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Ara-C affects formation of cancer cell DNA synthesome replication intermediates

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Abstract *Purpose*: An intact and fully functional multiprotein DNA replication complex (DNA synthesome) from human as well as from murine mammary carcinoma cells was first isolated and characterized in our laboratory. The human cell synthesome supports the in vitro origin-specific simian virus 40 (SV40) DNA replication reaction in the presence of the viral large T-antigen using a semiconservative mechanism and has been shown to contain all the proteins and enzymes required to support DNA synthesis. We are currently using the DNA synthesome as a unique model for analyzing the mechanism of action of anticancer drugs affecting DNA replication. The purpose of this study was to further investigate the mechanism of action of ara-C using the DNA synthesome isolated from the human breast cancer cell line MDA MB-468. *Methods*: Synthesome-mediated SV40 DNA replication was performed in the presence of various concentrations of ara-CTP (the active metabolite of ara-C) and the types of daughter DNA molecules produced were analyzed using neutral and alkaline gel electrophoresis. We also examined the effect of ara-C on intact MDA MB-468 cell DNA synthesis and on cell proliferation. In addition, we studied the effect of ara-CTP on the activity of some of the synthesome target proteins (the DNA polymerases α and δ). *Results*: Full-length daughter DNA molecules were obtained in the presence of low concentrations of ara-CTP while at higher concentrations, there was an inhibition of full-length daughter DNA synthesis. The findings suggest that specifically the initiation phase of DNA synthesis was inhibited by ara-CTP since the production of the short Okazaki fragments was suppressed at all concentrations of the drug above 10 μ*M*. In addition, it was found that the IC_{50} of ara-CTP for inhibition of synthesome-mediated in vitro DNA replication was comparable to that required to inhibit intact cell DNA synthesis. Further experimentation has shown that ara-CTP preferentially inhibits the activity of the synthesome-associated DNA polymerase α enzyme while the DNA polymerase δ seems to be resistant to the inhibitory effect of that drug. *Conclusions*: Our results indicate that ara-C's action on DNA replication is mediated primarily through DNA polymerase α and suggest that this enzyme plays a key role in DNA synthetic initiation events. The results also provide definitive support for the use of the DNA synthesome as a unique and powerful model for analyzing the mechanism of action of anticancer drugs which directly affect DNA replication.

Key words Ara-C · DNA synthesome · DNA replication · In vitro

Abbreviations *ara-C* 1-β-D-arabinofuranosylcytosine · *ara-CMP* 1-β-D-arabinofuranosylcytosine monophosphate · *ara-CTP* 1-β-D-arabinofuranosylcytosine triphosphate · *DTT* dithiothreitol · *EDTA* ethylenediaminetetraacetic acid · *EGTA* ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid ·

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HEPES *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) · *PBS* phosphate-buffered saline · *PEG* polyethylene glycol · *PMSF* phenylmethyl sulfonyl fluoride · *SV40* simian virus 40 · *Tris* Tris(hydroxymethyl)aminoethane

Introduction

Eukaryotic cell DNA replication is a highly complex process that involves the precise interactions of many proteins with one another and with the DNA template being replicated [23]. In the past few years, a general picture has emerged in which the DNA synthesizing machinery in mammalian cells is clearly organized into a multiprotein complex that is competent to coordinate the synthesis of both strands of the DNA template [2, 17, 18, 29–31, 36].

An intact, stable, and functional multiprotein DNA replication complex (designated DNA synthesome) from human [1, 5, 20, 26, 31] and murine mammary carcinoma cells [43] was first successfully isolated, purified and characterized in our laboratory. The human cell DNA synthesome is fully competent to support the SV40 origin-specific and large T-antigen-dependent in vitro DNA replication reaction. This reaction uses a semiconservative DNA replication mechanism to produce daughter DNA products which include form I (supercoiled) DNA, form II (nicked open circle) DNA, and the higher order topological intermediates [31]. We have shown that the DNA synthesome contains all the proteins and enzymes required to support DNA synthesis, including DNA polymerases α, δ, and ε, DNA primase, topoisomerases I and II, proliferating cell nuclear antigen, replication factor C (RF-C), replication protein A (RP-A), DNA helicase, and DNA ligase [1, 5, 20, 26].

We are currently using the DNA synthesome as a unique model for analyzing the mechanism of action of anticancer drugs affecting DNA replication. Coll et al. [4] have found that there is a close correlation between the IC_{50} value of camptothecin (a topoisomerase I poison) required to inhibit intact HeLa cell DNA synthesis and that required to suppress synthesome-mediated in vitro DNA synthesis. In a related study, Wills et al. [41] have demonstrated that the DNA synthesome has the ability to successfully incorporate ara-CMP into the replicating DNA at internucleotide positions in a manner similar to that observed using intact cells.

