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Inhibitory effect of conjugated eicosapentaenoic acid on mammalian DNA polymerase and topoisomerase activities and human cancer cell proliferation

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Abstract

Conjugated eicosapentaenoic acid (cEPA) selectively inhibited the activities of mammalian DNA polymerases (pols) and human DNA topoisomerases (topos) [Yonezawa Y, Tsuzuki T, Eitsuka T, Miyazawa T, Hada T, Uryu K, et al. Inhibitory effect of conjugated eicosapentaenoic acid on human DNA topoisomerases I and II. Arch Biochem Biophys 2005;435:197–206]. In this report, we investigated the inhibitory effect of cEPA on a human promyelocytic leukemia cell line, HL-60, to determine which enzymes influence cell proliferation. cEPA inhibited the proliferation of HL-60 cells (LD_{50} = 20.0 μM), and the inhibitory effect was stronger than that of non-conjugated EPA. cEPA arrested the cells at G1/S-phase, increased cyclin A and E protein levels, and prevented the incorporation of thymidine into the cells, indicating that it blocks the primary step of in vivo DNA replication by inhibiting the activity of replicative pols rather than topos. This compound also induced apoptosis of the cells. These results suggested the therapeutic potential of cEPA as a leading anti-cancer compound that poisons pols.

Keywords: Conjugated eicosapentaenoic acid (cEPA); DNA polymerase; Enzyme inhibitor; DNA replication; Cytotoxicity; Cell proliferation; Apoptosis

1. Introduction

Both DNA polymerases (pols) and DNA topoisomerases (topos) have recently emerged as important cellular targets for chemical intervention in the development of anti-cancer agents. Pol catalyzes the addition of deoxyribonucleotides to the 3′-hydroxyl terminus of primed double-stranded DNA molecules [1], and topo catalyzes the concerted breaking and rejoining of DNA strands and is involved in producing the necessary topological and conformational changes in DNA [1,2]. Therefore, there are no enzymatic similarities between the two enzymes, although they are both critical to many cellular processes, such as DNA replication, repair and recombination and may act in harmony with each other.

We have screened for inhibitors of pols [3,4], and found that mammalian pols α and β are inhibited by linear-chain fatty acids with the following characteristics: a hydrocar-

Abbreviations: EPA, eicosapentaenoic acid; cEPA, conjugated eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid; Ct, cycle threshold; dTTP, 2′-deoxythymidine 5′-triphosphate; DMSO, dimethylsulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PCR, polymerase chain reaction; pol, DNA polymerase (EC 2.7.7.7); topo, DNA topoisomerase

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bon chain containing 18 or more carbons, a free carboxyl end and double bonds with the *cis*-configuration, n-3 polyunsaturated fatty acid (PUFA) having the strongest inhibitory effect of any fatty acid tested [3,5]. These fatty acids also inhibit the activities of human topos I and II [6]. Epidemiological data indicate that consumption of fish oil rich in PUFA correlates with a reduced incidence of colon cancer [7]. Experimental studies indicate that fish oil plays a protective role in mice [8] and rats [9] and that perilla oil rich in n-3 PUFA (α-linolenic acid) inhibits colon carcinogenesis in rats [10]. n-3 PUFAs seem to be particularly important in the inhibition of colon carcinogenesis. Eicosapentaenoic acid (EPA; 5*Z*8*Z*11*Z*14*Z*17*Z*-20:5) and docosahexaenoic acid (DHA; 4*Z*7*Z*10*Z*13*Z*16*Z*19*Z*-22:6), both of which are n-3 PUFAs, exert significant inhibitory effects on colon carcinoma cell growth at the primary site and metastases [11,12]. However, it is unclear which class of n-3 PUFA is more potent in suppressing colon carcinogenesis.

