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# Programmed Cell Death (Apoptosis) of Mouse Fibroblasts Is Induced by the Topoisomerase II Inhibitor Etoposide

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**SUMMARY**

The mechanism by which etoposide, a topoisomerase II inhibitor, killed replicating mouse L929 fibroblasts was investigated. Etoposide at 10 μM killed 70% of the cells within 4 days, a result that was accompanied by DNA fragmentation. A characteristic "ladder" pattern of DNA fragmentation was confirmed by agarose gel electrophoresis. Simultaneous exposure of the cells to 10 μM etoposide plus 1 μM cycloheximide reduced both the extent of cell killing and the fragmentation of DNA. Delayed addition of cycloheximide protected cells only if cycloheximide was added 1–6 hr after exposure to etoposide. When added 6–24 hr after treatment with etoposide, cylcoheximide lost the ability to protect cells. Cell growth was completely inhibited by either etoposide or cycloheximide. Furthermore, DNA synthesis was inhibited by either etoposide or cycloheximide within 6 hr. Protein synthesis, however, was not inhibited by etoposide. Thus, the ability of cycloheximide to protect cells correlated with inhibition of protein synthesis, rather than inhibition of DNA synthesis. A 1-hr exposure to 2.5 mM *N*-methyl-*N*-nitrosourea similarly inhibited DNA synthesis within 6 hr, without affecting protein synthesis. However, no loss of viability accompanied *N*-methyl-*N*-nitrosourea treatment. Thus, an imbalance between protein synthesis and DNA synthesis cannot explain the cell killing by etoposide. H-7, a protein kinase C inhibitor, prevented the cell killing and DNA fragmentation, whereas aurintricarboxylic acid, an endonuclease inhibitior, reduced the extent of DNA fragmentation but did not have an effect on cell killing. The data document that the killing of replicating mouse fibroblasts by etoposide represents an example of programmed cell death (apoptosis) that depends on protein synthesis. Although protein synthesis is required during the first 24 hr of exposure to etoposide, cell death is delayed until several days later.

Etoposide is a clinically useful antineoplastic agent that inhibits the nuclear enzyme topoisomerse II of replicating cells (1–4). Topoisomerase II binds to DNA to form a "cleavable complex" of the enzyme with DNA. The enzyme modifies the topology of DNA by transiently breaking both strands and then passing a segment of double-helical DNA through this break (5). The severed strands are subsequently rejoined. Topoisomerase II inhibitors, such as etoposide, interfere with the DNA religation mechanism by stabilizing the covalently bound complexes formed between the enzyme and the 5′ cleaved ends of the DNA molecule (6).

There is a close relationship between the formation of stable cleavable complexes (protein-linked DNA strand breaks) and the cytotoxicity of topoisomerase II inhibitors (1–4, 6, 7). In most of the reports, cytotoxicity was expressed as an inhibition of cell proliferation, rather than as a loss of viability of the treated cells. Cell death is a form of toxic injury clearly distinct from the inhibition of cell growth.[2,3] Furthermore, although the formation of a stable cleavable complex may inhibit cell proliferation, its role in the mechanism of cell killing by inhibitors of topoisomerase II is not as clear.

Programmed cell death (apoptosis) is distinguished from conventional cell death (necrosis) by being an active process that requires protein synthesis (8, 9). Several reports have suggested that topoisomerase II inhibitors kill cells by apoptosis. Those studies used hematopoietic cells, including thymocytes (10), lymphocytes (11), and leukemic cells (12, 13), and defined apoptosis as cell death accompanied by DNA fragmentation. The mechanisms by which inhibition of topoisomerase II produced cell death and DNA fragmentation were not defined. Furthermore, it is not clear that the results of those studies are relevant to nonhematopoietic cells.

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[2] K. Mizumoto and J. L. Farber. ATP depletion is the critical event in the cell killing by alkylating agents: metabolic alterations that accompany poly(ADP-ribosyl)ation. Submitted for publication.

[3] K. Mizumoto and J. L. Farber. The inhibition of poly(ADP-ribose) polymerase has opposing effects on the interruption of cell growth and the loss of cell viability produced by *N*-methyl-*N*-nitrosourea. Submitted for publication.

