Role of Eukaryotic Topoisomerase I in Transient Cat Gene Expression, in HSV–1 Replication, and in HSV–1 Oris–Dependent Plasmid DNA Replication: Implications in Oncology

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Abstract **Objective** To study the inhibition effects of CPT on CAT gene expression, on HSV-1 replication and on HSV-1 origin-driven DNA replication. **Methods** CAT gene expression and activity assay, plaque titration technique and DpnI resistance assay were applied in the experiments. Different concentrations of CPT were used in these assays. **Results** CPT actively inhibited CAT gene expression, HSV-1 replication and HSV-1 origin-driven plamid DNA replication in cell cultures. The inhibitory effects were dose-dependent in all the experiments. **Conclusions** Eukaryotic DNA topoisomerase I is a key nuclear factor that regulate and control gene expression/transcription, viral genomic DNA replication. Furthermore, topoisomerase I is the eighth fundamental factor for a HSV-1origin of DNA replication to function in infected cells. This may provide molecular basis to understand the action mechanism of camptothecin, a specific inhibitor of the nuclear enzyme, in anti-cancer process.

Key Word Topoisomerase I; camptothecin; origin; replication; HSV-1

Today clinical cancer treatment consists of surgery, chemotherapy, radiotherapy, immunotherapy, gene therapy, and various combinations of single therapies. Drug resistance, defined as non-response to chemotherapy, is a common problem in the management of disseminated malignant disease. It may already be present at the beginning of therapy or it may develop during the course of a chemotherapy program by selection and/or induction. When it extends to structurally and functionally unrelated drugs (classically colchicine, doxorubicin, actinomycin D), it is called "multidrug resistance" (MDR)[1-3]. It has been shown that tumor cells which are resistant to adriamycin exhibit a cross resistance to vincristine, vinblastine and actinomycin D, but are equally sensitive to CPT. Thus, CPT which targets at eukaryotic topo I becomes extremely attractive in cancer chemotherapy since its potential contribution to overcome MDR.

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DNA topoisomerases (topos) are enzymes that change the topological state of DNA without altering the nucleotide sequence, and are classified into two types according to their mechanism of action. DNA topo I catalyzes the breaking and the rejoining of only one strand of DNA at a time, while DNA topo II works by passing a segment of DNA through a transient double-stranded break. DNA topo I is a universal enzyme in all living organisms. It is heavily concentrated in the nucleolus and catalytically active on rRNA genes where it is required for transcription of rRNA genes. Under the appropriate conditions it is capable of changing the topological state of covalently closed, circular DNA molecules by single strand breaking and rejoining. Accumulating evidence has shown that topo I plays an important role in DNA replication and is dose-associated with transcription in both prokaryotic and eukaryotic cells. In the present study, transient CAT gene expression, HSV-1 replication as well as HSV-1 oris-dependent plasmid DNA replication in cell cultures were studied to investigate the possible involvement of topo I which was probed by camptothecin (CPT). This report may provide basic data and means to further studies investigating the functions

of topo I and effects of CPT in neoplastic cells.

MATERIALS AND METHODS

Plasmid pST19

The plasmid was kindly provided by Dr ND Stow, Glasgow, UK, which contains 100 bp of HSV-1 oris[4].

CAT assay

CAT enzymatic activity assay (CAT assay) tests the conversion of ^{14}C chloramphenicol to ^{14}C acetyl chloramphenicol. pSV2cat was used in mammalian cell gene transfer and expression of chloramphenicol acetyltransferase (CAT) gene. pSV2cat was transfected by calcium phosphate precipitation method, followed by DMSO (25%) boost for one minute. At the same time of transfection, different concentrations (0.001, 0.01, and 1.0 μm) of CPT was added, which was maintained until cells were harvested 48 hrs post-transfection.

