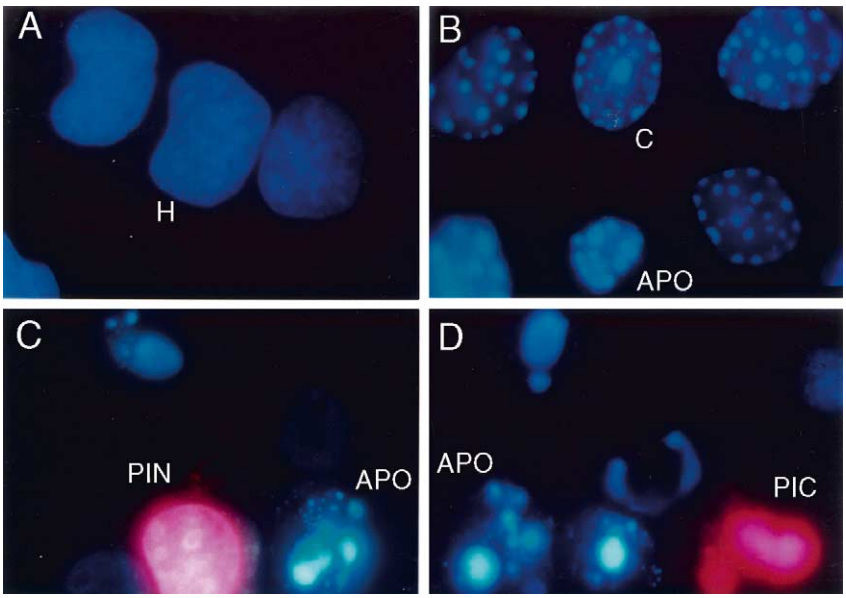

Fig. 1. Various types of chromatin condensation in hepatoma BEL-7402 cells determined by co-staining with the fluorescent dyes Hoechst 33342 and PI. (A) Control cells. (B) Cells treated with 1 μM LDM for 6 hr. (C) Cells treated with 100 μM Eto for 18 hr. (D) Cells treated with 30 μM MMC for 18 hr. H represents a living cell; APO, a cell with typical apoptotic chromatin condensation; C, a cell with "unique, atypical" chromatin condensation; PIC, a PI-positive cell with chromatin condensation; and PIN, a cell that was PI-positive and necrotic. Magnification: 1000×. This is a representative result from four separate experiments.

Table 1
Different types of chromatin condensation in human hepatoma BEL-7402 cells treated with MMC, Eto, or different concentrations of LDM, made apparent by co-staining with fluorescent dyes Hoechst 33342 and PI

