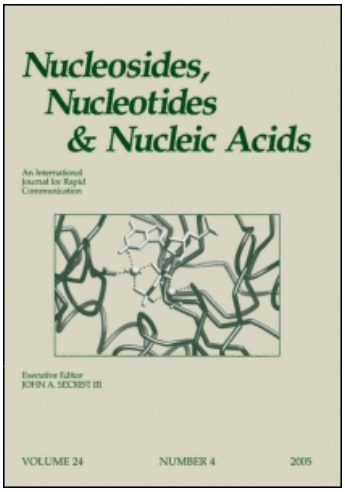

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THE CHARACTERIZATION OF CELL DEATH INDUCED BY 1-(3-*C*-ETHYNYL-ß-D-*RIBO*-PENTOFURANOSYL)CYTOSINE (ECyd) IN FM3A CELLS

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ABSTRACT: The characterization of cell death induced by 1-(3-*C*-ethynyl-ß-D-*ribo*-pentofuranosyl)cytosine (ECyd), a potent inhibitor of RNA synthesis, was performed using mouse mammary tumor FM3A cells *in vitro*. Accompanied with the cell death induced by ECyd (3.0 μM)-treatment, about 100-200 kbp-sized and internucleosomal DNA fragmentation were observed by orthogonal-field-alternation gel electrophoresis (OFAGE) and conventional gel electrophoresis, respectively. Protease inhibitors, carbobenzoxy-L-aspart-1-yl[(2,6-dichlorobenzoyl)oxy]methane (Z-Asp-CH_2-DCB), *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), effectively blocked the cell death, suggesting that the proteases inhibited by Z-Asp-CH_2-DCB, TLCK or TPCK were involved in the process of the cell death.

INTRODUCTION

In slow-growing solid tumors, the population of cells in S phase (the stage in which inhibitors of DNA synthesis are effective) is smaller than that in rapid-growing tumors. This is the main reason why pyrimidine analogues including 5-fluorouracil (5-FU) and 1-ß-D-arabinofuranosylcytosine (Ara-C) as DNA synthesis inhibitors, fail to show sufficient antitumor activity on slow-growing solid tumors. Thus, we have focused our attention to inhibition of RNA synthesis, as a good target for eliminating slow-growing tumor cells because RNA synthesis occurs mostly throughout the cell cycle (excluding M phase).

We have recently reported that newly synthesized ribo-nucleoside analogues, 1-(3-*C*-ethynyl-ß-D-*ribo*-pentofuranosyl)cytosine (ECyd) and 1-(3-*C*-ethynyl-ß-D-*ribo*-pentofuranosyl)uracil (EUrd), exert potent antitumor activity *in vitro* and *in vivo*[1, 2, 3]. After

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effective phospholylation of them, the resultant metabolites such as ECyd 5'-triphosphate (ECTP) and EUrd 5'-triphosphate (EUTP) inhibit RNA synthesis[1], resulting in induction of cell death accompanied with internucleosomal DNA fragmentation[3]. EUTP is characteristic in that it is further converted to a more toxic metabolite, ECTP, which is extremely stable in cells[1]. Due to their characteristic metabolism and action, these nucleoside analogues exert more potent antitumor activities on solid tumors relative to those of 5-FU and Ara-C[3, 4].

F28-7, a wild-type strain of mouse mammary tumor FM3A cells, was characterized as a strain to be committed to necrosis by 5-fluoro-2'-deoxyuridine (FdUrd)[16]. It is well noted that FdUrd induces apoptosis in tumor cells[5]. In F28-7 strain, however, 1.2 μM or lower concentration of FdUrd-treatment induced necrosis accompanied with swelling of the cells but not with apoptotic features, including internucleosomal DNA fragmentation and formation of apoptotic bodies (unpublished data). Interestingly, it was evidenced by us that ECyd induced apoptosis in F28-7 strain unlike FdUrd.

Recently, it has been well documented that proteases play a key role in the signal pathways of both apoptosis[6, 7-10] and necrosis[10, 11]. Mashima *et. al.* reported that carbobenzoxy-L-aspart-1-yl[(2,6-dichlorobenzoyl)oxy]methane (Z-Asp-CH_2-DCB) effectively prevented antitumor agent-induced apoptosis in leukemia cells. Here, we describe about the profile of the ECyd-induced cell death and the effects of protease inhibitors on this cell death.