Plaque titration

The standard plaque titration method was used for infectivity titration of herpes simplex virus type 1 (HSV-1) and compound test on its inhibition of viral replication. Vero cells, 1.5 x 10^5 cells in 0.5 ml growth medium, were seeded in 24 well plates. Cells were incubated at 37℃. On the next day, monolayers were confluent, medium was removed and cells washed with PBS twice without disturbing the monolayers. Virus stock solution was diluted in a series in 10-fold (10^{-1}, 10^{-2}, 10^{-3}, ⋯), and 0.3 ml of infection solution was given to each well. Adsorption was at 37℃, and lasted for 1-2 hrs. After infection, the infection solution was removed and cells washed twice with PBS, then 0.6 to 1.0 ml of overlay (1:1 v/v mixture of double concentrated EMEM and 1.6% carboxymethyl cellulose) was added. After incubation of 3 to 5 days, overlay was removed and culture washed with 0.5% crystal violet, and then washed with water, air-dried. The plaques in each well were counted.

DpnI resistance assay

DpnI resistance assay (Dpn assay) is based on the fact that restriction enzyme DpnI cleaves only methylated GATC sequences, therefore, DNA propagated from Dam+ strains (e.g., DH5α) of E. coli is methylated at the adenine residues within GATC and susceptible to DpnI digestion, while DNA replicated in eukaryotic cells is not methylated at GATC sequences and is thus resistant to DpnI cleavage. Pre-confluent cells were transfected with supercoiled plasmid DNA (pST19) by calcium phosphate transfection method, followed by DMSO shock at 4 hr post-transfection. Incubation was at 37℃ in DMEM containing 10% FCS. Six hours after transfection the cells were either mock-infected or super-infected with HSV-1, at a multiplicity of infection of 5 PFU/cell or more, and incubation continued at 37℃. Growth medium was removed when cytopathic effect (CPE) reached to 80-100%, and cells incubated at 37℃ for 4 hrs with 2 ml of lysis buffer (0.6% sodium dodecyl sulfate, 10 mM Tris-HCl pH7.5) containing 500μg proteinase K. NaCl was added to a final concentration of 200 mM, followed by sequential extractions with phenol and chloroform. RNase A (10μg/ml) was added and the samples incubated at 37℃ for more than 30 min prior to ethanol precipitation. The resultant total cell DNA (contains the input and replicated plasmid DNA) was sedimented by centrifugation and re-dissolved in H_2O. DNA was cleaved in pairs either with a selected enzyme alone (e.g., EcoRI) or together with DpnI, under conditions recommended by the manufacturer. Digested DNA fragments were separated on 0.8% agarose gel and transferred to nylon membrane. Probes were prepared by use of Phagemid vector pBluescript SK+/- with digoxigenin (DIG)-dUTP and a nonradioactive DIG DNA Labeling and Detection Kit. Nylon membranes were hybridized with the DIG-labeled probes.

Dpn−CPT assay

A combined assay of DpnI resistance and CPT inhibition (Dpn-CPT assay) was developed, based on DpnI resistance assay and the inhibiting role of topo I on replication of HSV-1. At the time of super-infection, an appropriate dose of CPT was delivered to the transfected cells. CPT was kept in the medium until harvesting total cell DNA so as for DpnI cleavage.

DIG−CL detection

DIG chemiluminescence (DIG-CL) detection protocol was according to manuals of DIG kit.

RESULTS

Inhibition of CAT gene expression by CPT

Inhibition effect of CAT gene transcription/expression by CPT was found, as compared with the panel of experiments without use of CPT in the CAT assay. As well the inhibition effect of CPT on CAT gene expression was dose-dependent, i.e., when CPT was delivered at 0.001, 0.01, 0.1 and 1.0μM, the CAT activity was found 34, 9, 0.8 and 0.1%, respectively.

Inhibition of HSV−1 replication by CPT

The inhibitory effect of CPT on HSV-1 replication can be observed when Vero cells were infected with the virus and exposed to CPT continuously. The inhibition was found dose-dependent, too. When Vero cells infected with HSV-1 were exposed to CPT at concentrations of 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5μM, the plaque assay revealed a titration reduction of 15, 27, 97, 98, 99, 100 and 99.5%, respectively.

Dpn Assay

To further investigate the action level of topo I on HSV-1 DNA replication, the experiments were designed to focus on the origin of HSV-1 DNA replication (ori). As a first step, Dpn assay was conducted. The plasmid pST19 was constructed with a 100-bp fragment specifying a functional HSV-1 oris replication origin inserted into vector pTZ19U at the SalI and SmaI sites. The plasmid can replicate in Vero cells by transfection of the plasmid DNA and super-infection of HSV-1. Figure 1A shows that the replicated plasmid DNA was DpnI-resistant. Furthermore, the amounts of the resistant DNA were increasing according to a time course (Figure 1A, panels 4, 6, and 8), while the input DNA was cleaved down as smaller fragments (Figure 1A, "DpnI fragments"). This system was used for the next step to study the possible role of topo I in the origin-dependent plasmid DNA replication.