Conjugated fatty acids are positional and geometrical isomers with several conjugated double bonds. One of these, conjugated linoleic acid (cLA), is found in meats from ruminants and in dairy products [13] and reportedly reduces colon cancer risk in rats [14]. cLA also reportedly inhibits growth of human colon cancer cells in culture [15,16]. As linoleic acid, an n-6 PUFA, accelerates the growth of colon cancer cells [12], the opposite effect of cLA is of particular interest. As n-3 PUFAs have been shown to have anti-carcinogenic activity, conjugated fatty acids converted from n-3 PUFAs may show higher tumor-inhibiting activity than cLA or n-3 PUFAs themselves. Fatty acids with conjugated double bonds exist in nature: seaweeds, such as red and green algae contain highly n-3 unsaturated conjugated fatty acids, i.e., conjugated EPA (cEPA; 5*Z*7*E*9*E*14*Z*17*Z*-20:5), bosseopentaenoic acid (5*Z*8*Z*10*E*12*E*14*Z*-20:5) and stellaheptaenoic acid (4*Z*7*Z*9*E*11*E*13*Z*16*Z*19*Z*-22:7) [17,18]. We realized the importance of the two classes of n-3 PUFA; EPA and DHA, normal and conjugated and the inhibitory effect of cEPA on both pols and topos was stronger than that of cDHA [19]. Thus, conjugated cEPA seems to be an ideal model for the study not only of the molecular mechanisms that inhibit pol and topo activities for the development of new anti-cancer drugs, but also of cellular proliferation processes, such as DNA replication.

2. Materials and methods

2.1. Materials

Eicosapentaenoic acid (EPA) was purchased from Nu-Chek-Prep Inc. (MN, USA). Nucleotides and chemically synthesized template-primers, such as poly(dA) and oligo$(dT)_{12–18}$ were purchased from Pharmacia. The radioisotope reagents, such as [^{3}H]-dTTP (2′-deoxythymidine 5′-triphosphate) (43 Ci/mmol), [methyl-^{3}H] thymidine, [5,6-^{3}H] uridine and L-[4,5-^{3}H] leucine were purchased from Amersham Biosciences (Buckinghamshire, UK). All other reagents were of analytical grade and purchased from Nacalai Tesque Inc. (Kyoto, Japan). HL-60, a human promyelocytic leukemia cell line (IFO50022) was supplied by the Health Science Research Resources Bank (Osaka, Japan).

2.2. Preparation of conjugated EPA by alkaline treatment

Conjugated EPA (cEPA) was prepared by alkaline treatment following the AOAC method with slight modifications [20]. Potassium hydroxide at a concentration of 6.6% or 21% (w/w) in ethylene glycol was prepared and the KOH solution was bubbled for 5 min with nitrogen gas. Ten milligrams of EPA or DHA was added to 1 ml of the 6.6% or 21% KOH solution in a test tube (10 ml volume). The mixture was bubbled with nitrogen gas and then screw-capped and allowed to stand for 5 or 10 min at 180 °C. The reaction mixture was cooled, and 1 ml of methanol was added. The mixture was acidified to below pH 2 with 2 ml of 6N HCl. After dilution with 2 ml of distilled water, the conjugated fatty acid was extracted with 5 ml of hexane. The hexane extract was then washed with 3 ml of 30% methanol and with 3 ml of distilled water before being evaporated under a nitrogen gas stream. The conjugated fatty acids were stored at −20 °C after being purged with nitrogen gas. UV–vis spectrophotometric analysis of the conjugated fatty acid was performed with a Shimadzu UV-2400PC. Spectrophotometric readings confirmed the conjugation of fatty acids of pentaene (345 nm) [20,21]. The cEPA and normal EPA were dissolved in dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 s.

2.3. Enzymes

DNA polymerase α (pol α) was purified from calf thymus by immuno-affinity column chromatography as described previously [22]. Recombinant rat pol β was purified from *E. coli* JMpβ5 as described by Date et al. [23]. The human pol γ catalytic gene was cloned into pFastBac. Histidine-tagged enzymes were expressed using the BAC-TO-BAC HT Baculovirus Expression System according to the supplier's manual (LIFE TECHNOLOGIES, Maryland, USA) and purified using ProBoundresin (Invitrogen Japan, Tokyo Japan) (Mizushina, et al., in preparation). Human pol δ and ε were purified from the nuclear fraction of human peripheral blood cancer cells (Molt-4) using the second subunit of pol δ and ε-conjugated affinity column chromatography, respectively [24]. Pol I (α-like) and II (β-like) from a higher plant, cauliflower inflorescence, were purified according to the methods outlined by Sakaguchi et al. [25]. The Klenow fragment of pol I from *E. coli* was purchased from

Worthington Biochemical Corp. (Freehold, NJ, USA). Taq pol, T4 pol, T7 RNA polymerase and T4 polynucleotide kinase were obtained from Takara (Kyoto, Japan). Bovine pancreas deoxyribonuclease I was bought from Stratagene Cloning Systems (LaJolla, CA, USA). Human recombinant DNA topoisomerases I and IIα (topos I and II) (2 units/ml) were purchased from TopoGen, Inc. (Columbus, OH, USA).