**ABBREVIATIONS:** MEM, minimum essential medium; MNU, *N*-methyl-*N*-nitrosourea; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; ATA, aurintricarboxylic acid; LDH, lactate dehydrogenase; PCA, perchloric acid; TCA, trichloroacetic acid; ABA, 3-aminobenzamide.

Inhibition of protein synthesis by cycloheximide has been reported to protect several cell lines against the cytoxtoxicity of etoposide (14). In that study, however, cytotoxicity was exclusively assessed with a colony-forming asssay. Inhibition of colony formation may reflect either inhibition of cell proliferation, cell killing alone, or a combination of the two. Thus, the specific relationship between protein synthesis and cell killing was not addressed in the study reported above (14).

In the present experiment, we have investigated the killing of mouse fibroblasts by etoposide. The data presented below indicate that etoposide kills replicating L929 cells by a mechanism that depends on protein synthesis and results in DNA fragmentation. The ability of cycloheximide to protect these cells from the toxicity of etoposide is not attributable to an inhibition of proliferation. Thus, the cell killing by etoposide seems to represent an example of programmed cell death (apoptosis).

## Materials and Methods

**Cell culturing conditions.** The L929 line of mouse fibroblasts (American Type Culture Collection, Rockville, MD) was maintained in 25-$cm^2$ flasks with 5 ml of MEM containing nonessential amino acids (GIBCO Laboratories, Grand Island, NY), 50 IU/ml penicillin, 50 $\mu$g/ml streptomycin, and 7% heat-inactivated fetal bovine serum, under an atmosphere of 95% air/5% $CO_2$. After trypsinization, $1.5 \times 10^6$ cells were plated into each flask. All experiments were performed 2 days after plating, at which time the cells were growing exponentially and had achieved a density of $2.5–3.5 \times 10^6$ cells/flask.

Etoposide (Sigma Chemical Co., St. Louis, MO) and MNU (Sigma) were dissolved in dimethylsulfoxide (0.25% final concentration) and added to the cultures at final concentrations of 10 $\mu$M and 2.5 mM, respectively. One hour before exposure to either etoposide or MNU, the cells were washed with isotonic HEPES-buffered saline (140 mM NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2$, 10 mM HEPES, pH 7.4) and then fresh medium was added. Cycloheximide was dissolved in $H_2O$ and added to the cultures at a final concentration of 1 $\mu$M. H-7 and ATA were dissolved in $H_2O$ and added to the cultures, simultaneouly with etoposide, at a final concentration of 100 $\mu$M. ABA was dissolved in dimethylsulfoxide and added to the cultures, simultaneously with etoposide, at a final concentration of 10 mM.

Cell viability was determined by the release of LDH into the culture medium (15). The loss of viability was expressed as the ratio of the LDH activity in the medium to the total LDH activity released upon treatment with Triton X-100. Cell viability measured in this manner correlated closely with that determined by trypan blue exclusion (data not shown). In the experiment shown in Fig. 4, cells were trypsinized and resuspended in MEM supplemented with fetal bovine serum and trypan blue, and then the total number of viable cells was counted in a hemocytometer. All experiments were performed on triplicate cultures and repeated at least three times. Throughout, the repetitions yielded similar results. Thus, in every case the data illustrated are a single experiment representative of the three performed.

**Determination of DNA fragmentation.** For the determination of DNA fragmentation, cells were lysed in buffer containing 5 mM Tris, pH 8.0, 20 mM EDTA, and 0.5% Triton X-100. After scraping, cell suspensions were centrifuged (28,000 × *g* for 20 min), and the pellet (nonfragmented DNA) was dissolved in 1 N PCA. The supernatant was made 1 N in PCA and centrifuged (28,000 × *g* for 20 min), and then the pellet (fragmented DNA) was dissolved in 1 N PCA. The DNA content of each fraction was determined by reaction with diphenylamine reagent at 70° for 20 min (16). The extent of DNA fragmentation is expressed as the percentage of total DNA in each sample.

The fragmentation of DNA also was demonstrated by agarose gel electrophoresis (17, 18). Briefly, lysates prepared as described above were brought to 50% isopropanol and 0.5 M NaCl and stored overnight at −20°. After centrifugation, the pellet was digested with 100 $\mu$g/ml proteinase K in 0.1 M EDTA, 10 mM NaCl, 0.5% sodium dodecyl sulfate, for 3 hr at 50°. DNA was extracted with phenol and then with phenol/chloroform and was precipitated with ethanol/sodium acetate at −20° overnight. Vacuum-dried DNA, dissolved in 10 mM Tris, pH 7.6, 1 mM EDTA, was separated by electrophoresis (0.8% agarose gel containing 0.4 $\mu$g/ml ethidium bromide) and visualized under UV light.

