FEBS Letters 441 (1998) 318–321 FEBS 21318

9-Hydroxyellipticine inhibits telomerase activity in human pancreatic cancer cells

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Received 19 October 1998; received in revised form 18 November 1998

Abstract There is increasing interest in identifying potent inhibitors of telomerase because the enzyme plays a crucial role in the development of cellular immortality and carcinogenesis. We hypothesized that 9-hydroxyellipticine (9-HE), an antitumor alkaloid, would inhibit telomerase activity because the drug has a unique mechanism of inhibiting phosphorylation of mutant p53 protein via inhibition of protein kinases, thereby restoring wild-type p53 function. This study was conducted to examine the effect of 9-HE on telomerase activity in human pancreatic cancer cells with differing p53 gene status. 9-HE treatment at relatively high concentrations resulted in rapid, complete inhibition of telomerase activity, irrespective of the p53 status. We conclude that 9-HE may exert a strong inhibitory effect on telomerase activity possibly through inhibition of protein kinases rather than through restoration of functional wild-type p53.

Key words: 9-Hydroxyellipticine; Telomerase activity; Inhibitor; Protein kinase

1. Introduction

Telomerase is a ribonucleoprotein enzyme that elongates and/or maintains telomeric DNA, and it is considered to play an important role in the development of cellular immortality and carcinogenesis [1,2]. Telomerase has been recognized as a potent diagnostic marker in a variety of human cancers because of its prevalence in most tumor cells; the majority of normal cells adjacent to tumors lack detectable levels of telomerase activity [3,4]. We reported previously that telomerase activity was highly elevated in 80% of the specimens from surgically resected lesions of pancreatic carcinoma [5,6] and in 75% of pancreatic juice samples obtained from patients with ductal carcinoma [7]. The specific association of telomerase activity with cancers has led researchers to target this enzyme for therapeutic application [8]. Several telomerase inhibitors have been reported, including inhibitors of retroviral reverse transcriptase [9], peptic nucleic acids [10], hammerhead ribozymes [11], antisense telomerase RNA component (hTR) [12], protein kinase C inhibitors [13], cisplatin [14], and *Oxytricha* telomere DNA binding proteins [15]. Studies of the mechanism of telomerase activity inhibition would facilitate the understanding of the functional role of this enzyme in tumor cell growth and its interaction with other components.

Recent studies have suggested a close linkage between the p53 tumor suppressor gene and telomerase activity. For instance, activation of telomerase was observed in immortalized human epithelial cells transfected with a mutant p53 [16] and in human keratinocytes transduced with human papillomavirus type 16 E6 protein, which mediates degradation of p53 protein [17,18]. These studies suggest that abrogation of p53 leads to activation of telomerase activity, which is normally downregulated in an indirect manner by the wild-type p53. Based on these studies, we hypothesized that 9-hydroxyellipticine (9-HE), an ellipticine derivative, would suppress telomerase activity because the drug has a unique anticancer mechanism of inhibiting phosphorylation of mutant p53 protein via inhibition of protein kinases, thereby restoring wild-type p53 function [19,20].

In the present study, we investigated the effect of 9-HE on telomerase activity in cultured human pancreatic cancer cells. Other chemotherapeutic agents, which act by different mechanisms, were tested for comparison with 9-HE. Our results demonstrated that of all the drugs tested, 9-HE alone caused specific inhibition of telomerase activity of treated cells regardless of the p53 gene status, thus implying that inhibition of protein kinases rather than restoration of wild-type p53 may be involved in the mechanism behind the effect.

2. Materials and methods

2.1. Cell lines

Six human pancreatic cancer cell lines were used in this study: MIA PaCa-2 (mutant p53) was provided by the Japanese Cancer Resource Bank (Tokyo, Japan); SUIT-2 (mutant p53), PANC-1 (mutant p53), KP-1N (mutant p53), AsPC-1 (p53-negative), and KP-3 (wild-type p53) were generously donated by Dr. H. Iguchi (National Kyushu Cancer Center, Fukuoka, Japan) [21]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml), and penicillin (100 U/ml) at 37°C in a humidified atmosphere containing 10% CO_2.