2.4. Enzyme assays

Activities of pols were measured by methods described previously [4,5]. Poly(dA)/oligo(dT)$_{12–18}$ and dTTP (2′-deoxythymidine 5′-triphosphate) were used as the template-primer DNA and nucleotide substrate, respectively. The activities of primase of calf pol α, T7 RNA pol, T4 polynucleotide kinase, bovine pancreas deoxyribonuclease I and human topos I and II were measured using standard assays according to Koizumi et al. [26], Nakayama and Saneyoshi [27], Soltis and Uhlenbeck [28], Lu and Sakaguchi [29] and Lui and Miller [30], respectively. The activity without the inhibitor was considered 100%, and the remaining activity at each concentration of inhibitor was determined relative to this value.

2.5. Investigation of cytotoxicity in cultured cells

HL-60 cells were cultured in RPMI 1640 medium supplemented with heat-inactivated 10% fetal bovine serum, sodium bicarbonate (2 g/l) and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. For the cell viability assay, cells were seeded in 96-well plates at a density of 3×10^5 cells per well with various concentrations of EPA and cEPA. These were dissolved in DMSO at a concentration of 30 mM as a stock solution. The stock solution was diluted to various concentrations with growth medium and applied to each well. After incubation for 24 h, the survival rate was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay [31].

2.6. Cell cycle analysis

HL-60 cells (3×10^5 cells in 35 mm dish) were collected by centrifugation. DAPI (4′,6-diamidino-2-phenylindole) was used to stain the DNA of individual nuclei by "Cystain DNA 2 steps" kit (Partec; Munster, Germany). The DNA content of 8000 stained cells was analyzed using a Partec Cell Counter Analyser (Partec, CCA Model; Munster, Germany) with Multicucle 3.11 software (Phoenix Flow Systems, San Diego, CA). The cell debris and fixation artifacts were gated out.

2.7. Measurement of inhibition of synthesis of DNA, RNA and protein

The effect of cEPA on DNA, RNA and protein synthesis was examined independently. HL-60 cells (1×10^4) were

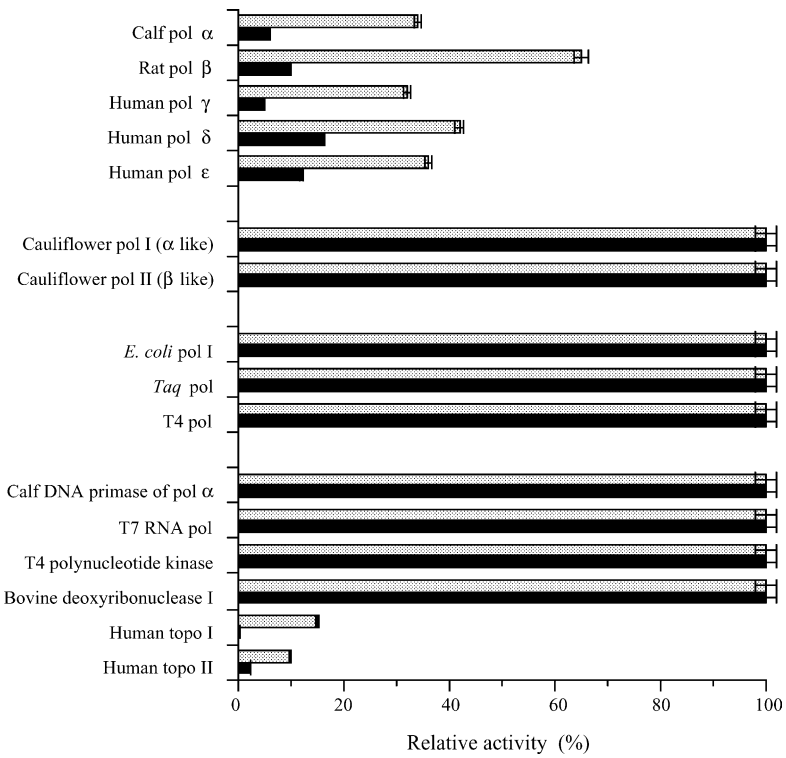

Fig. 1. Effects of normal and conjugated EPA on the activities of various DNA polymerases and other DNA metabolic enzymes. EPA and cEPA (50 μM each) were incubated with each enzyme. Percent relative activity is shown. The enzymatic activity was measured as described in Section 2. Enzymatic activity in the absence of the compound was taken as 100%. Each point represents the average of triplicate experiments and bars indicate S.D.