**Measurement of DNA synthesis.** DNA synthesis was measured as the incorporation of [$^3$H]thymidine into an acid-insoluble precipitate. Cells were incubated for 30 min at 37° in serum-supplemented MEM containing 0.25 $\mu$Ci/ml [$^3$H]thymidine (49 Ci/mmol; Amersham Corp., Arlington Heights, IL). After being washed with isotonic HEPES-buffered saline, labeled cells were incubated for 15 min at 4° with 3% TCA containing 2 mM thymidine and were then scraped from the flasks. The TCA-insoluble precipitate was collected by filtration onto glass microfiber filters (GF/A filters; Whatman International Ltd., Maidstone, UK). The precipitate on the filters was solubilized with 0.2 N NaOH for 1 hr, and the amount of radioactivity was determined by liquid scintillation counting.

**Measurement of protein synthesis.** Protein synthesis was determined by the incorporation of [$^3$H]leucine into an acid-insoluble precipitate. At the times indicated in Results, cells that had been washed with isotonic HEPES-buffered saline were incubated in 5 ml of a special serum-free MEM (leucine-free, lysine-free, methionine-free MEM; GIBCO) containing 0.5 $\mu$Ci/ml [$^3$H]leucine (5 Ci/mmol; DuPont-NEN, Boston, MA). After labeling for 30 min at 37°, cells were washed and lysed with 3% TCA containing 2 mM leucine. Radioactivity incorporated into protein was determined as detailed above for the incorporation of [$^3$H]thymidine into DNA.

**Statistics.** Statistical analysis was performed by the Student-Neuman-Keuls test, using the PC version of the SAS statistical package (version 6.04).

## Results

**Cell killing and accompanying DNA fragmentation.** Fig. 1 illustrates the time course of the fragmentation of DNA (Fig. 1, *upper*) and the killing (Fig. 1, *lower*) of mouse L929 fibroblasts by 10 $\mu$M etoposide. There was no DNA fragmentation or cell killing for the first 2 days. Within 3 days, >30%

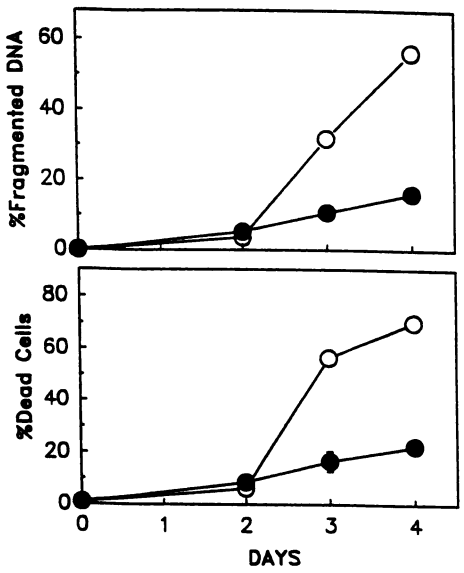


**Fig. 1.** Time course of the fragmentation of DNA (*upper*) and loss of viability (*lower*) of L929 fibroblasts treated continuously with 10 $\mu$M etoposide in the presence (●) or absence (○) of 1 $\mu$M cycloheximide. The results are the mean ± standard deviation of the determinations on three separate flasks.

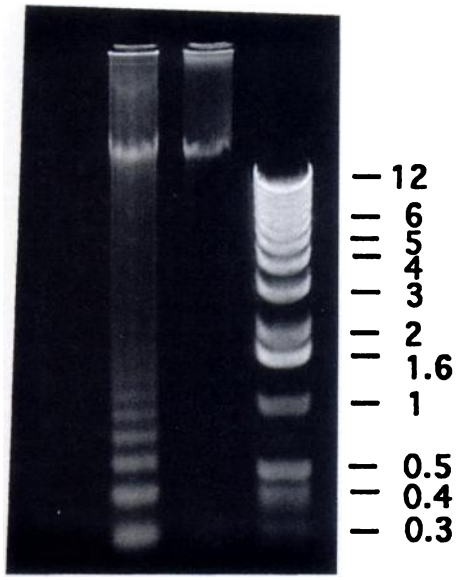


**Fig. 2.** DNA fragmentation revealed by agarose gel electrophoresis. *Lane A, (left)*, DNA from cells treated for 4 days with 10 μM etoposide; *lane B, (center)*, DNA from untreated controls; *lane C, (right)*, 1-kilobase DNA ladder.