In this study, we used the DNA synthesome isolated from the human breast cancer cell line MDA MB-468 to further investigate the mechanism of action of ara-C. The synthesome-mediated in vitro SV40 DNA replication reaction was performed in the presence of increasing concentrations of ara-CTP, and the types of daughter DNA molecules produced were analyzed using neutral and alkaline gel electrophoresis. In addition, we determined, for ara-CTP, the IC_{50} value required to inhibit the in vitro SV40 DNA replication reaction and compared this IC_{50} value to that of ara-C required for the inhibition of intact MDA MB-468 cell DNA synthesis. Furthermore, the effect of ara-CTP on the activity of the synthesome-associated DNA polymerases α and δ was studied. Our results provide definitive support for the use of the DNA synthesome as a unique and powerful model for analyzing the mechanism of action of anticancer drugs which directly affect DNA replication.

Materials and methods

Materials

Ara-C, ara-CTP, activated calf thymus DNA, and nonradioactive nucleotides were purchased from the Sigma Chemical Company (St. Louis, Mo.). Poly (dG-dC) poly (dG-dC) template was obtained from Pharmacia Biotec, and [α-^{32}P]dGTP, methyl ^{3}H-thymidine, and ^{3}H-TTP were obtained from NEN (Boston, Mass.).

Cell culture and harvest

Suspension cultures of MDA MB-468 cells were grown in Joklik's modified Eagle's medium supplemented with 5% each of irradiated calf and fetal bovine serum. Exponentially growing cells (5×10^5 cells/ml medium) were harvested and washed three times with PBS comprising 137 m*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8.8 m*M* Na_2HPO_4. The cells were then pelleted by low-speed centrifugation (1000 rpm, 5 min, 4 °C) and the cell pellets were stored at −80 °C until fractionated.

Isolation and purification of the DNA synthesome from MDA MB-468 cells

The DNA synthesome was isolated according to our published procedures [5, 31]. Briefly, MDA MB-468 cell pellets (10 g) were thawed and resuspended in two volumes of the homogenization buffer containing 50 m*M* HEPES (pH 7.5), 200 m*M* sucrose, 5 m*M* KCl, 5 m*M* $MgCl_2$, 2 m*M* DTT and 0.1 m*M* PMSF. The pellets were homogenized by 30 strokes of a loose-fitting Dounce homogenizer. The homogenate was centrifuged at 5000 rpm for 15 min to separate the cytosol (S-1) from the nuclei. The nuclear pellets were resuspended in two volumes of a buffer containing 50 m*M* HEPES (pH 7.5), 400 m*M* KCl, 5 m*M* $MgCl_2$, 5 m*M* EDTA, 2 m*M* DTT, and 0.1 m*M* PMSF and rocked for 2 h at 4 °C. The nuclear pellets were then separated by centrifugation at 40,000 rpm for 1 h and the supernatant (NE) was collected. EDTA and EGTA (5 m*M* final concentration of each) were added to the S-1 fraction and the mitochondria were then removed by centrifugation at 15,000 rpm for 15 min. The supernatant (S-2) was subjected to centrifugation at 40,000 rpm for 1 h to remove the microsomes, and the postmicrosomal fraction was collected and designated S-3.

The NE and S-3 fractions were combined, adjusted to 2 *M* KCl and 5% PEG (PEG 8000), and rocked for 1 h at 4 °C. The NE/S-3 fraction was then centrifuged at 15,000 rpm for 15 min to remove the PEG-precipitated materials and the supernatant was dialyzed for 2 h against a buffer containing 50 m*M* HEPES (pH 7.5), 250 m*M* sucrose, 1 m*M* DTT, 150 m*M* KCl, 0.1 m*M* PMSF, and 1 m*M* each of EDTA and EGTA. The dialyzed fraction was clarified by centrifugation at 15,000 rpm for 15 min and the resulting supernatant was layered onto a 2 *M* sucrose cushion and subjected to centrifugation at 40,000 rpm for 18 h at 4 °C. The supernatant solution (S-4) and the sucrose interphase (P-4) were removed. The P-4 fraction, which has the replication activity, was dialyzed against a buffer containing 20 m*M* HEPES (pH 7.5), 5 m*M* KCl, 1.5 m*M* $MgCl_2$, and 1 m*M* DTT. The dialyzed P-4 fraction was

subjected to centrifugation at 5000 rpm for 10 min and stored in aliquots at −80 °C.

Purification of SV40 large T-antigen

T-antigen was purified as described by Simanis and Lane [38] and it is the only virally encoded protein required for SV40 origin-dependent DNA replication in vitro [7].