inoculated into 96-well micro-plates and preincubated without cEPA for several hours. Then, medium containing cEPA diluted with 0.5% DMSO solution was applied to the cells, and this time point was taken as 0 h. At 0.5 h, as probes for DNA, RNA or protein synthesis, [methyl-^{3}H]-thymidine, [5,6-^{3}H]-uridine or L-[4,5-^{3}H]-leucine (final concentration, 3, 4 and 4 μCi, respectively) were added. At specified time points, incubation was stopped and cell lysate was prepared to measure the incorporated radioactivity as described for the cytotoxicity assay.

2.8. Real-time-PCR analysis

Real-time polymerase chain reaction (PCR) analyses were conducted by means of standard assays according to Heintel et al. [32]. RNA and cDNA were prepared from HL-60 cells using RNeasy MINI kit (Qiagen, valencia, CA, USA) and High-Capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA), respectively. Real-time-PCR was performed with the ABI Prism 7700 Sequence Detector (Applied Biosystems) according to the manufacturer's instructions. The expression profile of the endgenous control genes showed the $^{\Delta}$Ct (cycle threshold) value for β-actin (VIC labeled pre-developed TaqMans Assay reagent, Applied Biosystems), which was subsequently used as an endogenous control. PCR was carried out in a 50 μl reaction volume with 5 μl of a 1:10 cDNA dilution with primers for cell cycle-regulatory proteins (designed from sequences in exons 1 and 2, Assays-on-Demand Gene Expression system, Applied Biosystems), probes and TaqMans Universal Master Mix without AmpErasesUNG. Primers for cyclins A, B and E are listed as follows: Accession number (common name), Hs00171105 (CCNA1); Hs00259126 (CCNB1); Hs00233356 (CCNE1). The primers in this assay amplify cell cycle-regulatory proteins and several splice variants. All samples were compared using the relative Ct value. The Ct value, which is inversely proportional to the initial template copy number, is the calculated cycle number where the fluorescence signal emitted is significantly above background levels. The fold induction was measured by real-time-PCR several times and calculated after adjusting for actin using $^{\Delta}$Ct.

2.9. Western blot analysis

The cells were lysed in RIPA buffer (50 mM Tris–HCl (pH 7.2), 150 mM EDTA, 1% Nonidet P-40, 0.05% sodium dodecyl sulfate (SDS), 1 mM PMSF and 1 mM leupeptin). Cell lysates were centrifuged at 14,000 × *g* for 10 min at 4 °C. The supernatant was analyzed by SDS-PAGE, and then blotted on a polyvinyldenefluoride (PVDF) membrane. The blots were subsequently incubated with the desired primary antibody. A Zero-D scan was used for densitometric quantitation.

2.10. Detection of apotosis induced by cEPA

To analyze the DNA ladder formation on treatment with cEPA, HL-60 cells were washed twice with PBS. Cells (1×10^{6}) were lysed with 10 mM Tris–HCl (pH 7.4) containing 10 mM EDTA, 0.5% Triton X-100 and Rnase A (0.2 mg/ml), incubated at 37 °C for 1 h and digested with proteinase K (0.5 mg/ml) at 50 °C for 30 min. After the addition of a 1/2 volume of 10 M ammonium acetate, the DNA was precipitated with 2.5 volumes of ethanol, dissolved in gel loading buffer (40 mM Tris–5 mM sodium acetate–1 mM EDTA (pH 7.8)) and separated by electrophoresis in a 1.5% agarose gel. The gel was stained with EtBr, and the DNA bands were visualized under UV light.

