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Radiosensitization to X-ray radiation by telomerase inhibitor MST-312 in human hepatoma HepG2 cells

Yali Wang [a,b,c,d,1], Chao Sun [a,b,c,1], Aihong Mao [a,b,c], Xin Zhang [a,b,c], Xin Zhou [a,b,c], Zhenhua Wang [e], Hong Zhang [a,b,c,*]

[a] Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou 730000, People'sRepublic of China
[b] Key Laboratory of Heavy Ion Radiation Medicine of Chinese Academy of Sciences, Lanzhou 730000, People's Republic of China
[c] Key Laboratory of Heavy Ion Radiation Medicine of Gansu Province, Lanzhou 730000, People's Republic of China
[d] School of Pharmacy, Lanzhou University, Lanzhou 730000, People's Republic of China
[e] College of Life Sciences, Yantai University, Yantai 264005, People's Republic of China

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ABSTRACT

Aims: Previous studies in malignant cells have shown that irradiation-induced upregulation of telomerase activity, not only protected damaged telomeres, but also contributed to DNA damage repair by chromosomal healing and increased resistance to irradiation. The purpose of the present study was to investigate the radiosensitizing effect of telomerase inhibitor MST-312 and the corresponding mechanism in the human hepatoma cell line HepG2.
Main methods: Cell proliferation, telomerase activity, cell cycle distribution, DNA damage and repair, expression of p53, mitochondrial membrane potential, and cell apoptosis were measured with the MTT assay, real-time fluorescent quantitative PCR, flow cytometry, immunofluorescence, western blots, JC-1 staining, and Hoechst 33258 staining, respectively.
Key findings: MST-312 effectively inhibited telomerase activity and showed relative weak toxicity to HepG2 cells at 4 μM. Compared with irradiation alone, 4 μM MST-312 pretreatment, followed by X-ray treatment, significantly reduced clonogenic potential. Aggravated DNA damage and increased sub-G1 cell fractions were observed. Further investigation found that homologous recombination (HR) repair protein Rad51 foci nuclear formation was blocked, and expression of p53 was elevated. These led to the collapse of mitochondrial membrane potential, and enhanced the apoptotic rate.
Significance: These data demonstrated that disturbances of telomerase function could enhance the radiosensitivity of HepG2 cells to X-ray irradiation by impairing HR repair processes. In addition, telomerase inhibitor MST-312 may be useful as an adjuvant treatment in combination with irradiation.

Introduction

Telomeres are specialized structures at the chromosome ends that are composed of TTAGGG repeated sequences, which interact with specific proteins and provide the protective caps of linear chromosomes [14]. The main functions of telomeres are to protect chromosome ends from deleterious DNA repair or recombination events and maintain the stability of the chromosome [5]. Progressive telomere shortening, due to end replication problems, is one of the molecular mechanisms underlying aging of normal somatic cells [9]. Cells are prone to senescence when telomeres shorten below a critical length and lose their capacity to provide an adequate cap to the chromosome ends, which is termed the Hayflick limit [12,29]. However, in the case of continued proliferation, the cells with short telomeres might bypass senescence and enter a further growth cycle, by activating the telomerase, which could immortalize into cancer cells [23]. The reactivation of telomerase is necessary for the proliferation of cancer cells, and its activity has been detected in about 90% of all human cancer cases [16]. A previous study has shown that the majority of hepatocellular carcinomas (HCC) also exhibited a telomerase activity [34]. What is particularly intriguing is that radiotherapy could upregulate the telomerase activity of cancer cell lines at low dose [25]. Another study has shown similar results in human retinoblastoma Y79 cells [1]. The inherent and irradiation-induced telomerase activity not only protects damaged telomeres, but also may contribute to DNA damage repair by chromosomal healing and increased resistance to irradiation in malignant cells [1,2,25]. Thus, this raises the question as to whether the inhibition of telomerase activity could lead to the enhancement of radiosensitivity for cancer radiotherapy.

* Corresponding author at: Key Laboratory of Heavy Ion Radiation Biology and Medicine of Chinese Academy of Sciences, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou 730000, People's Republic of China. Tel.: +86 931 496 9344; fax: +86 931 496 9170.
E-mail address: zhangh@impcas.ac.cn (H. Zhang).
[1] These authors contributed equally.

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In order to address this question, we used telomerase inhibitor MST-312 which is a new compound derived from epigallocatechin gallate (EGCG). MST-312 inhibits telomerase activity in a dose-dependent manner [27]. Treatment of some tumors with MST-312 caused telomere dysfunction through two mechanisms. Continuous treatment resulted in progressive telomere shortening and eventual reduction of cell proliferation [27,36]. Short-time administration of MST-312 led to an acute effect, with DNA damage and reduced cell viability, which suggested that the telomerase complex might uncouple from DNA [28]. This hypothesis has been supported by reports that telomere dysfunction impaired DNA repair and enhanced sensitivity to ionizing radiation (IR) [10,33]. The pathogenic link between telomere dysfunction and impaired DNA repair suggested that a combination of telomerase inhibitors and radiotherapy could provide a rational strategy for the treatment of cancers. Whether a specific dose of MST-312 has radiosensitizing effects, and how this could impair the repair of irradiation-induced DNA damage remains less clear. In the present study, we investigated the radiosensitization effect of MST-312 and its underlying mechanisms in HepG2 cells.