Effect of Ara-C on MDA MB-468 cell growth

MDA MB-468 cells (5×10^4) were seeded in 60-mm cell culture plates and incubated for 48 h at 37 °C. The cells were then exposed to increasing concentrations of ara-C. After 24 h, the medium was removed, the cells were washed twice with PBS, and allowed to grow in drug-free medium for 5 days. The cells were then treated with trypsin/EDTA and quantified using a Coulter cell counter.

In vitro SV40 DNA replication assay

The reaction was carried out according to the procedures of Malkas et al. [31] with some modifications. The reaction mixture (25 µl) contained 30 m*M* HEPES (pH 7.5), 7 m*M* $MgCl_2$, 0.5 m*M* DTT, 1.5–3 µg SV40 large T-antigen, 50 µg synthesome protein fraction, 50 ng of the plasmid $pSVO^+$ containing a 200-bp insert of the SV40 replication origin DNA sequence [39], 1 µCi [α-^{32}P]dGTP (3000 Ci/mmol), 100 µ*M* each of dATP, and dTTP, 10 µ*M* each of dCTP and dGTP, 200 µ*M* each of rCTP, rGTP, and rUTP, 4 m*M* rATP, 40 m*M* phosphocreatine, and 1 µg creatine phosphokinase. The replication reaction was started by incubating the reaction mixture at 37 °C for 4 h. To determine the amount of radiolabel incorporated into the daughter DNA molecules, the reaction mixture was spotted on Whatman DE81 filters and quantified by liquid scintillation counting. For gel analysis of the replication products, the reaction was stopped by adding 100 µg yeast RNA in 1% sodium dodecyl sulfate followed by digestion for 1 h at 37 °C with 2 µg proteinase K. DNA replication products were then isolated by phenol/chloroform extraction followed by precipitation with 2-propanol in the presence of 2 *M* ammonium acetate. The isolated DNA was resuspended in 10 m*M* Tris/1 m*M* EDTA and electrophoresed in 1% agarose gels containing either TBE (90 m*M* Tris/90 m*M* boric acid/2 m*M* EDTA) or alkaline (50 m*M* NaOH/1 m*M* EDTA) buffers. Gels were dried and exposed to Kodak films at −80 °C followed by autoradiography.

Measurement of intact MDA MB-468 cell DNA synthesis

Exponentially growing MDA MB-468 cells (5×10^4) were incubated at 37 °C with increasing concentrations of ara-C for 24 h. The cells were then labeled by the addition of methyl ^{3}H-thymidine (1 µCi/ml) to the medium. After a 4-h incubation, the cells were lysed and the amount of radiolabel incorporated into DNA was measured by quantifying the amount of ^{3}H in acid-insoluble materials.

DNA polymerase α assay

The assay was carried out according to the procedure described by Malkas et al. [31]. Briefly, the reaction mixture (20 µl) contained 20 µg of synthesome fraction, 20 m*M* Tris-HCl (pH 8.0), 8 m*M* $MgCl_2$, 1 m*M* DTT, 0.2 m*M* each of dATP and dGTP, 0.01 m*M* dCTP, 2 µg activated calf thymus DNA template (Sigma), and 0.11 µCi ^{3}H-TTP (72.6 Ci/mmol). The reaction was started by incubating the reaction mixture in the absence or presence of increasing concentrations of ara-CTP (1, 10, 50, 100, 200, 800 and 1000 µ*M*) for 1 h at 37 °C. The amount of ^{3}H-TTP incorporated was quantified by liquid scintillation counting after spotting the reaction mixture on DE81 filters as described by Sambrook et al. [37].

DNA polymerase δ assay

The assay was carried out according to the method of Lee et al. [25] with some modifications. The reaction mixture (25 µl) contained 20 µg of synthesome fraction, 25 m*M* HEPES (pH 5.9), 10 m*M* $MgCl_2$, 0.2 mg/ml bovine serum albumin, 0.01 m*M* dCTP, 2 U/ml of poly (dG-dC) poly (dG-dC) template (Pharmacia Biotec), 5% glycerol, and 0.25 µCi of [α-^{32}P]dGTP (3000 Ci/mmol). The DNA template was denatured by heating for 5 min at 100 °C and chilled in ice. The reaction mixture was incubated in the absence or presence of 100, 200, 400, and 800 µ*M*, and 1 m*M* ara-CTP for 15 min at 37 °C. It was then spotted on DE81 filters and the amount of ^{32}P-dGTP retained on the filter was measured as described by Sambrook et al. [37].

