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Role of diallyl disulfide-mediated cleavage of c-Myc and Sp-1 in the regulation of telomerase activity in human lymphoma cell line U937

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ABSTRACT

Objective: Garlic (*Allium sativum*) has been considered a wonder herb for years with a reputation of disease prevention. Telomerase, a ribonucleoprotein enzyme responsible for telomere integrity, is strongly up-regulated in different types of cancers. The aim of this study was to reveal the role of diallyl disulfide (DADS), an organosulfur component of garlic, on telomerase activity in human lymphoma with an emphasis on key transcription factors c-Myc and Sp-1.
Methods: Human lymphoma cell line U937 was used as model cell line. Telomerase activity was measured by telomerase repeat amplification protocol assay, levels of related proteins and mRNAs were measured by Western blot and reverse transcriptase polymerase chain reaction, respectively. Moreover, in vitro binding assay was performed using radiolabeled double-stranded DNA having specific sequences to detect involvement of transcription factors in DADS-dependent modulation of telomerase activity.
Results: The present study demonstrated DADS-mediated decrease in telomerase activity in U937 cells with concomitant transcriptional down-regulation of human telomerase reverse transcriptase (hTERT) that is caused by reduced binding of c-Myc and Sp-1 to their respective binding sites on hTERT promoter. Lowering of DNA-binding activity of c-Myc and Sp-1 due to DADS treatment is caused by the deactivation of these transcription factors due to cleavage. Additionally, Mad1—the repressor protein of hTERT expression—is also overexpressed in DADS-treated U937 cells.
Conclusions: These findings strongly suggest that DADS down-regulate telomerase activity through c-Myc-, Sp-1-, and Mad1-dependent transcriptional down-regulation of hTERT.

Introduction

The life span of somatic cells is limited by the molecular erosion resulting from the end replication problem that leads to the loss of some information during each cycle of cell division. Telomeres, a six base-pair unique repetitive $(TTAGGG)_n$ DNA sequence present at the end of each eukaryotic chromosome, safeguard the chromosomal ends from degradation, end-to-end fusion, rearrangements, and loss [1,2]. Synthesis of telomeric repeats is thus an important phenomenon maintained by a ribonucleoprotein enzyme called telomerase. This ribonucleoprotein complex comprises a catalytic subunit called human telomerase reverse transcriptase (hTERT) [3,4], its template RNA, commonly known as human telomerase RNA or human telomerase RNA component (hTR/hTERC) [5,6], telomerase-associated proteins (TEP1) [7], and chaperone proteins (p23 and Hsp90) [8]. Telomerase, having a specialized reverse transcriptase activity, is barely detected in normal somatic cells but profoundly expressed in highly proliferating cells [9–11]. Loss of telomerase activity appears to be one of the deciding factors that distinguish mortal cells from immortal ones. Because telomerase is found to control sustained proliferation of majority of cancer cells and cellular senescence [12], it is a proposed target for cancer therapy.

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In all fairness, hTERT—the catalytic component of telomerase ribonucleoprotein complex—seems to be the major determinant of telomerase activity. In most cases of cancer initiation, progression, or immortalization, hTERT expression is noticed to be associated with telomerase activity. Although hTERT undergoes epigenetic modulation, expression of this catalytic subunit is tightly regulated at the level of transcription and occasionally by post-transcriptional modifications [13,14]. Unlike most promoters, hTERT promoter lacks TATA and CCAAT boxes but possesses an initiator sequence (CCTCTCC), which helps RNA polymerase II to identify transcription start site in absence of the TATA box. The core promoter encompasses the proximal 181 base-pair region upstream of transcription start site. It has been reported that c-Myc and Sp-1 bind specifically to the consensus motifs called E- and GC-boxes, respectively [15], within the core promoter region, playing distinct roles in transactivation of hTERT. Apart from these, several other binding sites are also present in the promoter region of telomerase that interact with variety of other regulatory proteins [16]. Members of c-Myc/-Max/Mad family proteins form dimers and bind to E-box of the promoter, playing a central role in either activating or repressing E-box containing promoter activity of hTERT. Actually, both c-Myc and Mad1 proteins can form heterodimeric complex with ubiquitously expressed Max. c-Myc/Max heterodimers activate gene expression by binding to E-box region, whereas Mad/Max heterodimers repress transcription doing the same [14]. Competition among c-Myc/Max and Mad/Max complexes to bind to E-box region of hTERT promoter becomes decisive factor for the transcriptional fate of hTERT. It has been reported that levels of hTERT mRNA are directly up-regulated by c-Myc overexpression [17,18] and gets down-regulated by siRNA directed against c-Myc [19]. Hence, c-Myc is believed to be a strong transcriptional regulator of hTERT. In addition to transcriptional regulation, hTERT can also be regulated post-translationally through Akt pathway, which was reported to enhance telomerase activity through phosphorylation of the hTERT at Ser^{824} [20].