MATERIALS AND METHODS

Materials and Cell Culture: ECyd was synthesized in the methods reported previously[12]. Z-Asp-CH_2-DCB, *i*-valeryl-L-Val-L-Val-(3*S*, 4*S*)-4-amino-3-hydroxy-6-methyl-heptanoic acid-L-Ala-(3*S*, 4*S*)-4-amino-3-hydroxy-6-methyl-heptanoic acid (pepstatin A), and [(S)-1-carboxy-2-phenylethyl]-carbamoyl-L-arginyl-L-valyl-argininal hydrochloride (antipain) were purchased from Peptide Institute (Osaka, Japan). *Nα-p*-Tosyl-L-lysine chloromethyl ketone (TLCK), *N-p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and 1, 10-phenanthroline were obtained from SIGMA (St. Louis, MO). All other chemicals were of the highest purity available.

Mouse mammary tumor FM3A cells (F28-7; wild type, JCRB0701) were obtained from the Japanese Cancer Research Resources Bank. The cells were grown at 37 °C in a 5.0% CO_2 atmosphere in ES medium (modified Eagle's minimum essential medium, Nissui Pharmaceutical, Tokyo, Japan) containing 2% heat-inactivated fetal bovine serum. The cell viability was monitored with trypanblue dye exclusion method[13].

DNA Fragmentation Assay: Orthogonal-field-alternation gel electrophoresis (OFAGE): The apparatus used for OFAGE was described previously[14]. Agarose blocks containing DNA of ECyd-treated cells were prepared with the partially modified method of Schwartz and Cator[13]. Briefly, following treatment with ECyd (3.0 μM), FM3A cells (2 x 10^6 cells) were placed in 0.6 ml of 0.6% low-melting agarose (Bio-Rad Laboratories, Richmond, CA) and then lysed in an aqueous solution containing 0.5 M EDTA, pH 9.0, 1% w/v sodium *N*-lauroylsarcosinate, and 1 mg/ml proteinase K for 2 days at 50 °C. The blocks were washed three times with 0.2 M EDTA, pH 8 and then plugged into wells of 1% agarose. The electrophoresis was performed with 0.6 x TBE (53 mM Tris, 53 mM boric acid, and 1.2 mM EDTA, pH 8.3) at 13 °C for 16 h using linear gradient pulses lasting 50 to 100 s. The agarose gel was stained with ethidium bromide to visualize DNA on a UV trans-illuminator.

Conventional gel electrophoresis: FM3A cells (1.5 x 10^6 cells) exposed to ECyd (3.0 μM) were suspended in 30 μl of the lysing solution containing 50 mM Tris-HCl (pH 7.8), 10 mM EDTA and 0.5% w/v sodium *N*-lauroylsarcosinate. After digestion of RNA (0.5 mg/ml of RNase A treatment at 50 °C for 30 min) and protein (0.5 mg/ml of proteinase K treatment at 50 °C for 60 min), ECyd-induced DNA fragmentation was analyzed using 2% agarose gel electrophoresis.

RESULTS

DNA fragmentation in ECyd-treated cells. In F28-7 strain, ECyd potently inhibited RNA synthesis. When the cells were incubated with ECyd (3.0 μM), capability of RNA synthesis was reduced to 30.0% at 4 h and 8.4% at 12 h (detail will be published elsewhere). After 12 h incubation, the reduction of cell viability was observed. The cell viability was time-dependently reduced to 34.3% at 24 h. DNA fragmentation in the cells was examined by OFAGE (FIG. 1A) and conventional gel electrophoresis (FIG. 1B). At 12 h of the treatment, a band of fragmented DNA, about 100-200 kbp-sized, was observed by OFAGE. The DNA fragments were accumulated time-dependently (FIG. 1A). Accompanying about 100-200 kbp-sized DNA fragmentation, internucleosomal DNA fragments were also observed at 12 h post-treatment by conventional gel electrophoretic analysis (FIG. 1B). FIG. 2 shows the comparison of DNA fragmentation between FdUrd (0.12 μM)- and ECyd (3.0 μM)-treated cells at 24 h of incubation by conventional gel electrophoresis. In FdUrd-treated cells, about 100-200 kbp-sized DNA fragments were observed by OFAGE as well as ECyd-treated cells (data not shown). Internucleosomal DNA fragmentation, however, was not observed in FdUrd-treated cells (FIG. 2). The viability of FdUrd-treated was 27.6%. At that time point in FdUrd-treated cells, morphological findings evidenced onset of necrosis but not apoptosis.