Treatment	Hours of treatment	Types (%)				
		C	APO	PLC	PIN	H
Control	6	0	0.2 ± 0.4	0	5.1 ± 2.8	94.7 ± 3.8
	12	0	0.8 ± 0.7	0.2 ± 0.4	7.0 ± 2.6	92.0 ± 2.8
	18	0	0.2 ± 0.4	0.5 ± 0.7	4.7 ± 2.9	94.6 ± 3.9
	24	0	0.2 ± 0.4	0.2 ± 0.3	5.0 ± 2.8	94.6 ± 3.6
MMC (30 μM)	6	0	3.3 ± 1.9	0	7.5 ± 2.4	89.2 ± 3.7
	12	0	51.5 ± 3.8	0.4 ± 0.6	4.4 ± 1.6	43.7 ± 4.6
	18	0	87.8 ± 4.5	6.0 ± 2.8	4.6 ± 2.5	1.6 ± 0.9
	24	2.0 ± 1.1	61.8 ± 4.6	34.8 ± 4.5	1.4 ± 1.5	0
Eto (100 μM)	6	0	3.9 ± 2.8	0	5.6 ± 1.5	90.5 ± 4.5
	12	0	7.4 ± 2.3	0.3 ± 0.5	5.2 ± 2.7	87.1 ± 4.5
	18	0	25.2 ± 3.6	3.2 ± 2.7	4.5 ± 2.9	67.1 ± 5.3
	24	0	27.1 ± 4.9	5.3 ± 2.6	4.1 ± 1.1	63.5 ± 4.8
LDM (1 μM)	6	84.8 ± 3.9	5.4 ± 4.7	3.0 ± 1.8	3.4 ± 2.5	3.4 ± 2.0
	12	72.4 ± 5.7	9.4 ± 3.7	12.9 ± 4.3	3.3 ± 2.3	2.0 ± 1.8
	18	38.7 ± 5.5	31.0 ± 4.9	29.0 ± 2.6	0.8 ± 0.7	0.5 ± 0.8
	24	29.9 ± 4.8	5.7 ± 2.4	62.6 ± 5.7	1.5 ± 1.4	0.3 ± 0.4
LDM (0.1 μM)	6	74.8 ± 4.9	6.7 ± 2.1	1.5 ± 0.5	3.3 ± 2.2	13.7 ± 3.3
	12	75.7 ± 8.3	6.9 ± 2.3	7.9 ± 3.2	4.9 ± 2.9	4.6 ± 2.6
	18	52.1 ± 4.7	12.9 ± 3.7	29.9 ± 4.7	3.8 ± 2.8	1.3 ± 1.3
	24	35.0 ± 5.2	22.8 ± 4.3	39.4 ± 3.8	2.5 ± 1.0	0.3 ± 0.7
LDM (0.01 μM)	6	2.1 ± 1.2	14.6 ± 3.5	0.2 ± 0.4	3.7 ± 2.4	79.4 ± 4.7
	12	14.9 ± 3.1	28.8 ± 5.0	5.2 ± 2.3	4.8 ± 2.0	46.3 ± 5.1
	18	19.2 ± 3.5	46.3 ± 4.3	13.4 ± 3.0	2.8 ± 1.4	18.3 ± 5.7
	24	19.6 ± 2.3	50.0 ± 5.9	17.4 ± 3.2	2.6 ± 1.0	10.4 ± 2.8

Each type of cell was counted under a fluorescence microscope. C, a cell with a "unique, atypical" chromatin condensation; APO, a cell with typical apoptotic chromatin condensation; PIC, a PI-positive cell with chromatin condensation; PIN, a PI-positive cell and necrotic; and H, a living cell. Results are means ± SD and were from three independent experiments.

cells were as high as 74.8 and 84.8% in BEL-7402 cells treated with 0.1 and 1 μM LDM for 6 hr, respectively, but were not present in 6 hr MMC- or Eto-treated cells. Fewer apoptotic cells (less than 5%) were also detected with MMC or Eto treatment at this time period. However, a low concentration of LDM (0.01 μM) led to the generation of 14.6% of "APO"-type cells, and the number of "APO" cells increased to 28.8, 46.3, and 50% after 12, 18, and 24 hr of incubation, respectively (Table 1). This is consistent with our previous findings that incubation of HL-60 cells, a human promyelocytic leukemia cell line, with very low concentrations of LDM (as low as 0.1 to 10 nM) led to induction of typical apoptosis, the "APO"-type cells [15]. When using higher concentrations of LDM (0.1 and 1 μM) for longer treatment times, the percentages of "C"-type cells decreased from 70–80% at 6- and 12-hr incubation to about 30% after 24 hr (Table 1). The small "dots" of condensed chromatin in most of the "C"-type cells did not merge into large "dots" or form any intermediate type of condensed chromatin; instead they became PI-positive, and were designated "PIC" (PI-positive with chromatin condensation) (Table 1 and Fig. 1D). As the number of "C"-type cells decreased over time, the number of "PIC"-type cells increased with 0.1 or 1 μM LDM treatment, respectively. The number of PIN (PI-positive, necrotic) cells remained low (<5%) under all treatment conditions (Table 1).