3. Results and discussion

3.1. Effects of conjugated EPA on various DNA metabolic enzymes

Fig. 1 shows the inhibitory effects of normal or conjugated eicosapentaenoic acid on the various DNA metabolic enzymes tested. At 50 μM, these compounds significantly inhibited the activities of mammalian DNA polymerases and human DNA topoisomerases, and cEPA was a two-fold stronger pol inhibitor than EPA. cEPA consists of conjugated double bonds, and this structural feature may stimulate the inhibition of pol and topo activities. The inhibitory effect of cEPA was stronger than that of aphidicolin [33] or dideoxyTTP [34], well-known inhibitors of replicative pols, such as pol α and β, respectively.

EPA and cEPA had little inhibitory effect on the activities of higher plant (cauliflower) pols I (α-like pol) and II (β-like pol), prokaryotic pols, such as the Klenow fragment

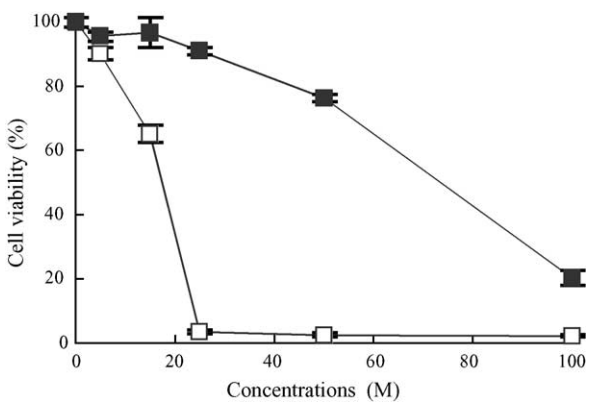

Fig. 2. Effect of normal and conjugated EPA on the proliferation of a human cancer cell line, HL-60. The data show the dose–response curves of growth inhibition of the human promyelocytic leukemia cell line HL-60 incubated with various concentrations of EPA (closed square) and cEPA (open square) for 24 h. Cell proliferation was determined by MTT assay [31]. Values are shown as means ± S.E.M. ($n = 4$).

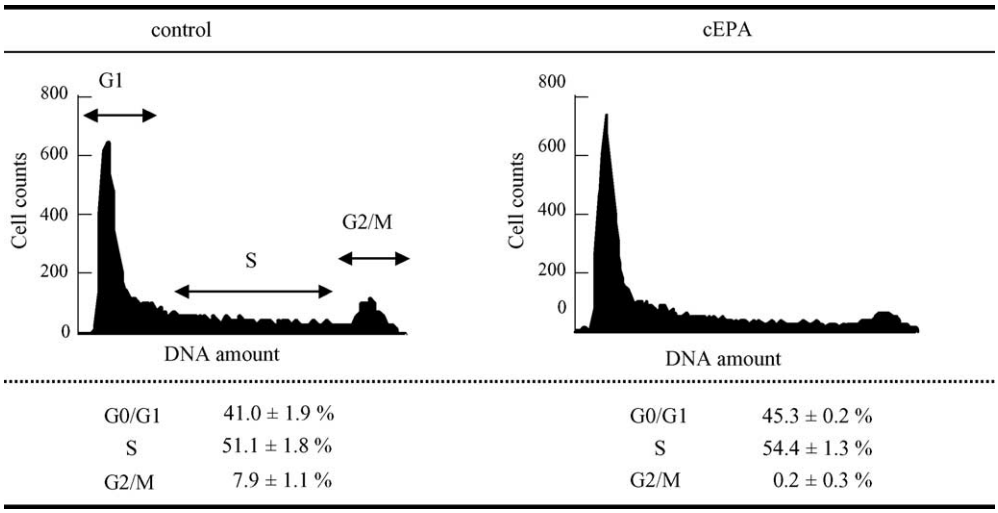

Fig. 3. The effect of cEPA on the cell cycle. HL-60 cells were incubated without (control) or with 13 μM cEPA for 24 h. The cell cycle distribution was calculated as the percentage of cells that were in the G0/G1, S and G2/M phase. All experiments were performed three times.

of *E. coli* pol I, Taq pol and T4 pol and DNA metabolic enzymes, such as calf RNA primase of pol α, T7 RNA pol, T4 polynucleotide kinase and bovine deoxyribonuclease I (Fig. 1). Therefore, EPA and cEPA might be potent inhibitors of mammalian pols and topos, which could be molecular, targets for anti-cancer chemotherapy. We therefore investigated the mechanism of the inhibitory effect of EPA and cEPA on human cancer cells.