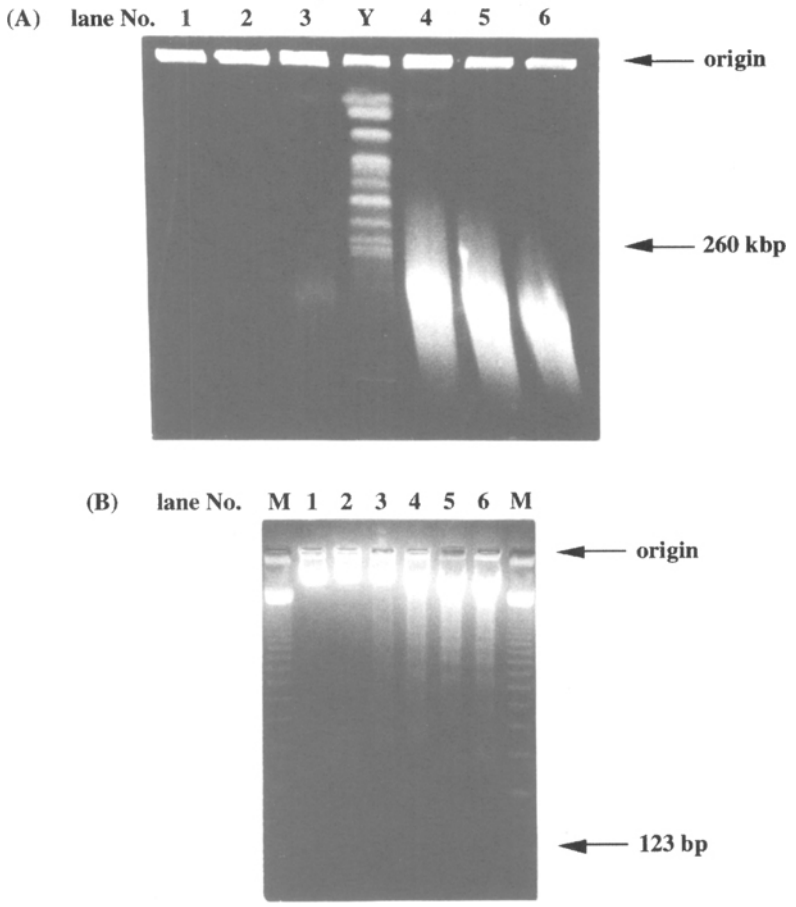

FIG. 1. Time-course changes of DNA fragmentation induced by ECyd (3.0 μM). The DNA from cells was analyzed by both OFAGE (A) and conventional gel electrophoresis (B). (A) Agarose blocks containing zero time control cells (lane 1) and cells treated with ECyd were prepared at 8 h (lane 2), 12 h (lane 3), 16 h (lane 4), 20 h (lane 5), and 24 h (lane 6) as described in "MATERIALS AND METHODS." Lane Y is DNA size markers (yeast chromosomal DNA). (B) Zero time control cells (lane 1) and ECyd-treated cells were harvested at 8 h (lane 2), 12 h (lane 3), 16 h (lane 4), 20 h (lane 5), and 24 h (lane 6) and prepared for the samples of conventional gel electrophoresis as described in "MATERIALS AND METHODS." Lanes M are 123 bp-sized ladder markers.