3.2. Simultaneous appearance of mitochondria and condensed chromatin in LDM-treated cells

Untreated BEL-7402 cells exhibited non-condensed chromatin (fluoresced light green) in their nuclei and mitochondria (fluoresced red) in their cytoplasm (Fig. 2A). In contrast, MMC-treated apoptotic cells contained condensed chromatin in their nuclei but lacked mitochondria in their cytoplasm (Fig. 2B). However, both mitochondria and condensed chromatin were present in BEL-7402 cells treated with 1 μM LDM for 6 hr (Fig. 2C), underscoring the dramatic morphological differences between LDM- and MMC-treated cells. Similar morphological features were observed in LDM-treated MCF-7 cells (Fig. 2A and C vs E and F).

3.3. Induction by LDM of direct cleavage of genomic DNA in both BEL-7402 and MCF-7 cells

The rapid chromatin condensation induced by 1 μM LDM prompted us to perform DNA fragmentation experiments in

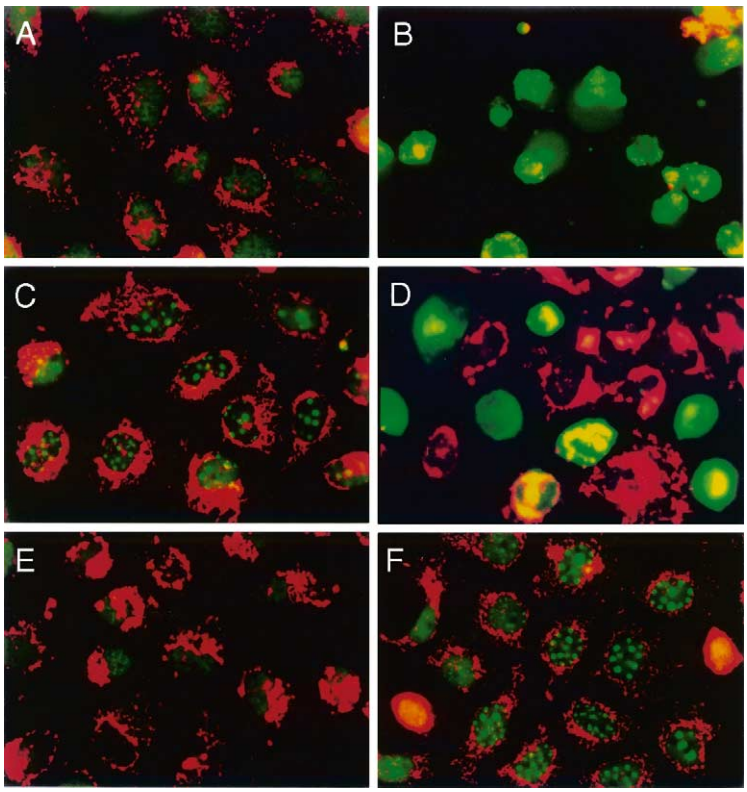

Fig. 2. Co-staining of human hepatoma BEL-7402 cells and human breast carcinoma MCF-7 cells with the mitochondria-specific dye Mitosensor™ and the DNA-specific dye Hoechest 33342. (A) Untreated BEL-7402 cells, control. (B) BEL-7402 cells in suspension and treated with 30 μM MMC for 12 hr. (C) BEL-7402 cells treated with 1 μM LDM for 6 hr. (D) BEL-7402 cells treated with 1 μM LDM for 12 hr; (E) untreated MCF-7 cells, control; (F) MCF-7 cells treated with 1 μM LDM for 6 hr. Magnification: 400×. This is a representative result from three separate experiments.