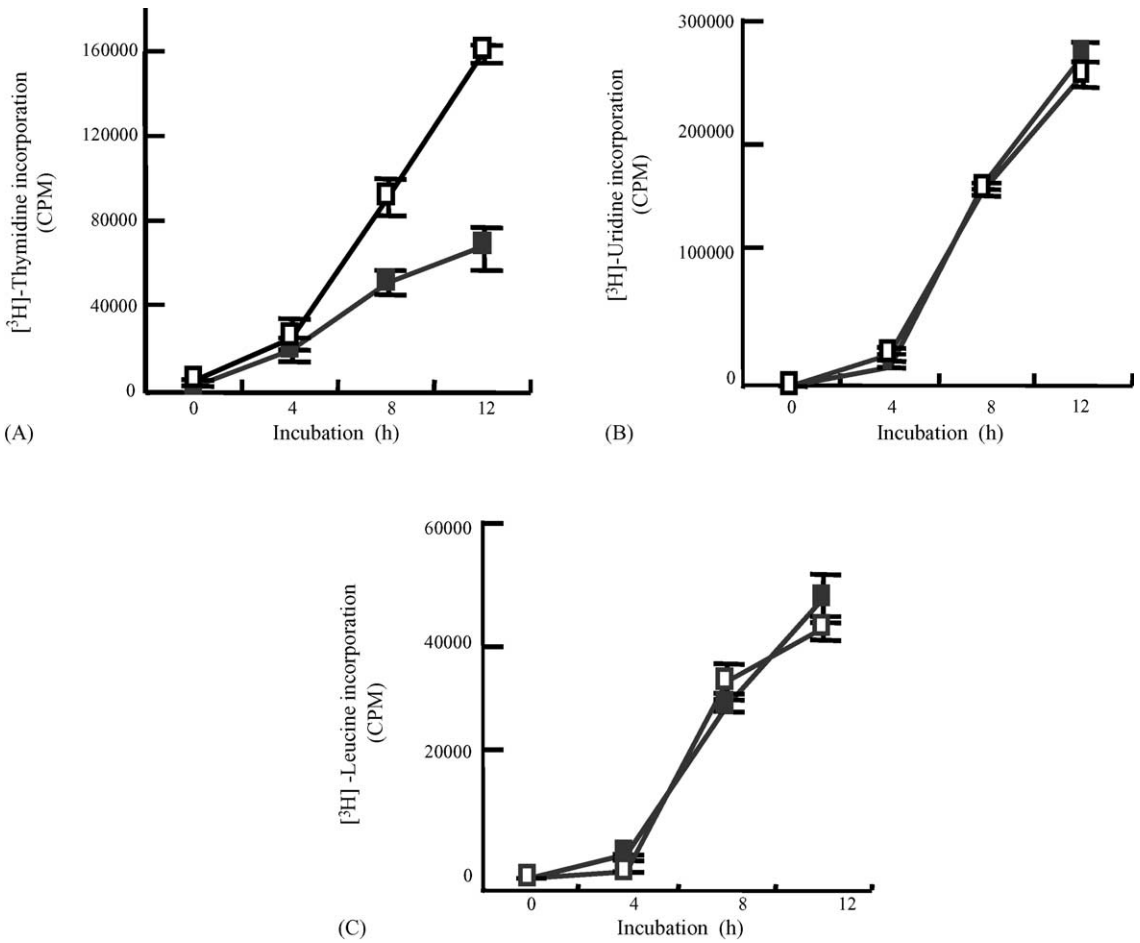

Fig. 4. Measurement of DNA, RNA and protein synthesis in HL-60 cells incubated with cEPA. HL-60 cells (1×10^4) were incubated without (control, open square) or with 13 μM cEPA (closed square) from 0 h, and radiolabeled thymidine, uridine or leucine was added at 0.5 h. (A–C) Panels showed that the incorporation of radiolabeled thymidine, uridine and leucine, respectively. Each point represents the average of triplicate experiments and bars indicate S.D.

3.2. Effects of EPA and cEPA on inhibition of cancer cell growth

To determine the effects of EPA and cEPA on cultured cancer cells, we tested their influence on cell growth in HL-60, a promyelocytic leukemia cell line. EPA and cEPA efficiently inhibited cell growth in a dose-dependent manner. After 24 h of treatment, the LD_{50} values of EPA and cEPA were 73 and 13 μM, respectively (Fig. 2). cEPA was more cytotoxic than normal EPA to HL-60 cells, and this tendency of cell growth was the same as the inhibitory effect of mammalian pols and topos. We investigated in more detail which inhibition by cEPA is effective for cancer cell proliferation, that of pols or topos.