Results

Effect of Ara-C on MDA MB-468 cell proliferation

To determine the effect of ara-C on the growth of MDA MB-468 cells, the drug was added for 24 h to exponentially growing cells at concentrations ranging from 10 n*M* to 1 m*M*. The medium was then removed and the cells were allowed to grow in drug-free medium for 5 days at which time the number of growing cells was quantified. Figure 1 shows that a logarithmic increase in ara-C concentration was accompanied by a steady decrease in the ability of cells to proliferate. The concentration of ara-C required to inhibit cell growth by 50% was approximately 1 µ*M*. This result agrees

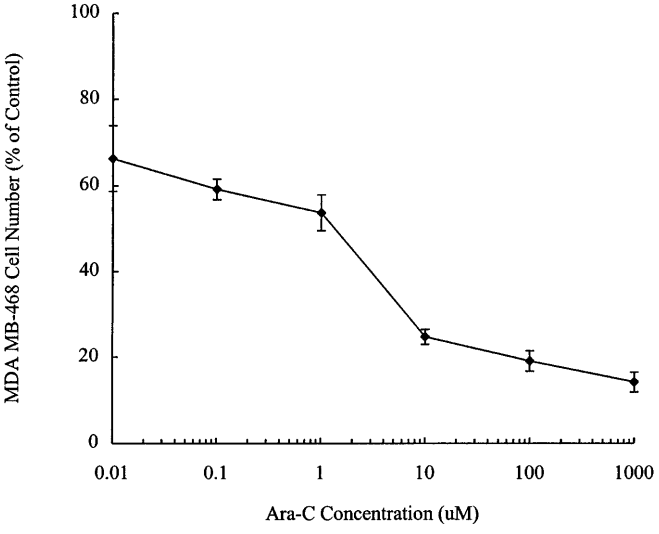

Fig. 1 Effect of ara-C on MDA MB-468 cell growth. MDA MB-468 cells (5×10^4) were seeded onto 60-mm cell culture plates and incubated for 48 h at 37 °C. The cells were exposed to increasing concentrations of ara-C. After 24 h, the medium was removed and the cells were allowed to grow in drug-free medium for 5 days. The cells remaining in the plate were then treated with trypsin/EDTA and the viable cells were counted. Each point on the graph represents the mean ± SEM (standard error of the mean) of three to five independent experiments. Cells grown in the absence of ara-C provided the control cultures, with which the number of cells in plates containing ara-C were compared

clearly with the previously reported data for HL-60 cells [27].

Ara-C inhibits both intact MDA MB-468 cell DNA synthesis and the synthesome-mediated in vitro SV40 DNA replication reaction

To validate the usefulness of the synthesome as an in vitro model system for studying the mechanism of action of ara-C, we determined whether the concentration of ara-C that effectively inhibits MDA MB-468 intact cell DNA synthesis was comparable to the concentration of ara-CTP required to suppress the synthesome-driven in vitro SV40 DNA replication reaction. Exponentially growing MDA MB-468 cells were exposed to increasing concentrations of ara-C for 24 h and then labeled with ^{3}H-thymidine for 4 h. The IC_{50} of ara-C required to inhibit intact cell DNA synthesis was 115 μM (Fig. 2). For the in vitro study, the synthesome-mediated in vitro SV40 DNA replication reaction was carried out in the absence or presence of increasing concentrations of ara-CTP. The reaction mixture was incubated at 37 °C for 4 h and the level of DNA synthesis was determined by quantifying the amount of ^{32}P-dGTP incorporated into the replication products. The concentration of ara-CTP required to cause 50% inhibition of in vitro DNA synthesis was 65 μM (Fig. 3). This result indicates that there was a reasonable correlation between the IC_{50} of ara-CTP for inhibition of the synthesome-mediated in vitro DNA replication and that of ara-C required to inhibit intact MDA MB-468 cell DNA synthesis. A similar correlation between the IC_{50} values for the inhibition of intact cell DNA synthesis and in vitro replication has been found in a previous study carried out in our laboratory using camptothecin and ara-C with HeLa cells [4, 28]. This result further supports the usefulness of the synthesome as a powerful model system for analyzing the mechanism of action of anticancer drugs which directly affect DNA replication.