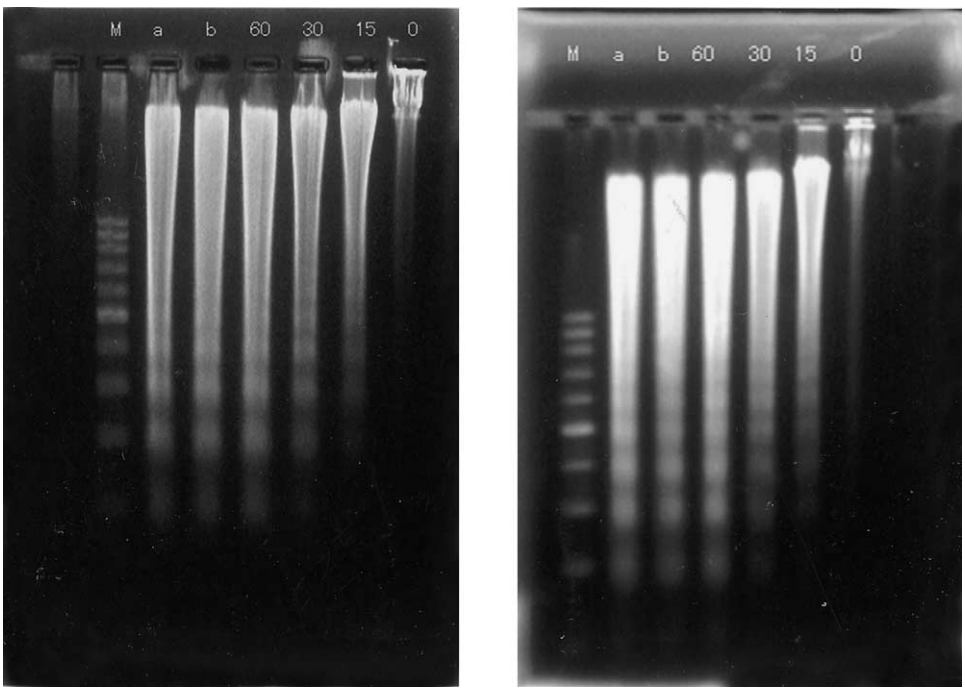

Fig. 3. Induction by LDM of the DNA ladder patterns in human hepatoma BEL-7402 (left panel) and human breast carcinoma MCF-7 (right panel) cells. The cells were treated with or without 1 μM LDM for 15, 30, or 60 min, respectively. M represents the standard DNA marker at 200-bp intervals; lanes a and b represent DNA patterns induced by incubation of LDM with the caspase-3 inhibitor DEVD-fmk or the caspase inhibitor VAD-fmk, respectively. This is a representative result from three separate experiments.

whole living cells. DNA ladder patterns were observed in both cell lines, and the action of LDM was very rapid. Fragmentation appeared as early as 15 min and was completed within 60 min after incubation with 1 μM LDM (Fig. 3). This is very unique, especially for MCF-7 cells in which DNA fragmentation and chromatin condensation were not easily detectable following exposure of the cells to a variety of antitumor agents [27–29]. Interestingly, the DNA ladder pattern was not changed after co-incubation of the cells with LDM and the caspase-3 inhibitor DEVD-fmk or the caspase inhibitor VAD-fmk (Fig. 3, lanes a and b). These results suggest that LDM can directly cleave genomic DNA of both BEL-7402 and MCF-7 cells, and this effect is not dependent upon caspase activities.

3.4. Degradation of histone H1 induced by LDM

The LDM-induced cleavage of genomic DNA into a ladder pattern in whole tumor cells suggests that LDM may specifically cleave the internucleosomal chromatin structure. It has been reported that other members of the enediyne antibiotic family, such as kedarcidin, neocarzinostatin, and maduropeptin, have protease activities, the optimal substrate of which is histone H1 [25,26]. It would be interesting to determine whether LDM has protease activity and can also use histone H1 as a substrate. As shown in Fig. 4, after incubation with histone H1 for 12 hr *in vitro*, LDM induced the degradation of histone H1 into smaller fragments. With increasing concentrations of LDM, degradation became more complete, with 0.5 μM inducing minimal and 10 μM inducing maximal histone H1 degradation (Fig. 4, top panel). LDM had no effect on bovine albumin fraction V (Fig. 4, middle panel), indicating its specificity towards histone H1. Moreover, the degradation of histone H1 could not be inhibited by the protease inhibitor leupeptin (Fig. 4, bottom panel).