TABLE 1

**Effect of time of exposure to etoposide on cell killing**

L929 fibroblasts were exposed to 10 μM etoposide for the times indicated, washed with isotonic HEPES-buffered saline, and incubated in fresh medium. Cell killing was determined after 3 days. The results are the mean ± standard deviation of the determinations on three separate flasks.

| Length of exposure to etoposide | Dead cells |
|---|---|
| | % |
| 0 hr | 1.7 ± 1.0 |
| 1 hr | 7.6 ± 0.4 |
| 3 hr | 9.3 ± 0.4 |
| 6 hr | 11.5 ± 1.6 |
| 24 hr | 36.4 ± 4.7 |
| 3 days | 54.4 ± 4.0 |

of the DNA was fragmented and almost 60% of the cells had died. At the end of 4 days, 70% of the cells were dead and almost 60% of the cellular DNA was fragmented. Greater concentrations of etoposide did not increase either the rate or the extent of the cell killing (data not shown).

Fig. 2 shows the characteristic ladder pattern of DNA fragmentation in the fibroblasts after 4 days of continuous treatment with etoposide. At the same time, there was no evidence of DNA fragmentation in the control cells.

Continuous exposure to etoposide was not necessary for cell killing to occur at the end of 3 days (Table 1). Cells exposed to etoposide for 24 hr, washed, and then incubated in the absence of etoposide were still killed at the end of 3 days. However, exposure to etoposide for periods of <24 hr resulted in progressively less cell killing (Table 1). Finally, as much as 1 mM etoposide had no effect on the viability of cultured rat hepatocytes, which are nonproliferating cells (data not shown).

**Protection by cycloheximide.** The killing of L929 fibroblasts, as well as the fragmentation of DNA, could be prevented by cylcoheximide, an inhibitor of protein synthesis. Fig. 1 shows that after 3 days 1 μM cycloheximide reduced the number of dead cells from almost 60% to <20% of the cells. After 4 days, cell killing was reduced from 70% to slightly more than 20% of the cells. Whereas almost 60% of the DNA was fragmented after 4 days in the absence of cycloheximide, in its presence only 16% of the DNA was fragmented (Fig. 1). Cycloheximide alone had no effect on either the viability or the fragmentation of DNA (Fig. 3). Doses of cycloheximide of >1 μM were toxic to the cells (data not shown).

Fig. 3 illustrates the effect of delaying the addition of cycloheximide from 1 to 24 hr after exposure of the fibroblasts to 10 μM etoposide. The data reflect the extent of both DNA fragmentation and cell killing 4 days after addition of etoposide. Again, the simultaneous addition of both etoposide and cycloheximide reduced the extent of both DNA fragmentation (Fig. 3, *upper*) and cell killing (Fig. 3, *lower*) by 10 μM etoposide. There was no significant difference in the extent of protection when cycloheximide was added 1 or 3 hr after etoposide. Cycloheximide was slightly less effective in preventing both the DNA fragmentation and cell killing when it was added 6 hr after etoposide ($p < 0.001$). Delaying the addition of cycloheximide for 12 hr further reduced the protection determined after 4-day exposure. When added 24 hr after etoposide, cycloheximide did not prevent cell killing. Interestingly, when cycloheximide was added 24 hr after exposure to etoposide there was still significant reduction ($p < 0.001$) in the extent of DNA fragmention after 4 days.