2.2. Drug treatment

Exponentially growing cells were plated at a density of 1×10^5 cells/well in 24-well plates. After overnight incubation, the cells were continuously exposed to various concentrations (1–10 μM) of 9-HE, which was kindly donated by M. Ohashi (Tanabe Seiyaku Co. Ltd., Tokyo, Japan). For comparison, MIA PaCa-2 cells were treated with other chemotherapeutic agents in the same manner: VP-16 (10 μM), cisplatin (30 μM), mitomycin C (5 μM), 5-fluorouracil (300 μM), and vincristine (5 μM). The concentrations of these chosen drugs correspond to the concentrations required to achieve 40–50% cell killing, which is almost equivalent to the killing rate encountered in 9-HE treatment at a concentration of 10 μM, measured by the cell killing assay (see below) on day 5 of treatment. Antiproliferative effects were evaluated daily based on cell number as assessed in a particle distribution analyzer, CDA500 (Sysmex, Kobe, Japan).

2.3. Cell killing assay

Cytotoxicity was evaluated using a multiwell fluorescence scanner

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PII: S0014-5793(98)01571-3

by the method described by Nieminen et al. [22] with minor modifications. This method allows an indirect measurement of cell killing and is based on the binding of propidium iodide (PI) to the nuclei of cells whose plasma membranes have become permeable due to cell death. Briefly, 10 μl of a 3.06 mM solution of PI was added to each well for a final concentration of 30 μM. After a 60-min incubation at 37°C, initial fluorescence unit from each well was measured in a CYTOFLUOR II (PerSeptive Biosystems Inc., Framingham, MA, USA) using 530-nm excitation and 645-nm emission filters. After reading, 20 μl of 32.54 mM digitonin (600 μM) was added to each well to permeabilize all cells and label all nuclei with PI. After a 30-min incubation at 37°C, fluorescence was measured again to obtain a value corresponding to 100% cell death. The percentage of dead cells was calculated as the proportion of a fluorescence unit of dead cells to that of total cells.

2.4. Telomerase activity assay

Cells were harvested by centrifugation, resuspended in CHAPS lysis buffer (10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol), and incubated for 30 min on ice. After centrifugation at 15 000 × *g* for 20 min at 4°C, the supernatants were transferred to fresh tubes and used as the cell extracts for telomerase assay. Telomerase activity was measured by the PCR-based telomeric repeat amplification protocol (TRAP) assay [2] with minor modifications. In brief, 2 μl of the cell extracts (equivalent to 1000 cells) were incubated in a reaction mixture containing 50 mM deoxynucleotide triphosphate, 2 units of Taq DNA polymerase (Promega Corp., Madison, WI, USA), and 0.1 μg of TS primer (5′-AATCCGTCGAGCAGAGTT-3′) at 20°C for 30 min. After incubation, 0.1 μg of CX primer (5′-CCCTTACCCTTACCCT-TACCCTAA-3′) was added, and the reaction mixture was subjected to a 31-cycle PCR amplification. The PCR products were resolved by electrophoresis on a non-denaturing 12% polyacrylamide gel and the gel was stained with SYBR green DNA stain (FMC BioProducts, Rockland, ME, USA). Signal intensity of the 6-base ladder was measured by NIH image, version 1.60 (NTIS, Springfield, VA, USA) and used for quantitative analysis. The relative density in each treated sample was expressed as its percentage of untreated control after subtraction from background.

3. Results

Changes in total cell number of MIA PaCa-2 cells treated with 9-HE (10 μM), VP-16 (10 μM), cisplatin (30 μM), mitomycin C (5 μM), 5-fluorouracil (300 μM), and vincristine (5 μM) are shown in Fig. 1. Compared with untreated cells (control), 9-HE treatment completely inhibited cell growth, as

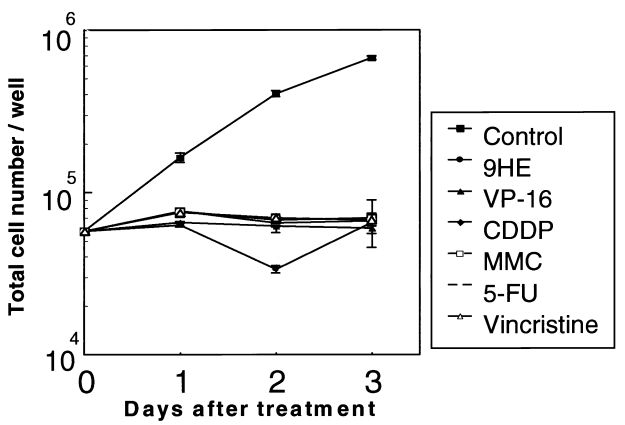

Fig. 1. The daily changes in total cell number of MIA PaCa-2 cells treated with 9-HE (10 μM), VP-16 (10 μM), cisplatin (30 μM), mitomycin C (5 μM), 5-fluorouracil (300 μM), or vincristine (5 μM). Cell numbers shown are mean ± S.D. of three independent wells for each agent.