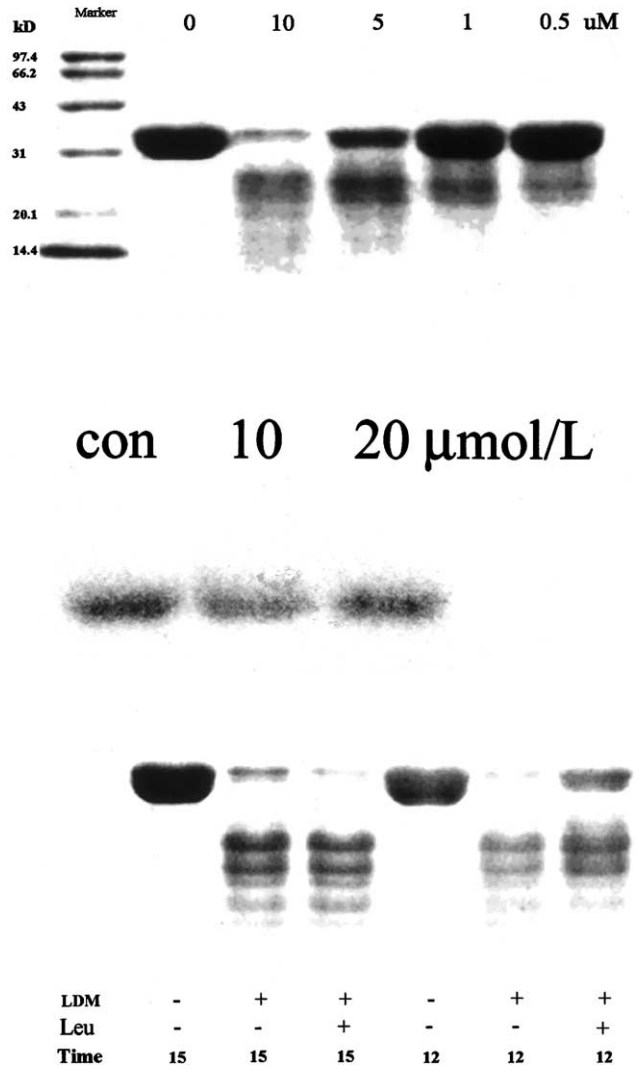

Fig. 4. Degradation of histone H1 caused by LDM *in vitro*. (Top panel) Degradation of histone H1 induced by LDM at 0.5, 1, 5, or 10 μM. (Middle panel) Inefficiency of LDM on degradation of bovine serum albumin V. (Bottom panel) Effect of the protease inhibitor leupeptin (Leu) on the degradation of histone H1 caused by 10 μM LDM for 12 and 15 hr. The results were assayed on 17 or 10% SDS–PAGE and are representative of three separate experiments.