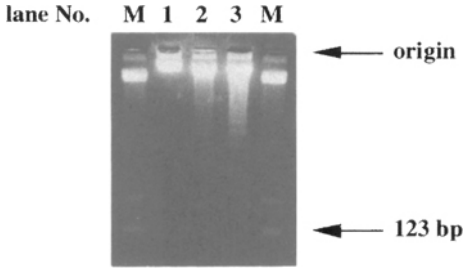

FIG. 2. Comparison of DNA fragmentation between FdUrd (0.12 μM)-treated and ECyd (3.0 μM)-treated cells by conventional gel electrophoresis. Zero time control cells (lane 1), FdUrd-treated (lane 2) and ECyd-treated cells at 24 h (lane 3) were prepared for the samples of conventional gel electrophoresis as described in "MATERIALS AND METHODS." Lanes M are 123 bp-sized ladder markers.

Effects of protease inhibitors on the cell death induced by ECyd treatment. To ascertain the role of proteases in induction of the cell death in ECyd-treated cells, we investigated the effects of protease inhibitors on ECyd-induced cell death. As shown in TABLE 1, Z-Asp-CH_2-DCB (a caspase inhibitor)[6], TLCK and TPCK (serine protease inhibitors), effectively blocked the cell death, whereas 1, 10-phenanthroline (a metalloprotease inhibitor), pepstatin A (an aspartic protease inhibitor) and antipain (a cysteine protease inhibitor) failed to exert such inhibitory effects. On gel electrophoretic analysis, Z-Asp-CH_2-DCB (100 μM) prevented DNA fragmentation including about 100-200 kbp-sized (FIG. 3A) and internucleosomal fragments (FIG. 3B). On the other hand, antipain (100 μM) failed to prevent both the types of DNA fragmentation.

DISCUSSION

We have recently reported that ECyd and EUrd potently inhibited RNA synthesis resulting in cell death[1, 2, 3]. These nucleosides are expected to be promising candidates as new antitumor agents because of their potent antitumor activities without serious side effects[3, 4].

In the present study, we investigated the profile of cell death induced by ECyd in FM3A cells. Exposure to ECyd was associated with the cell death, accompanied with the relevant DNA fragmentation. In ECyd-treated cells, RNA synthesis were potently inhibited within 4 h. At 12 h of treatment we observed about 100-200 kbp-sized DNA fragmentation and internucleosomal DNA fragmentation indicative of a well-noted feature of apoptotic cell death[15]. In our laboratory, the mechanism of cell death induced by FdUrd has been studied with use of the F28-7 strain of FM3A cells. The cells were

TABLE 1. Effects of protease inhibitors on the cell death induced by ECyd (3.0 μM) treatment

Protease inhibitors	Concentration of protease inhibitors (μM)	Viability (%)[a]
None	——	34.3 ± 1.8
Z-Asp-CH2-DCB	100	98.5 ± 0.6
TLCK	200	91.0 ± 0.4
TPCK	5.0	80.9 ± 4.1
Pepstatin A	100	52.4 ± 2.8
1, 10-Phenanthroline	100	43.5 ± 3.4
Antipain	100	29.2 ± 1.2

a : At 24 h after the incubation, the viability of cells were assessed with trypanblue dye exclusion method. At zero time and 24 h after the incubation, the viability of control cells were more than 99.0%.

characterized as the strain which was committed to necrosis by FdUrd. Internucleosomal DNA fragmentation was not observed in FdUrd-treated cells (FIG. 2). Interestingly, ECyd induced the apoptosis accompanied with internucleosomal DNA fragmentation (FIG. 1B and FIG. 2), formation of apoptotic bodies and chromatin condensation (data not shown). Given all this, it was postulated that the F28-7 strain of FM3A cells might possess at least two types of systems related to cell death. Addition of 2 μg/ml of cycloheximide to the medium at 8 h of FdUrd-treatment could reverse the cell death[16], but rather augmented cell death induced by ECyd (unpublished observation). Martin *et. al.* also reported that apoptosis induced by actinomycin D did not require protein synthesis[17]. Thus, in contrast to FdUrd, ECyd might activate the proteases participating in signaling pathway of cell death without protein synthesis.