**Growth inhibition by etoposide or cycloheximide.** Etoposide interacts with the cleavage complex transiently present during DNA replication (5). In turn, cycloheximide has been shown to inhibit cell replication, an effect that could reduce sensitivity to etoposide. Fig. 4 details the effects of both 10 μM etoposide and 1 μM cycloheximide on the growth of L929 fibroblasts. The control cells grew exponentially, and the number of cells increased 3-fold over the 48-hr time course of the experiment. In contrast, treatment with either etoposide or cycloheximide prevented an increase in the number of cells over the course of the same 48 hr. Because no cell killing

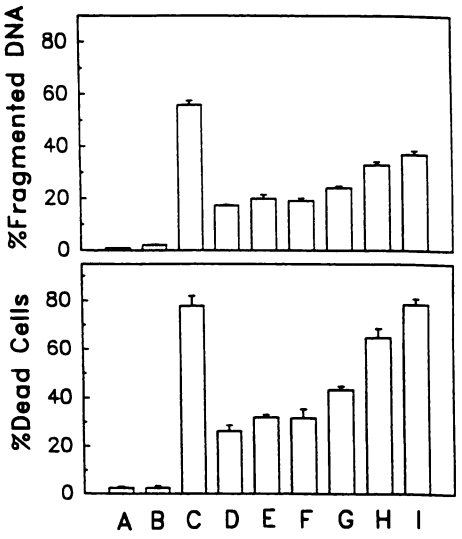


**Fig. 3.** Effect of delaying the time of addition of cycloheximide on the cytotoxicity of etoposide. The extent of DNA fragmentation (*upper*) and cell killing (*lower*) was determined 4 days after addition of etoposide. *A*, Untreated control; *B*, 1 μM cycloheximide only; *C*, 10 μM etoposide only; *D*, simultaneous addition of etoposide and cycloheximide; *E–I*, addition of cyclohexmide to the cells 1, 3, 6, 12, or 24 hr, respectively, after exposure to 10 μM etoposide. The results are the mean ± standard deviation of the determinations on three separate flasks.

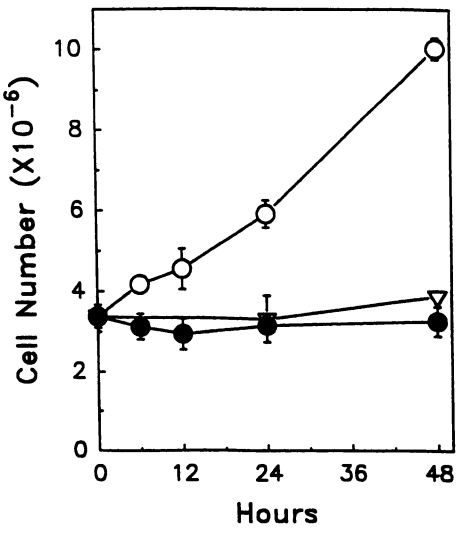


**Fig. 4.** Time course of the growth inhibition by etoposide or cycloheximide. L929 fibroblasts were treated with 10 μM etoposide (●) or 1 μM cycloheximide (▽). Control cells (○) were untreated. At the times indicated, the number of viable cells was determined as described in Materials and Methods. The results are the mean ± standard deviation of the determinations on three separate flasks.

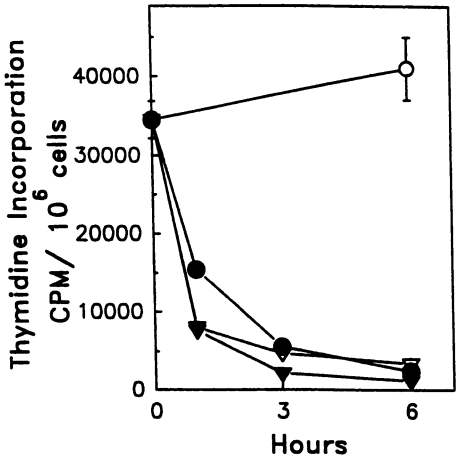


**Fig. 5.** Inhibition of DNA synthesis by etoposide and cycloheximide. L929 fibroblasts were exposed to 10 μM etoposide (●), 1 μM cycloheximide (▽), or a combination of the two (▼). Control cells (○) were untreated. The extent of [$^3$H]thymidine incorporation into DNA during a 30-min pulse was determined at the times indicated. The results are the mean ± standard deviation of the determinations on three separate flasks.

occurred during the first 48 hr after exposure to etoposide (Fig. 1), the data in Fig. 4 indicate that 10 μM etoposide completely inhibits cell proliferation. Similarly, 1 μM cycloheximide inhibited the growth of the cells.