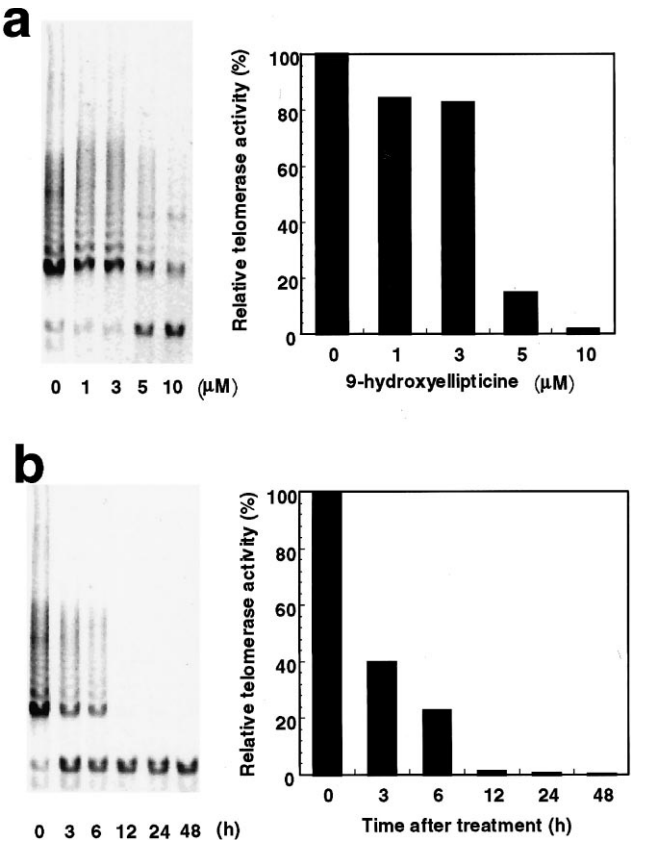

Fig. 2. Inhibition of telomerase activity in MIA-PaCa 2 cells treated with 9-HE. a: Cells treated for 48 h with 9-HE at a concentration of 0 (control), 1, 3, 5, and 10 μM. b: Cells treated with 9-HE for 0 (control), 3, 6, 12, 24, and 48 h at a concentration of 10 μM. Telomerase activity was measured using a PCR-based telomerase assay as described in Section 2. The relative densities of the telomeric ladders in each treated sample are expressed as a percentage of the untreated control after subtraction from background (plotted in bar graphs).

did the other drugs. Using the PCR-based TRAP assay, we first assessed whether 9-HE inhibits telomerase activity in MIA PaCa-2 cells in culture. 9-HE significantly inhibited telomerase activity of treated cells in a concentration-dependent manner (Fig. 2a). Whereas relatively low concentrations of 9-HE (1, 3 μM) resulted in almost no inhibition, treatment at the highest concentration, 10 μM, completely inhibited activity. Time courses of telomerase activity in MIA PaCa-2 cells upon exposure to 10 μM 9-HE are shown in Fig. 2b. The inhibition of telomerase activity was detectable as early as 3 h after the beginning of drug exposure. At 12 h after exposure, the telomerase ladders were almost undetectable, indicating complete inhibition. The in vitro direct effect of 9-HE on telomerase activity was analyzed to test the possibility that 9-HE could inhibit the PCR reaction. When MIA PaCa-2 cell extracts were exposed to 10 μM 9-HE for 3 h before primer addition, no telomerase inhibition was observed (data not shown). Therefore, neither a direct influence of 9-HE on telomerase activity in a cell-free system nor an inhibition of PCR reaction seems to be involved in the decline of the enzyme activity observed after drug treatment.