Protease inhibitors such as Z-Asp-CH2-DCB, TLCK and TPCK, could block ECyd-induced cell death (TABLE 1). On the other hand, 1, 10-phenanthroline, pepstatin A and antipain failed to prevent the cell death. According to electrophoretic analysis, Z-Asp-CH2-DCB, which could prevent ECyd-induced cell death, blocked both types of DNA fragmentation (FIG. 3A and 3B), suggesting that caspases play a key role in a sequence of

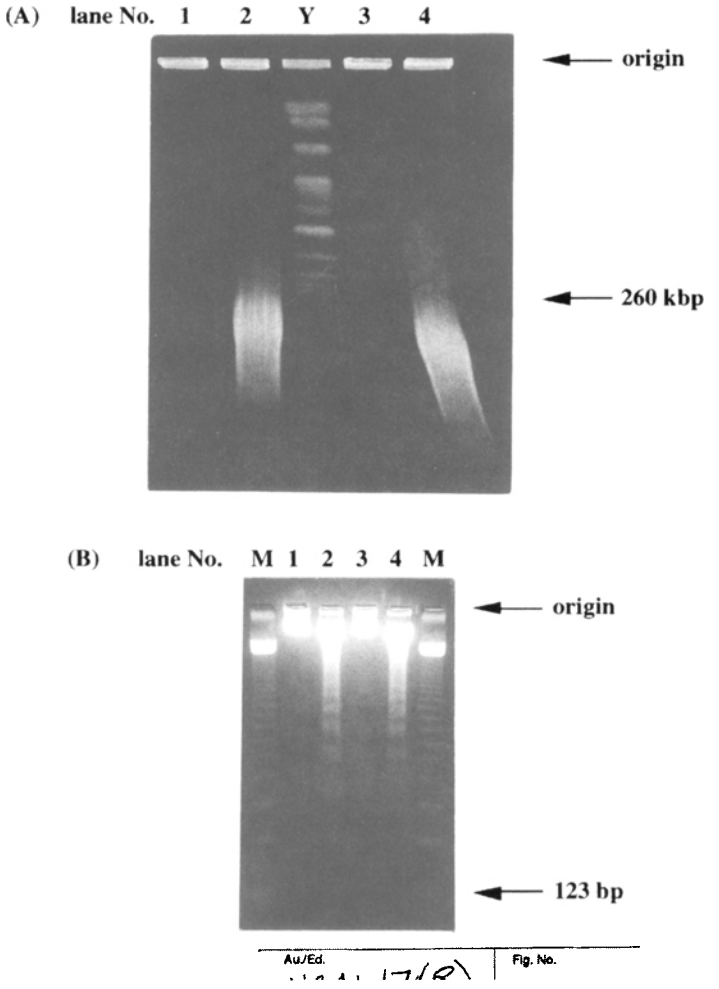

FIG. 3. The effects of Z-Asp-CH_2-DCB (100 μM) and antipain (100 μM) on DNA fragmentation induced by ECyd (3.0 μM)-treatment for 24 h. (A) OFAGE : The cells treated with ECyd in the presence of Z-Asp-CH_2-DCB (lane 3) or antipain (lane 4). Control cells (lane 1) and the cells treated with ECyd in the absence of these protease inhibitors (lane 2). Lane Y is DNA size markers (yeast chromosomal DNA). (B) Conventional gel electrophoresis : The cells treated with ECyd in the presence of Z-Asp-CH_2-DCB (lane 3) or antipain (lane 4). Control cells (lane 1) and the cells treated with ECyd in the absence of these protease inhibitors (lane 2). Lanes M are 123 bp-sized ladder markers.

cell death events which end up with DNA fragmentation *i.e.* giving mortal damages to the cells. We have already observed that FdUrd-induced cell death was also blocked by Z-Asp-CH_2-DCB, TLCK or TPCK, but not by 1, 10-phenanthroline, pepstatin A, nor antipain (these data will be published elsewhere). The analysis of proteases participating in the signal pathways in ECyd-treated cells is now under way for further studying in our laboratory.

ECyd-treatment could trigger the cell death in a different way from these after FdUrd-treatment, leading cells to apoptosis. Comparison of these two types of cell death may provide us with the key to open the black box installed with the change-circuit switch to decide future fates of cells either to necrosis or to apoptosis. The new antitumor nucleoside, ECyd may be able to induce apoptosis in the cells which are usually resistant to induction of apoptosis even after treatment with antitumor agents commonly used in clinical fields.

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