**Effects of etoposide and cycloheximide on DNA synthesis.** The cessation of proliferation in cells treated with either etoposide or cycloheximide (Fig. 4) reflects rapid inhibition of DNA synthesis. Fig. 5 details the effects of etoposide, cycloheximide, and a combination of the two on the incorporation of [$^3$H]thymidine into DNA. Within 1 hr, thymidine incorporation was inhibited by 55% and 77% by etoposide and cycloheximide, respectively. Together, the combination inhibited DNA synthesis by 78%. After 3 hr and 6 hr, thymidine incorporation was inhibited by 90% or more by either etoposide, cycloheximide, or the two together.

**Effects of etoposide and cycloheximide on protein synthesis.** Whereas both etoposide and cycloheximide inhibited DNA synthesis, only cycloheximide inhibited protein synthesis. Fig. 6 illustrates the effects of etoposide, cycloheximide, and a combination of the two on the incorporation of [$^3$H]leucine into protein. Within 1 hr, 1 μM cycloheximide inhibited protein synthesis by almost 60%. In contrast, etoposide was without effect on the incoroporation of leucine into protein. This difference persisted for up to 6 hr after treatment of the cells with either agent. The effect on protein synthesis of the combination of cycloheximide and etoposide was the same as that of cycloheximide alone. Thus, the protective effect of cycloheximide against the cell killing by etoposide was better correlated with the inhibition of protein synthesis than with that of DNA synthesis.

**DNA synthesis and protein synthesis after treatment with MNU.** The data in Figs. 5 and 6 show that etoposide causes an imbalance between protein and DNA synthesis. It might be argued that the continued synthesis of protein in the absence of DNA synthesis contributes to the loss of viability. We have shown previously that a brief exposure of L929 fibroblasts to the alkylating agent MNU inhibits their proliferation for up to 2 weeks without any loss of viability.[3] Thus, it was of interest to compare the effects of MNU on the synthesis of DNA and protein with those of etoposide.

Fig. 7 illustrates the time course of the incorporation of [$^3$H] thymidine into DNA and of [$^3$H]leucine into protein after treatment of fibroblasts with MNU. Cells were exposed to 2.5 mM MNU for 1 hr, washed, and placed in fresh medium. The extent of incorporation of a 30-min pulse of either [$^3$H]thymidine or [$^3$H]leucine was determined immediately after washing of the cells or after continued incubation for the times indicated.

Although transient inhibition occurred after 1 hr, no inhibition of protein synthesis was evident after 3 hr. In contrast, within 3 hr of exposure to MNU incorporation of [$^3$H]thymidine into DNA was inhibited by >95%. This difference between the rates of DNA and protein synthesis was also evident 6 hr after exposure to MNU. Thus, the cell killing with etoposide cannot

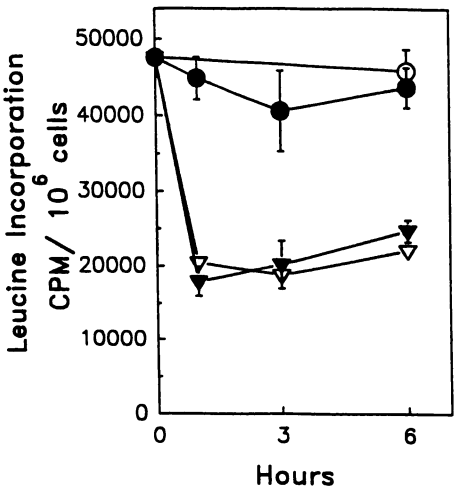


**Fig. 6.** Effects of cycloheximide and etoposide on protein synthesis. L929 fibroblasts were exposed to 10 μM etoposide (●), 1 μM cycloheximide (▽), or combination of the two (▼). Control cells (○) were untreated. The incorporation of [$^3$H]leucine into protein during a 30-min pulse was determined at the times indicated. The results are the mean ± standard deviation of the determinations on three separate flasks.

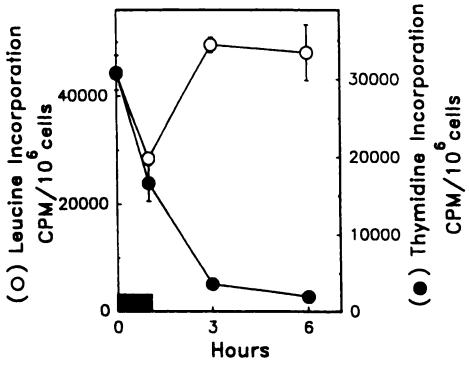


**Fig. 7.** Effects of MNU on DNA and protein synthesis. L929 fibroblasts were exposed to 1 mM MNU for 1 hr, washed, and placed in fresh medium. Thymidine incorporation (●) and leucine incorporation (○) were determined at the times indicated. *Shaded area*, period during which the cells were exposed to MNU. The results are the mean ± standard deviation of the determinations on three separate flasks.