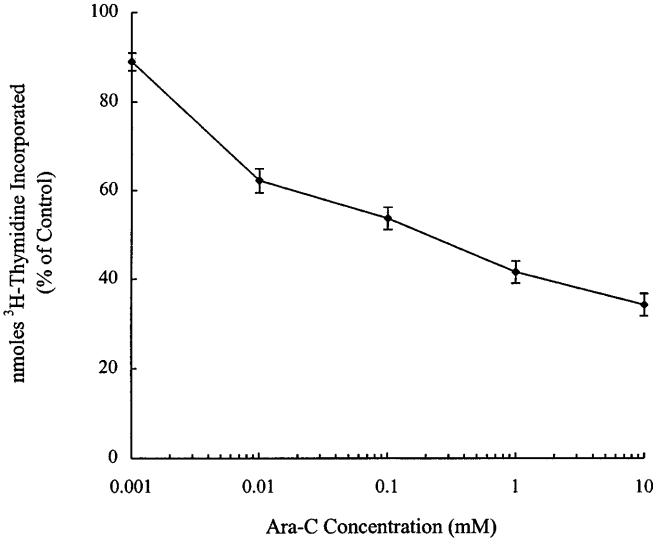

Fig. 2 Effect of ara-C on intact MDA MB-468 cell DNA synthesis. MDA MB-468 cells (5×10^4) were seeded onto 60-mm cell culture plates and incubated for 24 h at 37 °C. The cells were exposed to increasing concentrations of ara-C for 24 h and then labeled with ^{3}H-thymidine (1 μCi/ml of medium). After a 4-h incubation, the cells were lysed and the level of DNA synthesis was determined by quantifying the amount of ^{3}H-thymidine present in acid-insoluble material. Each point on the graph represents the mean ± SEM of three to five independent experiments. Controls consisted of plates of cells grown in the absence of ara-C for 24 h prior to labeling with ^{3}H-thymidine

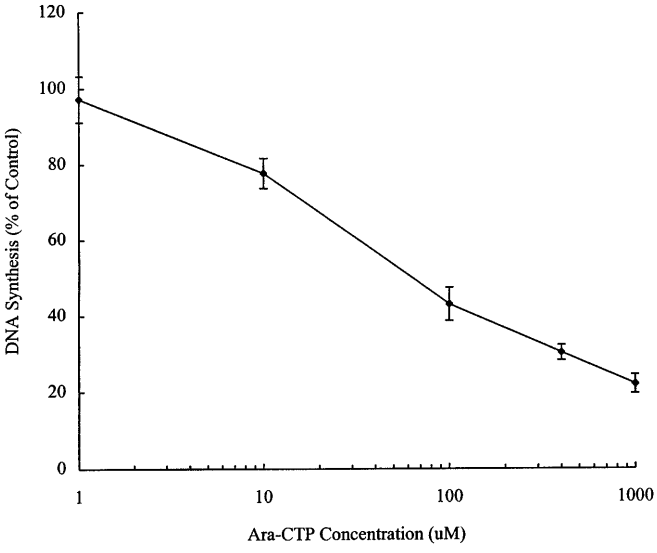

Fig. 3 Effect of increasing concentrations of ara-CTP on the synthesome-mediated in vitro SV40 DNA replication reaction. The replication reaction was initiated by incubating the reaction mixture at 37 °C for 4 h in the absence or presence of increasing concentrations of ara-CTP under the conditions described in the text. To determine the amount of radiolabeled ^{32}P-dGTP incorporated into the daughter DNA molecules, the reaction mixture was spotted on Whatman DE81 filters which were then washed, and the amount of radiolabeled nucleotide retained on the each filter quantified by liquid scintillation counting [37]. One unit of in vitro SV40 DNA synthesis activity is equivalent to 1 nM of total [α-^{32}P]dNMP incorporated into SV40-origin DNA per hour at 37 °C. Each point represents the mean ± SEM of three to five experiments. Control reactions were performed in the absence of ara-CTP, and the incorporation of ^{32}P-dGTP was quantified as described in Materials and methods

Ara-CTP affects the formation of the DNA synthesome replication intermediates

The synthesome-mediated in vitro SV40 DNA replication reactions were performed in the absence or presence of increasing concentrations of ara-CTP (Materials and methods) and the types of daughter DNA molecules produced in the in vitro reaction were analyzed using neutral and alkaline gel electrophoresis.

We have previously shown that the DNA synthesome isolated from HeLa cells supports the in vitro origin-specific T-antigen-dependent SV40 DNA replication reaction [31, 41]. The results of this study clearly demonstrate that the DNA synthesome isolated from human