To determine whether the observed inhibition of telomerase activity by 9-HE is due to drug-induced cell killing, we performed a cell killing assay of MIA PaCa-2 cells treated with

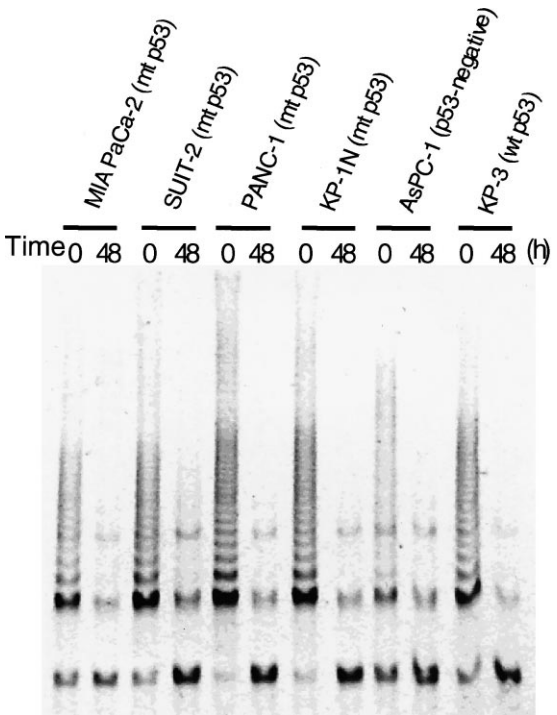

Fig. 3. Effect of 9-HE treatment on telomerase activity in six pancreatic cancer cell lines with differing p53 statuses. Cells were treated with 9-HE for 48 h at a concentration of 10 μM and telomerase activity was assessed.

9-HE. The percentages of dead cells at 6, 24, 48, 72, and 120 h after treatment with 10 μM 9-HE were 1.0 ± 0.4, 2.7 ± 0.5, 3.8 ± 0.6, 17.4 ± 3.7, and 42.0 ± 5.0%, respectively. Therefore, 9-HE treatment had little or no effect on the rate of cell death until 72 h after the start of exposure, at which time the inhibition of telomerase activity was already apparent.

When other pancreatic cancer cells (SUIT-2, PANC-1, KP-1N, AsPC-1, KP-3) with different p53 gene statuses were treated with 10 μM 9-HE, they all showed similar inhibitory effects at 48 h after drug exposure (Fig. 3). Furthermore, in AsPC-1 (p53-negative) and KP-3 (wild-type p53) cells, the inhibition of telomerase activity was detectable from 3 h after drug treatment, which is a similar finding to that encountered in MIA PaCa-2 (mutant p53) cells (data not shown). These findings indicate that telomerase inhibition caused by 9-HE is not related to the p53 status of tumor cells.

To examine the specificity of telomerase inhibition by 9-HE, the effect of other cytotoxic agents was investigated under the same conditions. In contrast to 9-HE, VP-16, cisplatin, 5-fluorouracil, mitomycin C, and vincristine had no inhibitory effect on telomerase activity of treated cells. In fact, cells treated with VP-16 and 5-fluorouracil had slightly elevated levels of telomerase activity than that of control cells (Fig. 4).

4. Discussion

In the present study, we have clearly demonstrated that 9-HE, an ellipticine derivative, significantly inhibited telomerase activity in a time- and concentration-dependent manner in human pancreatic cancer cells. In contrast, other chemotherapeutic agents had no effect on the enzymatic activity of treated cells. These findings suggest that the telomerase inhibition caused by 9-HE could contribute to its therapeutic action, which the other drugs lack. 9-HE has potent anticancer activities in a variety of tumors possibly as a result of intercalation with DNA and/or inhibition of topoisomerase II [23–25]. Recently, it has been shown that 9-HE has an additional anticancer mechanism of inhibiting phosphorylation of mutant p53 protein via inhibition of protein kinases, such as cdk2 kinase and DNA-activated kinase, which are responsible for the phosphorylation of p53 [19,20]. Inhibition of mutant p53 phosphorylation results in a large amount of dephosphorylated mutant p53, thereby restoring wild-type p53 function. In other experiments, we found that a relatively low concentration (1 μM) of 9-HE was sufficient to restore the