TABLE 2

**Modulation of DNA fragmentation and cell killing**

Cells were exposed to 10 μM etoposide alone or with 100 μM H-7, 100 μM ATA, or 10 mM ABA. The percentage of fragmented DNA and the extent of cell killing were measured 4 days later. The results are the mean ± standard deviation of the determinations on three separate flasks.

| Treatment | Fragmented DNA | Dead cells |
|---|---|---|
| | % | % |
| Control | 1.5 ± 0.6 | 2.6 ± 0.3 |
| H-7 | 4.5 ± 0.7 | 9.8 ± 1.0 |
| ATA | 1.7 ± 0.2 | 5.9 ± 1.5 |
| ABA | 5.1 ± 1.0 | 4.3 ± 0.1 |
| Etoposide | 50.1 ± 0.3 | 72.5 ± 1.0 |
| Etoposide + H-7 | 16.2 ± 0.2[a] | 34.4 ± 0.9[a] |
| Etopside + ATA | 18.0 ± 0.3[a] | 71.9 ± 2.6 |
| Etoposide + ABA | 51.4 ± 0.6 | 70.8 ± 0.5 |

[a] Significantly different from etoposide, $p < 0.001$.

be explained as an unbalanced synthesis of protein in the presence of an inhibition of DNA synthesis.

**Protection by an inhibitor of protein kinase C.** Activation of protein kinase C has been implicated in the mechanisms of programmed cell death (19). Table 1 shows that the protein kinase C inhibitor H-7 reduced both the killing of L929 fibroblasts and the extent of DNA fragmentation. In contrast, the endonuclease inhibitor ATA was without effect on the cell killing by etoposide. Nevertheless, ATA substantially reduced the extent of DNA fragmentation (Table 2). Finally, ABA, an inhibitor of poly(ADP-ribose) polymerase, an enzyme involved in DNA repair, was without effect on either the cell killing or DNA fragmentation (Table 2).

## Discussion

The data presented above indicate that the topoisomerase II inhibitor etoposide kills cultured L929 mouse fibroblasts by a mechanism that is accompanied by the fragmentation of DNA and that depends on protein synthesis. These two features are typical of what has been called programmed cell death or, more generally, apoptosis (20–23).

The dependence of cell killing by etoposide on protein synthesis was shown by the ability of cycloheximide to prevent the loss of viability. At the doses used in the present study, cycloheximide stopped the proliferation of the fibroblasts (Fig. 4) and inhibited the incorporation of [$^3$H]thymidine into DNA (Fig. 5).

The cytotoxicity of etoposide has been closely correlated with its effect on DNA synthesis. Thus, the protective effect of cycloheximide could be explained in at least two ways. By stopping the synthesis of DNA and inhibiting the proliferation of the cells, cycloheximide may simply have made these cells insensitive to the toxicity of a topoisomerase II inhibitor such as etoposide. Cultured hepatocytes do not proliferate and were insensitive to as much as 1 mM etoposide. Alternatively, cycloheximide may inhibit the synthesis of a protein that mediates the death of the fibroblasts and that is made in response to the inhibition of DNA synthesis by etoposide. The data presented above clearly discriminate between these two interpretations of the action of cycloheximide. It is concluded that cycloheximide acts to prevent the synthesis of a protein that is required for the cells to die in response to etoposide.

Etoposide alone completely inhibited the proliferation of the cells in a manner indistinguishable from the effect of cycloheximide alone (Fig. 4). Thus, inhibition of cellular proliferation cannot be the mechanism by which cycloheximide protected the cells. Furthermore, etoposide alone rapidly inhibited the incorporation of [$^3$H]thymidine into DNA. Again, cycloheximide similarly inhibited the synthesis of DNA (Fig. 5), and within 3 hr this effect was identical to that observed with etoposide (Fig. 5). It is noteworthy that the addition of cycloheximide could be delayed, without a loss of its ability to protect, to a time (3–6 hr) at which etoposide had already completely inhibited DNA synthesis. Thus, the protective effect of cycloheximide cannot be explained as preventing the effect of etoposide on DNA metabolism.