3.3. Effects on the cell cycle progression of cEPA

Next, we analyzed whether cEPA affected the cell cycle distribution of cEPA-treated cells (Fig. 3). The cell cycle fraction was recorded after 24 h of treatment with each test compound, and was compared with cells incubated in medium alone. Consequently, among cells treated with cEPA, the population of cells in the G1 and S phases increased (41.0–45.3% and 51.1–54.4%, respectively). But the percentage of cells in the G2/M phase decreased from 7.9% to 0.2%. These results suggested that the actions of cEPA block an early step of S phase in HL-60 cells. Dehydroaltenusin, which is a specific pol α inhibitor, inhibited the cell cycle at S phase [35], and classical topo inhibitors, such as etoposide arrested the cell cycle at G2 phase [36]. cEPA, therefore, might be more effective in the inhibition of pols than topos in the cell, although cEPA inhibited the activities of mammalian pols and human topos, and the inhibitory effect for topos was stronger than that for pols in vitro (Fig. 1).

Table 1
The table gives fold induction values of genes significant by real-time-PCR

Accession no.	Description	Fold induction				
		0 h	3 h	6 h	12 h	24 h
Hs00171105	Cyclin A	1.0	1.0	1.1	1.1	1.1
Hs00259126	Cyclin B	1.0	1.0	1.1	1.1	1.1
Hs00233356	Cyclin E	1.0	1.0	1.1	1.1	1.1

The cells were treated with 13 μM cEPA for 0, 3, 6, 12 and 24 h. The expression levels of mRNA at 0 h were set as 1.0.

3.4. Effects of inhibition of synthesis of DNA, RNA and protein by cEPA

The results of cell cycle analysis were more directly confirmed by the incorporation experiment. Fig. 4A–C shows the incorporation of [^{3}H]-labeled thymidine, [^{3}H]-uridine and [^{3}H]-leucine into HL-60 cells, respectively. cEPA inhibited only the incorporation of [^{3}H]-thymidine into the cells. The [^{3}H]-thymidine incorporation was decreased by 42% of the control level in the presence of 10 μM cEPA (Fig. 4A). Neither [^{3}H]-uridine nor [^{3}H]-leucine incorporation was affected by cEPA (Fig. 4B and C). These observations indicated that cEPA must inhibit cell growth by blocking the primary step in the replication of DNA. The effect of cEPA on HL-60 cells in Figs. 3 and 4 would suggest that cEPA is distributed in the nucleus, and the mechanism of its action was the inhibition of replicative pols rather than topos. Therefore, cell cycle regulation was induced by the inhibition of pol activity by cEPA.

3.5. Effect on mRNA and protein expression of cyclins by cEPA

We examined whether the cell cycle effect of cEPA was associated with the expression of mRNA of cyclins using