A prompt degradation of telomeric repeats triggering apoptotic death is manifested in several systems like HeLa cells treated with cisplatin [21], murine melanoma cells, and human pharynx carcinoma cells treated with paclitaxel [22,23], HL60 cells treated with DNA damaging agents like camptothecin, etoposide, and ultraviolet radiation [24]. Different compounds of herbal origin also exhibit potent cytotoxic effect against cancer cells and are found to induce apoptosis through inhibition of telomerase—a potential target for cancer therapy. Butein and Platycodin D, two common herbal compounds, demonstrate apoptosis in leukemic cells by down-regulating telomerase activity through suppression of hTERT expression [25,26]. Diallyl disulfide (DADS), the major component of garlic, effectively induces apoptosis in different cancers [27–32]. A previous study also accentuated DADS-induced nuclear factor-κB–mediated transient G2/M arrest followed by apoptosis in human leukemic cell lines [33]. Because resistance to apoptosis and maintenance of telomeric integrity by activation of telomerase are key features of cancerous cells, a possible underlying link between telomeres, telomerase, and apoptosis has been investigated in DADS-treated lymphoma cell line U937.

To our knowledge, the present study showed for the first time that telomerase activity is modulated by DADS in lymphoma cell line U937. To investigate the molecular mechanisms involved in DADS-induced transcriptional regulation of hTERT, the expression levels of c-Myc, Sp-1, and Mad along with their binding to respective *cis*-elements in hTERT promoter, has been investigated.

Materials and methods

Materials

DADS was purchased from Fluka Chemika Co. (Bucha, Switzerland); MTT from Hi-Media (Bangalore, India); RPMI-1640, and fetal bovine serum (FBS), penicillin-streptomycin, and amphotericin B purchased from Pan Biotech (Germany). Bovine serum albumin and dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, MO, USA); Triton-X from Hi-media; and Bradford reagent from Amresco (Solon, OH, USA). Antibodies like anti-Sp-1 (#5931), anti-Mad1 (#4682) were purchased from Cell-Signaling (Danvers, MA, USA), anti-hTERT [(H-231): Sc-7212], anti-c-Myc [(9 E 10): Sc-40], anti-β-actin [(C4): Sc-47778], and luminol reagent from Santa-Cruz Biotechnology (Santa Cruz, CA, USA). Trizol was purchased from Invitrogen (Carlsbad, CA, USA); RNase-A, random hexamer, reverse transcriptase (RT), and reagents for RT polymerase chain reaction (PCR) from Fermentas (Burlington, Canada); polyvinylidene difluoride membrane from Thermo-Scientific, (Waltham, MA, USA) film, developer; and fixer from Kodak (Tokyo, Japan). Other chemicals and reagents were of analytical grade and purchased locally.

Cell culture

Human histiocytic lymphoma cell line U937[1] (purchased from NCCS, Pune, India) were maintained in RPMI-1640 supplemented with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin-B at 37°C in a humidified atmosphere containing 5% CO_2. Doubling time of U937 cells were around 24 h. Cells (3×10^5 cells/mL) were treated with either different concentration of DADS for 24 h or 50 μM DADS for different time periods (as mentioned in the figures). All cell culture assays were done within 30 to 40 passage numbers and were repeated in triplicate.

Peripheral blood mononuclear cells (PBMCs) from healthy human were used as control. PBMCs were isolated from healthy human blood using the method as described previously [34]. The cell morphology was visualized under microscope and was treated with 50 μM of DADS for 24 h. All experiments were performed in triplicate.

Telomeric repeat amplification protocol assay

U937 cells (4×10^6) were treated with different concentration of DADS for 24 h and with 50 μM DADS for different time. Cell extracts were prepared using telomeric repeat amplification protocol assay (TRAP)-buffer (10 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% NP40, 10% glycerol), protein content were estimated by Bradford Assay [35]. Telomerase activity was determined by TRAP assay using the method described previously [36]. Telomeric substrate (TS) was extended using cell extract (1 μg) in TRAP reaction mixture (20 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 68 mM KCl, 0.0005% Tween-20, 1 mM EGTA, 50 μM dNTP, 0.04 ng/μL TS and two units of Taq polymerase) in 25 μl at 25°C for 30 min followed by PCR using reverse primer (CX): 5′-CCCTTACCCTTACCCTTACCCTTA-3′. TSNT: 5′-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3′ and NT: 5′-ATCGCTTCTCGGCCTTTT-3′ were used as the internal control primers as described previously [37]. The samples were resolved on 10% nondenaturing polyacrylamide gel electrophoresis (PAGE) and stained with ethidium bromide (EtBr; 0.5 mg/mL). Telomeric products were visualized and quantified using gel documentation (Bio-Rad, Herculus, CA, USA) and Quantity-One software.

RNA extraction and RT-PCR

Total RNA from U937 cells was extracted using trizol (as manufacturer's instruction). Two μg of RNA were reverse transcribed to prepare cDNA. PCR was performed with specific primers using GeneAmp PCR system 9700 (Applied Biosystems, Waltham, MA, USA). For each pair of primers, the number of PCR cycles were standardized and kept well below the saturation level. PCR products were separated in 2% agarose gel, stained with EtBr (0.5 mg/mL) and quantified by gel documentation. RT-PCR with each primer set was performed in triplicate. Two negative controls were done for each set of sample, one without cDNA (i.e., with RNA) and other without both cDNA and RNA.

Western blot analysis

Total cell extracts were prepared from DADS-treated U937 cells (5×10^6) using cell lysis buffer (BD Pharmingen, San Diego, CA, USA). Proteins were estimated by Bradford assay. Proteins (80–100 μg) were separated by sodium dodecyl sulphate-PAGE, transferred on PVDF membrane, blocked overnight in tris-buffered saline containing 5% skim milk, incubated with primary antibodies

[1] U937(ATCC CRL1593.2).