Importantly, the action of cycloheximide differed from that of etoposide in a very clear way. With etoposide, the inhibition of cellular proliferation (Fig. 4) and DNA synthesis (Fig. 5) was not accompanied by an inhibition of protein synthesis (Fig. 6). In contrast, cycloheximide inhibited protein synthesis at the same time that it stopped the proliferation of the fibroblasts (Fig. 6). Thus, prevention by cycloheximide of the cell killing by etoposide correlates with inhibition of protein synthesis rather than with inhibition of DNA synthesis.

It is possible that in the etoposide-intoxicated cells the continued synthesis of protein in the presence of inhibition of cell replication, so-called unbalanced growth, is itself a cause of injury. Such an interpretation was ruled out by the action of another agent that damages DNA and inhibits the proliferation of L929 fibroblasts. A brief exposure of these cells to MNU stops their proliferation.[3] In this situation, there is inhibition of DNA synthesis with continued synthesis of protein (Fig. 7). However, the cells are not killed.[3] Thus, the difference between etoposide and MNU effects must relate to the qualitative pattern of protein synthesis, rather than simply the persistence of protein synthesis in the absence of DNA synthesis.

It is concluded that, as a consequence of the inhibition of topoisomerase II activity, etoposide causes the synthesis of a protein that is not normally synthesized and that is needed, in a still undefined manner, for the subsequent loss of viability of the cells. Furthermore, the data presented above indicate that the synthesis of this critical protein is required during the first 24 hr after exposure to etoposide (Fig. 3; Table 1), despite the fact that there is complete inhibition of DNA synthesis within 3 hr (Fig. 5) and no loss of viability for at least the next 2 days (Fig. 1).

Programmed cell death is an active process that is exempli-

fied by the elimination of tissue that occurs during embryonic development (20, 21). The process is accompanied by an internucleosomal fragmentation of DNA that is widely held to distinguish programmed cell death from toxic injury and necrosis (22–24). Recently, the concept of programmed cell death has been broadened to include a variety of injuries characterized by DNA fragmentation and referred to as apoptosis. The requirement for protein synthesis and the accompanying DNA fragmentation allow the killing of L929 fibroblasts by etoposide to be included under the rubric of programmed cell death or apoptosis.

Although DNA fragmentation closely parallels the loss of viability with etoposide, there is evidence that the two can be dissociated. The addition of ATA, an endonuclease inhibitor, to the culture medium simultaneously with etoposide had no effect on the extent of cell killing over the next 4 days. However, ATA did significantly reduce the extent of DNA fragmentation (Table 2). A similar result was seen after the delayed addition of cycloheximide (Fig. 3). Addition of this protein synthesis inhibitor 24 hr after exposure of the cells to etoposide provided no protection from the cell killing at 4 days but did signficantly reduce the extent of DNA fragmentation. These data indicate that the mechanism by which etoposide kills the cells does not necessarily depend on the fragmentation of DNA. A similar dissociation of programmed cell death from the fragmentation of DNA has been observed in the killing of cultured hepatocytes with microtubule antagonists (25).

The precise mechanism by which etoposide induces the programmed cell death of L929 fibroblasts remains to be defined. However, a few comments are in order. In particular, the difference in the fate of the cells when DNA synthesis is interrupted by etoposide as opposed to MNU needs to be considered. Why do the cells die with etoposide and not with MNU? This difference may reflect the real significance of programmed cell death.

MNU inhibits DNA synthesis as a consequence of the alkylation of its constituent bases. This DNA damage can be repaired, and thus the inhibition of cellular proliferation is eventually reversible. In contrast, the DNA damage resulting from the action of etoposide is not reversible after 24 hr (Table 1). Thus, it would seem that proliferating cells, such as L929 mouse fibroblasts, have a mechanism that discriminates between different types of DNA damage. If the damage cannot be repaired (as with etoposide), a program is expressed that leads to the death of the cells. If the damage can be repaired (as with MNU), this program remains repressed. Such an hypothesis can be assessed by further elucidation of the mechanisms by which the inhibition of DNA synthesis by etoposide is coupled to a change in the pattern of protein synthesis and identification of the protein(s) whose action, in turn, relates to the loss of viability.

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