Materials and methods

Reagents and chemicals

Telomerase inhibitor MST-312 (Sigma, USA) was dissolved in DMSO with 20 mg/ml concentration and diluted with Dulbecco's Modified Eagle's Medium (DMEM) medium to a concentration of 4 μM. DMEM medium was purchased from GIBCO Company (USA). Fetal bovine serum (FBS) was purchased from Hyclone Company (USA). Other unmentioned reagents and solutions in our experiments were purchased from Sigma Aldrich Company.

Cell culture and X-ray irradiation

Human hepatoma HepG2 cell line was obtained from ATCC and maintained in DMEM medium supplemented with 10% FBS and antibiotics (penicillin 100 units/ml, streptomycin 100 μg/ml). X-ray irradiation was performed with a cabinet X-ray irradiation system at the Institute of Modern Physics, Chinese Academy of Sciences. X-ray was generated with Faxition43885D X-ray machine at 50 kVp energy, and the dose rate was set to about 0.534 Gy/min.

Cytotoxicity assay

The cytotoxicity of MST-312 was evaluated in HepG2 cells by MTT assay. Briefly, cells were seeded in 96-well plates (0.5×10^5 cells/well in 100 μl of medium) and cultivated at 37 °C overnight. Then cells were exposed to various concentrations of MST-312 for 12and 24 h. Thereafter, 20 μl MTT (0.5 mg/ml) was added into 96-well plates directly and continued to incubate for 4 h. Afterwards, the medium was aspirated off and MTT formazan was dissolved in 150 μl of DMSO. The optical absorbance was measured at 490 nm using a microplate reader of Tecan Infinite M200 (Austria). Experimental data was expressed as the percentage of the control group.

Real-time fluorescent quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) and cDNA was synthesized with the Thermo Fisher K1622 (USA). The PCR amplification mixture contained 1 μl cDNA, 10 μl SYBR Green Master Mix buffer (TOYOBO, Japan), and forward and reverse primers (20 μM each) in 20 μl reaction volume. The following primer pairs were used: hGAPDH-sense (5′-CATCTTCTTTTGCGTCGCCA -3′) and hGAPDH-antisense (5′-TTAAAAGCAGCCCTGGTGACC -3′) for hGAPDHand hTERT-sense (5′-TGGCTGATGAGTGTGTACGT-3′) and hTERT-antisense (5′-TGTCTGATTCCAATGCTTTG-3′) for hTERT. Real-time fluorescent quantitative PCR was carried out with the ABI Step one plus Real time-PCR system (USA). Every assay was performed in triplicate and all experiments included analysis of GAPDH mRNA levels as internal standard. Relative quantification approach (ΔΔCt) was used according to the method previously described [17].

Colony forming assay

Cells in exponential growth were seeded at 1000 cells per 60 mm culture dishes and allowed to attach overnight for colony formation. Cells were treated with 4 μM MST-312 or left untreated for 2 h, and exposed to 2 Gy X-ray irradiation. Cells were given fresh medium with 4 μM MST-312 every three days and further incubated. After 8 days, the colonies were fixed with methanol and stained with 1% methylene blue solution. The colonies were determined manually. Only colonies containing more than about 50 cells were scored.

Immunofluorescence

Rad51, XRCC4 and γ-H2AX were detected by immunofluorescence. Briefly, HepG2 cells were grown on the surface of glass cover slips in DMEM medium overnight. Cells were treated with 4 μM MST-312 or left untreated for 2 h, and exposed to 2 Gy X-ray irradiation. The medium was aspirated followed by three PBS washes. Cells were then fixed onto cover slips using 4% paraformaldehyde for 30 min. Cells were washed three times with PBS and permeated with 0.5% Triton X-100 for 10 min, followed by three PBS washes. The 1% albumin from bovine serum was used as a blocking solution for 30 min at room temperature. The appropriate antibody was added and incubated overnight at 4 °C. Cells were washed with PBS three times followed by the addition of the appropriate secondary antibody for 30 min. After PBS washes, the slides were counterstained with DAPI (4′,6′-diamidino-2-phenylindole). All images were captured with a fluorescent confocal microscope (Zeiss LSM700, GER).

Analysis of cell cycle

Cell cycle status was analyzed by propidium iodide (PI) staining and flow cytometry (Becton Dickinson, USA). Briefly, cells were treated with 4 μM MST-312 or left untreated for 2 h, and exposed to 2 Gy X-ray irradiation. Then, cells were collected, washed in cold PBS and fixed in 70% cold ethanol at 4 °C overnight. The fixed cells were then washed twice with cold PBS and resuspended in 500 μl of fluorochrome solution (PI at 50 mg/l in 0.1 mg/ml RNAase and 0.1% Triton X-100) and incubated at room temperature for 30 min in the dark. Twenty thousand events were measured per sample using flow cytometry. Cell cycle distribution was quantified using cell cycle analysis software (Flowjo 7.6). The sub-G1 fraction was determined and considered as cell death and apoptosis.

Western blot analysis

The protein level of p53 was detected by western blot. Proteins were extracted in lysis buffer and concentrations were measured using a BCA protein assay kit (Pierce, USA). Equal amounts of protein (20 μg protein each lane) were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% BSA in PBS/Tween 20 (0.05%, v/v) for 2 h at room temperature, followed by incubation with primary antibodies against p53 (Bioworld technology, USA)at 4 °C overnight. After washing with PBST for 30 min, the membranes were incubated with an appropriate HRP-conjugated secondary antibody that was diluted in 5% BSA for 1 h at room temperature. Membranes were washed three times for 15 min each with PBST. Reactive proteins were detected using a chemiluminescence kit (Bioworld technology, USA) according to the manufacturer's manual. Data were presented as a relative protein level normalized to β-actin, and the ratio of control samples was taken as 100% [32].