Dpn–CPT assay

To study the role that topo I plays in the HSV-1 oris-dependent plasmid DNA replication, as a second step, Dpn-CPT assay was performed. Plasmid pST19 and virus HSV-1 were used for the assay. CPT was added at a concentration of 0.4 μM to cells transfected and super-infected, and continuous exposure of CPT was maintained. An inhibition effect of CPT on the plasmid DNA replication was found. Figure 1B shows a nearly complete inhibition of HSV-1oris-denpendent plasmid DNA replication in eukaryotic cells (Vero cells) with helper function provided by HSV-1.

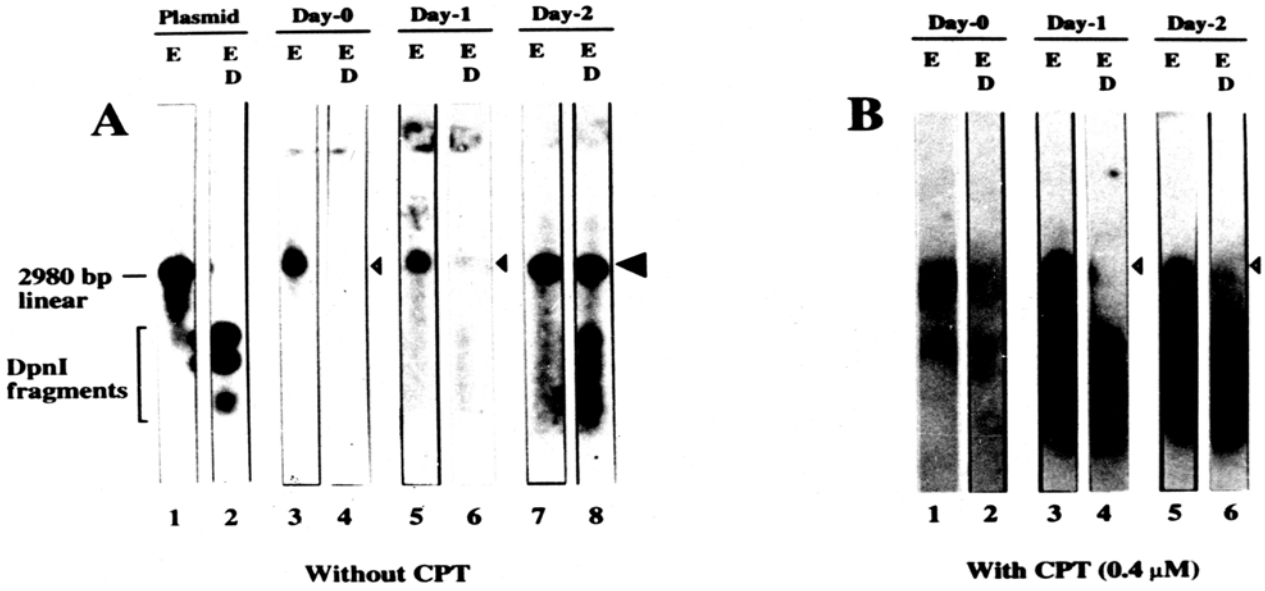

Fig. 1 Dpn-CPT assay. (A) DpnI assay of pST19. Time course of plasmid pST19 amplification was studied. Vero cells were transfected with pST19 and superinfected with HSV-1(F) at moi of 5 pfu per cell or more. DNA prepared from cells at the indicated time was analyzed following cleavage with RcoRI or EcoRI plus DpnI. The fragments were separated by agarose gel electrophoresis, transferred to a membrane and hybridized to DIG-pBS probe. DIG-CL detection was performed. Plasmid pST19 DNA isolated from E.coli DH5α serves as markers for linear form (cleaved by EcoRI, 2980 bp) and for DpnI fragments as indicated on the left. Empty arrowhead indicates the plasmid DNA was not replicated. Filled arrowheads (lanes 6 and 8) indicate replicated, DpnI-resistant pST19 DNA, of which the relative amounts are expressed by the sizes of the filled arrowheads. (B) Effect of continuous CPT treatment on pST19 DNA replication. In parallel to the experiments of (A), another panel of experiments was performed with addition of 0.4 μM CPT to cells immediately after super-infection. The same time course was studied. The empty arrowheads indicate nearly no replication of plasmid pST19 DNA. Restriction enzymes: E, EcoRI; D, DpnI.