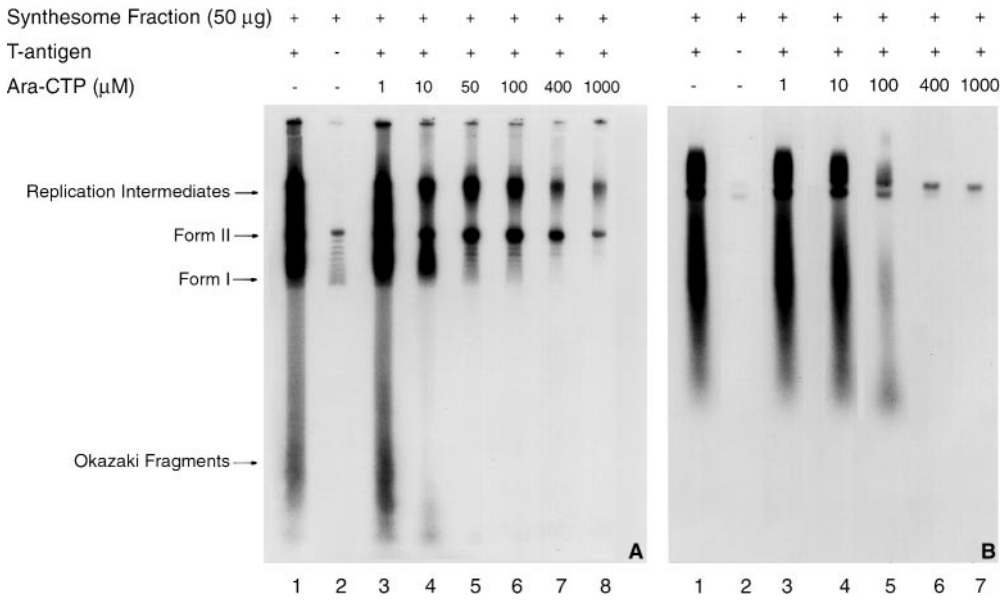

Fig. 4A,B Neutral (**A**) and alkaline (**B**) gel analysis of the synthesome-mediated in vitro SV40 DNA replication products formed in the absence or presence of increasing concentrations of ara-CTP. The DNA replication products formed in the in vitro DNA replication reaction were isolated by phenol/chloroform extraction, followed by precipitation with 2-propanol in the presence of 2 *M* ammonium acetate. The isolated DNA was resuspended in 10 m*M* Tris/1 m*M* EDTA and electrophoresed through 1% agarose gels containing either TBE (**A**) (90 m*M* Tris/90 m*M* boric acid/2 m*M* EDTA) or alkaline (**B**) (50 m*M* NaOH/1 m*M* EDTA) buffers. Gels were dried and exposed to Kodak XAR-5 film at −80 °C for autoradiographic analysis of the resolved DNA replication products

breast cancer cells (MDA MB-468) was fully competent to produce newly replicated form I (superhelical) DNA, form II (nicked open circular) DNA, as well as higher order topological intermediates. The production of these DNA replication products was dependent on the presence of T-antigen (Fig. 4A,B; compare lanes 1 and 2).

Full-length daughter DNA molecules were obtained in the presence of low concentrations of ara-CTP (1 and 10 µ*M*; Fig. 4A,B, lanes 3 and 4) while at higher concentrations, there was an inhibition of full-length daughter DNA synthesis (Fig. 4A, lanes 5–8; Fig. 4B, lanes 5–7). These results are in accordance with our previous results using the DNA synthesome isolated from HeLa cells [41]. Our results also showed that specifically the initiation phase of DNA synthesis was inhibited by ara-CTP since the production of the short Okazaki fragments was suppressed at concentrations of the drug exceeding 10 µ*M* (Fig. 4A,B).

The synthesome-associated DNA polymerase α is sensitive to Ara-CTP while DNA polymerase δ is resistant to the inhibitory effect of that drug

Studies were initiated to determine the effect of ara-CTP on the activities of synthesome-associated DNA polymerases α and δ. Enzyme assays were carried out in the absence or presence of increasing concentrations of ara-CTP (Materials and methods). The concentration of ara-CTP required to inhibit 50% of the activity of the synthesome-associated DNA polymerase α was approximately 40 µ*M* (Fig. 5). This concentration was comparable to that required to effectively inhibit the in vitro SV40 DNA replication reaction (65 µ*M*; Fig. 3). Our results also clearly demonstrated that the synthesome-associated DNA polymerase δ seems to be resis-