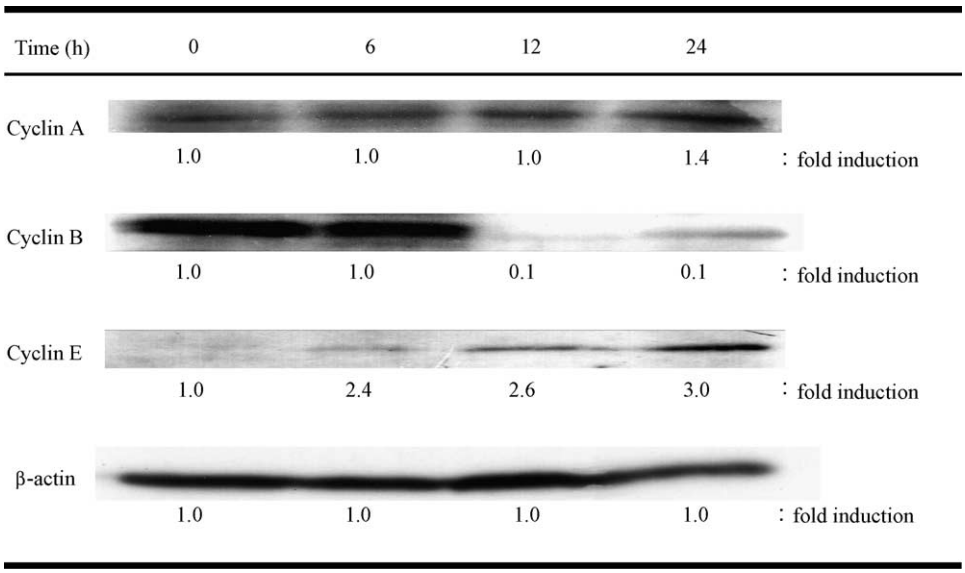

Fig. 5. Cyclin expressions was analyzed using Western blotting. Cell extracts of the nuclear fraction were prepared from cells treated with 13 μM cEPA. Cyclin A, B, E and β-actin (control) were detected with specific antibodies. Densitometric assay of the proteins was performed and fold induction was calculated.

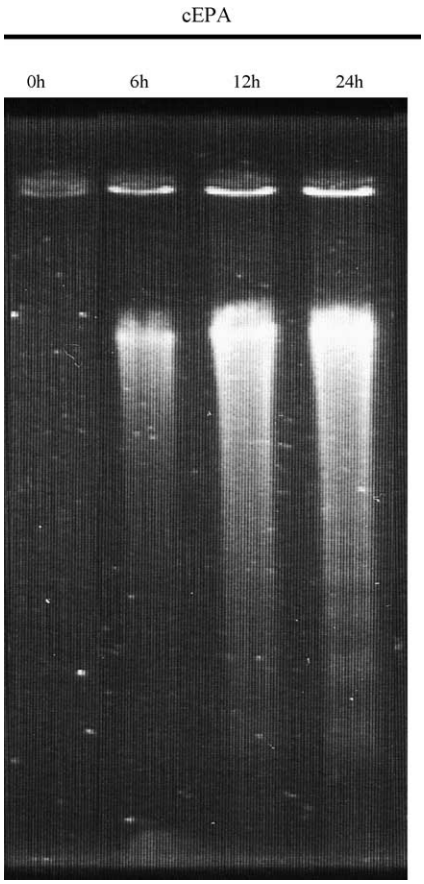

Fig. 6. Detection of intracellular DNA ladder formation in HL-60 cells treated with 13 μM cEPA several times. Following cell lysis, total DNA was extracted and analyzed by agarose gel electrophoresis.

real-time-PCR (Table 1). Cyclin E protein is present from the end of G1 to early S phase, and cyclin A protein keeps accumulating from the G1 to S phase [37,38]. It was suggested that cyclins A and E link the transition mechanism from the G1 to S phase, and appear when the cells start to synthesize DNA [37,38]. On the other hand, cyclin B was associated with the mechanism of G2/M transition [38]. Since the ratios of $^{\Delta}$Ct values of all cyclins were unchanged, the transcription levels of cyclins were not influenced by cEPA. Next, we examined whether the effect of cEPA was associated with the expression of cyclin proteins using Western blotting (Fig. 5). Cyclin A proteins slightly increased and cyclin E proteins significantly increased with cEPA treatment, but cyclin B significantly decreased. These results suggested that cEPA induced protein expression of cyclins A and E, and then the cyclins arrested human cancer cells in the G1/S phase. The inhibition of pol activity by cEPA might lead to the translation of cyclin proteins.

3.6. Effects of cEPA on apoptosis

To examine whether the decrease in cell numbers caused by cEPA was due to apoptosis, DNA fragmentation was analyzed by electrophoresis. A DNA ladder formation was dose-dependently observed in HL-60 cells treated with the LD_{50} value of cEPA for 24 h (Fig. 6). Therefore, apoptotic effects were evident in the cells, and ladders were apparent at 6 h. In the cell cycle analysis, cEPA-treated cells were in the sub-G1 phase (data not shown). The effect of cEPA must involve a combination of growth arrest and cell death. Since cEPA did not influence the proliferation of normal cells (data not shown), cEPA should be considered the lead compound of potentially useful cancer chemotherapy agents.

In this report, the mechanisms by which cEPA suppresses cancer cell growth were investigated, and it was revealed that the inhibition of pol activity by cEPA, which is an inhibitor of mammalian pols and topos, influenced not only the cell proliferation but also the cell cycle.

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