3.5. Rate of increase in caspase activities in LDM-treated cells

It is well known that successive activation of caspase-9, -3, -7, and -6 is required in a caspase-dependent apoptotic pathway [18,19,30]. Whether or not caspase activation was involved in LDM-induced apoptosis remained to be determined. Caspase-9, -3/7, and -6 activities were measured in BEL-7402 cells. As shown in Fig. 5A, caspase-9, -3/7, and -6 activities remained at background level after 1 hr of LDM incubation; the activity increased gradually to about 3-, 3.6-, and 3.9-fold, respectively, after 6 hr of LDM treatment. A similar pattern was seen in MCF-7 cells treated with LDM (Fig. 5B). MCF-7 lacks functional caspase-3 [31] and, therefore, we only measured caspase-7 activity (Fig. 5B), which increased 2.6-fold after 6 hr of LDM treatment compared with untreated MCF-7 cells. Since chromatin condensation and DNA fragmentation occurred very early after LDM treatment (Figs. 1–3), we compared the kinetics of chromatin condensation and caspase activation (Fig. 5A and B). Interestingly, while the majority of cells (about 85–95%) displayed chromatin condensation after 0.5 and 1 hr of 1 μM LDM treatment, minimal caspase activities were detected at this time period. While chromatin condensation plateaued, the caspase activities began to increase gradually to 2- to 3-fold from 1 to 6 hr of LDM treatment compared with the untreated cells (Fig. 5). These results demonstrate that chromatin condensation occurred very early and preceded the increase in caspase activities, suggesting that LDM-induced apoptosis was caused directly by DNA cleavage at an early stage of the apoptotic process.

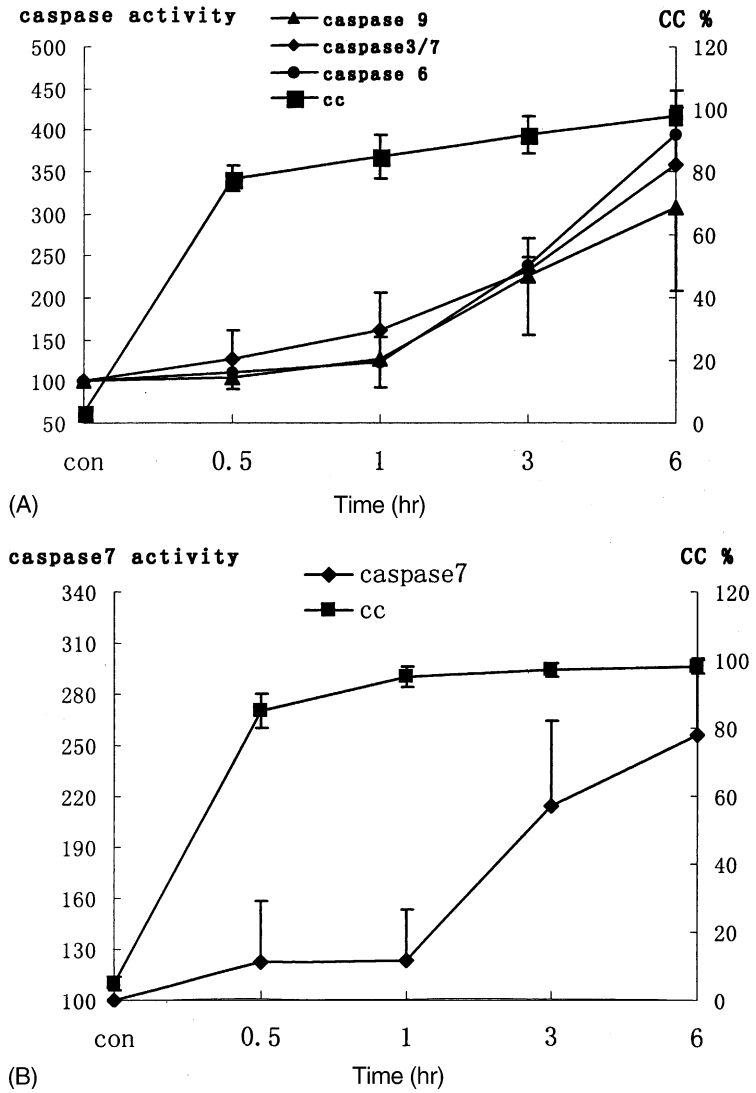

Fig. 5. Kinetics of chromatin condensation (cc) and caspase activation in BEL-7402 (A) and MCF-7 (B) cells after 1 μM LDM treatment for 0.5, 1, 3, and 6 hr, respectively. Con represents untreated control cells. Data are presented as means ± SD from three separate experiments.