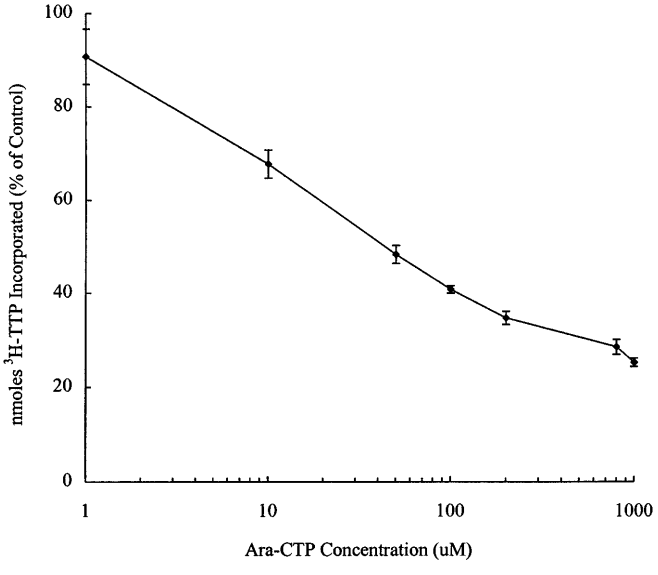

Fig. 5 Effect of different concentrations of ara-CTP on the synthesome-associated DNA polymerase α activity. The reaction was started by incubating the reaction mixture (20 µl) in the absence or presence of increasing concentrations of ara-CTP (1, 10, 50, 100, 200, 800, and 1000 µM) for 1 h at 37 °C. The reaction mixture was spotted onto DE81 filters, and the amount of ^{3}H-TTP retained on the filters was determined as described by Sambrook et al. [37]. Each point represents the mean ± SEM of three to five experiments. Control reactions were performed in the absence of ara-CTP

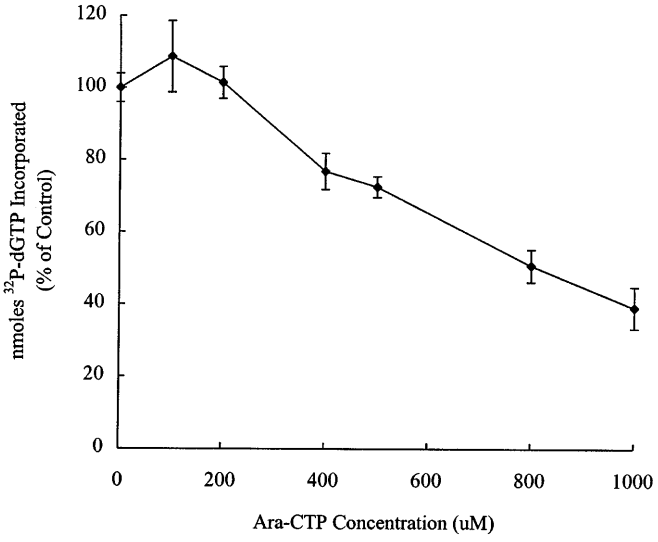

Fig. 6 Effect of ara-CTP on the synthesome-associated DNA polymerase δ activity. The assay was performed as described in the text and the amount of ^{32}P-dGTP incorporated into the DNA template was determined by binding to Whatman DE81 filters [37]. Each point represents the mean ± SEM of three to five experiments. Control reactions were performed in the absence of ara-CTP

tant to the inhibitory action of that drug (Fig. 6). These results are in agreement with those obtained by Han et al. [16] using the DNA synthesome isolated from HeLa and MCF-7 cells. The close correlation between the IC_{50} for ara-CTP-mediated inhibition of the synthesome-driven in vitro SV40 DNA replication reaction and that required to inhibit the synthesome-associated DNA polymerase α activity indicates that ara-C's action on DNA replication is mediated primarily through its inhibition of DNA polymerase α and suggests that this enzyme plays a key role during the initiation of DNA synthesis and Okazaki fragment formation.

Discussion

Ara-C is one of the most effective nucleoside analogues used in the treatment of acute myelogenous leukemia [8, 22, 32]. Ara-C, a deoxycytidine analogue, must be activated in the cell by the phosphorylation of the nucleoside to the monophosphate form (ara-CMP). This phosphorylation is catalyzed by the enzyme deoxycytidine kinase. Ara-CMP is then activated by conversion to the triphosphate form (i.e. ara-CTP) which is the biologically active form of the drug [6]. Ara-C inhibits cell proliferation and DNA replication in mammalian cells [13]. Yet despite a series of extensive studies, the mechanisms by which ara-C exerts its cytotoxic effect remain to be fully elucidated. Several hypotheses have been proposed to explain the inhibitory effect of ara-C on DNA replication. Previous studies have indicated that ara-CTP competes with dCTP for the binding sites on DNA polymerase α, inhibiting its enzymatic activity [11, 14]. However, it has been found that incorporation of ara-C into the DNA is the major mechanism by which the drug exerts its lethal effect [24, 27, 44] and that there is a strong correlation between the incorporation of ara-C into DNA and the loss of clonogenic potential [27]. Incorporation of ara-C into DNA has several effects. Fridland [9, 10] and Bell and Fridland [3] have demonstrated that incorporation of ara-C into DNA inhibits the initiation of new DNA synthesis. Other studies [33–35], however, have shown that the presence of ara-CMP at the 3′ terminus of DNA interferes with the elongation of a replicating DNA strand and not the initiation of synthesis.