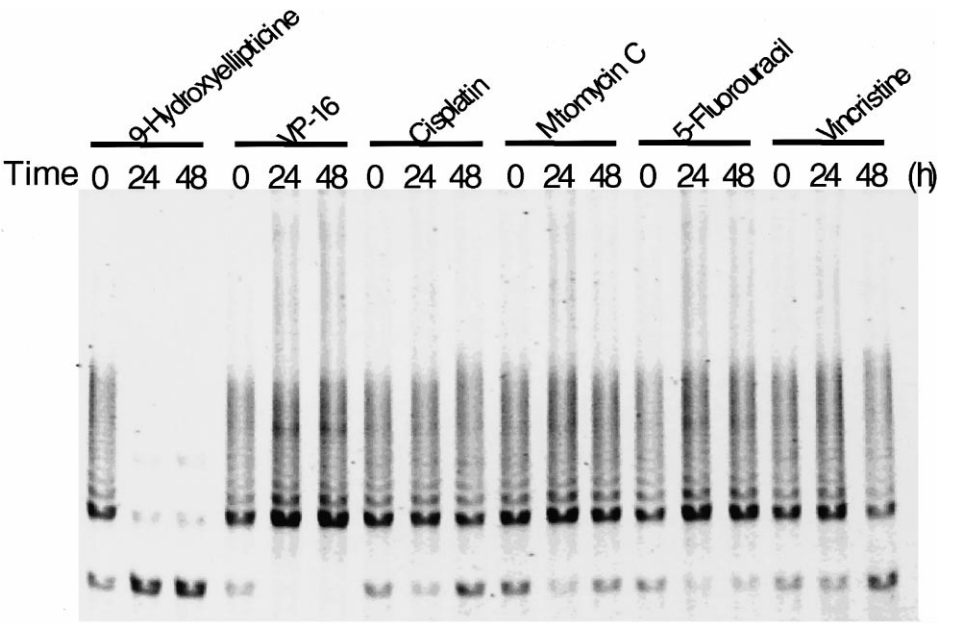

Fig. 4. Effects of chemotherapeutic agents on telomerase activity in MIA-PaCa 2 cells. Cells were treated with VP-16 (10 μM), cisplatin (30 μM), mitomycin C (5 μM), 5-fluorouracil (300 μM), and vincristine (5 μM) for 24 and 48 h, and telomerase activity was assessed.

wild-type p53 function and that this effect was specific for cells containing mutant p53 (unpublished data). Therefore, we hypothesized that the restoration of wild-type p53 or the accumulation of dephosphorylated mutant p53 contributes to the decline in telomerase activity observed after treatment with 9-HE.

Recently, the relation between the p53 tumor suppressor gene and telomerase activity has received much attention. One finding suggesting a linkage between p53 and telomerase activity is that normal human breast epithelial cells transfected with a p53 mutant became immortalized and were reactivated for telomerase [16]. In addition, primary human keratinocytes transduced with the human papillomavirus type 16 E6 gene, which binds to the cellular p53 protein and promotes its degradation, expressed significant telomerase activity [18]. These studies imply that inactivation of p53 leads to activation of telomerase, which is normally downregulated in an indirect manner by the wild-type p53. More recently, it has been reported that human non-small-cell lung cancer cells (wild-type p53) that were stably transfected with sense wild-type p53 cDNA showed a significant reduction in telomerase activity [26]. These findings are consistent with our hypothesis that 9-HE might inhibit telomerase activity via the restoration of wild-type p53. However, in our present study, telomerase inhibition was almost undetectable after exposure to 9-HE at a concentration of 1 μM, which is sufficient to restore the wild-type p53 function in cells harboring p53 mutations. Furthermore, the telomerase inhibition was found not only in mutant p53 expressing cells but also to a similar extent in p53-negative (AsPC-1) or wild-type p53 (KP-3) cells, thus the inhibition was not dependent on p53 status. Restoration of wild-type p53 could not explain the mechanism of inhibiting telomerase activity, at least in p53-negative or wild-type p53 expressing cells.

Several reports have described a relation between telomerase activity and protein kinases. For example, the protein kinase C inhibitors bisindolylmaleimide I and H-7 were found to inhibit telomerase activity in human nasopharyngeal cancer cells in culture [13]. This effect on telomerase activity was specific for PKC inhibitors and was not observed in cells treated with various other chemotherapeutic agents, which is consistent with our present results. Furthermore, protein phosphatase 2A inhibits nuclear telomerase activity in human breast cancer cells, which implies that telomerase is reversibly regulated by protein phosphorylation and dephosphorylation through protein kinases and the protein phosphatase 2A [27]. In the light of these findings, we assume that inhibition of protein kinases such as cdk2 kinase and DNA-activated kinase may account for the telomerase inhibition caused by 9-HE treatment. However, it is not clear how these protein kinases and protein phosphatase are involved in controlling telomerase activity in cancer cells. Further investigation of the molecular relation between telomerase and protein kinases should provide a new anticancer strategy.

We conclude that 9-HE may exert a potent inhibitory effect on telomerase activity in cultured pancreatic cancer cells, probably through inhibition of protein kinases, although the precise molecular mechanism of telomerase inhibition remains to be elucidated.

Acknowledgements: We are very grateful to Dr. H. Iguchi for kindly supplying the pancreatic cancer cell lines and to Dr. M. Ohashi for donating 9-hydroxyellipticine.

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