4. Discussion

In this study, we have demonstrated for the first time that the enediyne antibiotic LDM can induce non-caspase-mediated DNA cleavage and rapid chromatin condensation, a unique type of apoptosis. In comparison with the typical apoptosis induced by MMC or Eto, LDM-mediated apoptosis possessed the following distinct features: (a) Chromatin condensation occurred very early and showed different morphology as designated by the ''C''-type versus the ''APO''-type of typical apoptosis (Figs. 1 and 2). (b) LDM-induced DNA ladder patterns appeared as early as 15 min, which was extremely rapid compared to typical apoptosis in which DNA fragmentation only occurs after many hours of apoptosis induction. In addition, caspase inhibitors did not inhibit LDM-mediated DNA cleavage, which was also different from that of typical apoptosis. (c) In contrast to typical apoptosis in which DNA fragmentation and chromatin condensation usually occur at a late stage of the apoptotic process, which can be detected by annexin V binding or TUNEL staining [32,33], LDM-mediated apoptotic cells were negative for annexin V-GFP binding and TUNEL staining (data not shown). Moreover, mitochondria (stained with Mitosensor^{TM}) could be detected at the stage of chromatin condensation in LDM-treated cells (Fig. 2). (d) Although caspase activities were increased after 1 hr of LDM treatment, DNA fragmentation and chromatin condensation occurred much earlier than full caspase activation, which is in contrast to typical apoptosis where apoptotic features appear after full caspase activation. Therefore, LDM treatment induced a very unique type of apoptosis, and the early phases of chromatin condensation and DNA fragmentation were independent of caspase activities.

These unique apoptotic features have not been reported for other enediynes, such as neocarzinostatin (Neo) [34–38] or any other apoptosis-inducing agents. No DNA ladder

was detected in human SK-N-SH neuroblastoma cells after they were treated with 10 μM Neo for 1 hr [34]. Our previous study also revealed that the DNA cleaving properties of LDM are 100 times stronger than those of Neo [39]. Therefore, compared with other anticancer agents, LDM is highly toxic to tumor cells, and its ability to cleave DNA rapidly and directly may contribute to its high cytotoxicity. The protease activity of LDM to degrade histone H1 is consistent with that of other enediyne members such as kedarcidin and maduropeptin [25,26], suggesting that the DNA cleavage activity of the enediyne antibiotics is due to their specific orientation to internucleosomal components. However, LDM-mediated histone H1 degradation could not be inhibited by leupeptin, a protease inhibitor that could block Neo- or kedarcidin-induced histone H1 degradation [25]. In agreement with this result, LDM has been shown to have an aminopeptidase activity *in vitro* that could not be inhibited by leupeptin [40]. The selective protease activities of enediyne antibiotics may reflect their complex toxicity spectrum toward tumor cells, and LDM, being the most potent to date in this family, may solely rely on its ability to cleave DNA rapidly through caspase-independent pathways for its toxicity. However, as shown in Fig. 5, after 1 hr of LDM treatment, caspase activities also increased gradually, indicating that multiple apoptotic pathways, including typical apoptotic pathways, were activated. The apoptosis that occurred at this later time frame also contributed, at least in part, to the cytotoxicity of LDM. Different apoptosis pathways (unique or typical) seemed to also depend on both the LDM concentration and its incubation time with the tumor cells (Table 1, Fig. 2D). In general, ''APO''-type cells became more evident after 18 hr of incubation with higher concentrations of LDM (0.1 to 1 μM), although ''APO''-type cells could be detected earlier with a lower concentration (0.01 μM) of LDM. This reflects the complex mechanisms of LDM-mediated apoptosis. Taken together, rapid and direct cleavage of DNA as well as the initiation of the caspase pathways both contribute to the high antitumor activity of LDM.

Acknowledgments

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