Using the purified human DNA synthesome which supports in vitro SV40 origin-specific DNA replication in the presence of the viral large T-antigen, we have found that the concentration of ara-C required to inhibit intact human breast cancer cell DNA synthesis, for the cell line MDA MB-468, was comparable to that required for the inhibition of synthesome-mediated in vitro SV40 DNA replication. The reason the concentration of ara-C required to inhibit intact cell DNA synthesis (115 μ*M*) is larger than the concentration required to inhibit in vitro DNA synthesis (65 μ*M*) is most likely due to one or more of the following factors. First, a larger dose of ara-C may be required than is needed to directly inhibit the purified DNA synthesome because ara-C must be transported across the cell membrane. Second, a higher concentration of ara-C may be required to compensate for that portion of drug that is inactivated by degradative enzymes (such as deoxycytidine deaminase). Third, the effective cellular concentration may be lower than the extracellular concentration of ara-C since the drug is readily exported from within the cell. Fourth, a larger dose of ara-C may be required for the intact cell study because ara-C must be converted to its active metabolite (ara-CTP) in order to produce its cytotoxic effect. Therefore, the concentration of the active metabolite that reaches the target site (i.e. the DNA synthetic apparatus in the nucleus) is probably less than that of the drug placed in the medium. However, these factors are not of consequence to the in vitro studies since a direct effect of the active metabolite of the drug (ara-CTP) on DNA synthesis was measured. Our results are therefore in agreement with those of a previous study in our laboratory examining the DNA synthesome isolated from HeLa cells [28].

The IC_{50} value of ara-C for inhibition of MDA MB-468 cell proliferation was found to be much lower than that for inhibition of either intact cell DNA synthesis or in vitro DNA synthesis (1 μ*M* compared to 115 or 65 μ*M*, respectively). Ara-C's mechanism of action includes not only inhibition of DNA polymerase and incorporation into DNA but also the generation of reactive oxygen intermediates [19], endoreduplication [42], alteration of the concentration of intracellular lipid second messengers such as ceramide [40], which is a potent inducer of apoptosis, and damaging the genomic DNA [15, 21]. Therefore, it is clear that the effect of ara-C on cell growth involves several mechanisms that collectively lead to inhibition of cell proliferation.

In our current study, the synthesome-mediated in vitro SV40 DNA replication reaction was performed in the presence of increasing concentrations of ara-CTP, and the types of daughter DNA molecules were analyzed using neutral and alkaline agarose gel electrophoresis. Our results clearly demonstrated that full-length daughter DNA molecules were produced in the presence of low concentrations of ara-CTP; however, at higher concentrations, the drug suppressed the formation of daughter DNA synthesis. Moreover, our results show that the initiation phase of DNA synthesis was specifically inhibited by ara-CTP since the production of short Okazaki fragments was suppressed at concentrations of the drug exceeding 10 μM. These results are consistent with those reported by Wills et al. [41] who found that the DNA synthesome isolated from HeLa cells was able to incorporate ara-CMP into internucleotide linkages, and to add deoxynucleotides at the 3′ terminus of a DNA strand containing incorporated ara-CMP, leading to the production of full-length daughter DNA molecules. Recently, Gmeiner et al. [12] studied the effect of ara-C substitution on the structural and thermodynamic properties of a model Okazaki fragment and found that the drug inhibits lagging strand DNA synthesis by destabilization of the interaction between the nascent DNA and the DNA template being replicated on the lagging strand of the replication fork.

Using highly specific antibodies recognizing DNA polymerases α or δ, Han et al. [16] demonstrated that the assay conditions for the synthesome-associated DNA polymerases α and δ were very specific for each enzyme and permitted the activity of each DNA polymerase to be measured while it was in the presence of the other polymerase (i.e., in the DNA synthesome). Our results have shown that ara-CTP preferentially inhibited the activity of the synthesome-associated DNA polymerase α enzyme, while the DNA polymerase δ appeared to be resistant to the inhibitory action of that drug. This result is in agreement with that of Han et al. [16] using the human cell DNA synthesome isolated from HeLa and MCF-7 cells. Our results indicate that the action of ara-C on DNA replication is mediated primarily through DNA polymerase α, and suggests that this enzyme plays a key role during the initiation of DNA synthesis and Okazaki fragments formation.

Taken together, our results suggest that the DNA synthesome can be a highly effective, powerful, and unique in vitro model system for studying the mechanism of action of ara-C and other nucleoside analogues that directly inhibit the DNA replication process.

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