DISCUSSION

Topo I and topo II are nuclear enzymes functioning to resolve DNA topological problems during replication, transcription, and other DNA processes[5]. The effects of CPT and VP16 (a specific inhibitor of topo II) have been tested on DNA replication of parvoviruses LuIII and H-1. It is found that viral DNA synthesis is suppressed by CPT but not VP16. Similar results were obtained in studies on herpesvirus with CPT, as reported by the present study. Another study has shown CPT remarkedly reduced the level of IE HSV-2 mRNA, therefore, it was proposed that eukaryotic topo I may play an important role in the process of transcription. It was also reported that HIV-1 replication in vitro can be inhibited by non-cytotoxic doses of CPT[6], and that the replication of another member of lentiviridae subfamily of retroviruses in chronically infected cells can be inhibited.

The initiation of replication takes place at a particular sequence in the parental DNA, designated the origin. The isolation of replication origins assists in the identification and purification of initiation factors and mutagens that enhance or inhibit the origin function. Since easier to handle, models of DNA replication have been established with plasmids and viruses, and the origins of bacterial and yeast chromosomes have been transplanted into small plasmids to facilitate their analysis. Origins of DNA replication may be grouped into (1)eukaryotic virus and organelle origins including those of SV40, HSV viruses (type 1 and 2); (2) eukaryotic chromosome origins; and (3) prokaryotic origins including those of E. coli, phage T7. Two approaches to identify origins are commonly used. The initiation sites can be (1) physically mapped on replication intermediates or (2) functionally mapped by determining the cis-acting sequences required for initiation of replication on a DNA molecule.

In the present paper we describe a combined functional ori assay---DpnI-CPT assay. The cis-acting sequence of HSV-1 has been identified and sequenced, and DpnI resistance assay was applied to this sequence and showed it functional when cloned into plasmid. Followed a series application of camptothecin together with CAT assay, plaque titration assay, DpnI-CPT assay was developed.

In addition to the basic ori sequence and seven HSV-1 elements including UL5, UL8, UL9, dbp, pol, UL42 and UL52, the present experiments confirmed that eukaryotic topo I is the eighth fundamental factor for an origin of DNA replication being functional. It may have putative application value in studying origins of DNA replication in human genome, especially in malignant situation.

In the field of oncology, origin(s) of DNA replication of human genome is one of the areas that have not yet been explored, largely because the human genome is extremely huge, and cloning and sequencing of the genome is surely time consuming. Efforts are needed in the future to identify and clone origins of human genomic DNA especially in cancer cells, therefore, functional ori assays could be applied to those human genomic origins of DNA replication. These studies may provide more information for a better understanding of human cellular DNA replication especially at its initiating sites, which will be the selective targets for developing new drugs of chemotherapy.

REFERENCES

1. Geng Yi, Jiang Sen, Li jiafu, et al. Molecular Basis of MD R(I). Current Advances in Obstetrics and Gynecology, 1995, 4 (3): 288-294.
2. Geng Yi, Jiang Sen, Li jiafu, et al. Molecular Basis of MD R (II). Current Advances in Obstetrics and Gynecology, 1996, 5 (1): 89-93.
3. Geng Yi, Jiang Sen, Li jiafu, et al. Molecular Basis of MD R (III). Current Advances in Obstetrics and Gynecology, 1996, 5 (2): 188-193.
4. Stow ND and McMonagle EC. Characterization of the TRs/IRs origin of DNA replication of herpes simplex virus type 1. Virology, 1983, 130: 427-438.
5. Alberts B. DNA replication and recombination. Nature, 2003, 421: 431-435.
6. Priel ED, Showalter D, and Blair DG. Inhibition of human immunodeficiency virus (HIV-1) replication by non-cytotoxic-doses of camptothecin, a topoisomerase I inhibitor. Hum Retroviruses, 1991, 7: 65-72.