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Comparative binding of antitumor drugs to DNA containing the telomere repeat sequence

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Abstract

Telomeres are the ends of the linear chromosomes of eukaryotes and consist of tandem GT-rich repeats in telomere sequence *i.e.* 500-3000 repeats of 5'-TTAGGG-3' in human somatic cells, which are shortened gradually with age. The G-rich overhang of telomere sequence can adopt different intramolecular fold-backs and tetra-stranded DNA structures, *in vitro*, which inhibit telomerase activity. In this report, DNA binding agents to telomere sequence were studied novel therapeutic possibility to destabilize telomeric DNA sequences. Oligonucleotides containing the guanine repeats in human telomere sequence were synthesized and used for screening potential antitumor drugs. Telomeric DNA sequence was characterized using spectral measurements and CD spectroscopy. CD spectrum indicated that the double-stranded telomeric DNA is in a right-handed conformation. Polyacrylamide gel electrophoresis was performed for binding behaviors of antitumor compounds with telomeric DNA sequence. Drugs interacted with DNA sequence caused changes in the electrophoretic mobility and band intensity of the gels. Depending on the binding mode of the anticancer drugs, telomeric DNA sequence was differently recognized and the efficiency of cleavage of DNA varies in the bleomycin-treated samples under different conditions. DNA cleavage occurred at about 1% by the increments of 1 μM bleomycin-Fe(III). These results imply that the stability of human telomere sequence is important in conjunction with the cancer treatment and aging process.

Keywords: antitumor drugs, bleomycin, DNA damage, gel mobility, telomere DNA

Introduction

The life span of an organism is attributed to the sum of deleterious changes and counteracting repair responding to the damage, which may be related to many diseases, such as cancers. Interestingly, telomeres have been known to maintain the integrity of the genome and to involve in cellular aging and cancer (Mergny and Helene, 1998). Cellular senescence may be predictable for the nuclear-transfer derived animal by analyzing the telomere lengths in a cloned sheep (Shiels *et al.,* 1999). The telomeric complex may tell natural chromosomal ends from random DNA breaks by capping the chromosomal terminus (Griffith *et al.,* 1999). Repairs of damage to both the bases and the backbone of DNA in living cells require several distinct repair pathways involving numerous enzymes. In repairing DNA breaks with damaged ends, removal of 3'-blocking groups is essentially an early step in the process. Many apurinic/apyrimidinic (AP) endonucleases are found to remove 3'-phosphoglycolates at internal breaks and some also retains activity toward 3'-blocks on double-strand break ends (Suh *et al.,* 1997).

Telomeres are the ends of the linear chromosomes of eukaryotes. In most eukaryotes, telomeres consist of tandem GT-rich repeats. Particularly, telomeres have 500-3000 repeats of 5'-TTAGGG, in human somatic cells, which are shortened gradually with age *in vivo* and *in vitro* (de Lange 1994). Telomere shortening, thus, could be a molecular clock that signals the replicative senescence. Shortening of telomeric ends of chromosomes causes limited replication of human cells in culture. This limited replicative capacity of a critical telomere length activates the senescence program by p53 pathway (Chin *et al.,* 1999). In contrast, the telomeres of germ line and cancer cells do not shorten: several human cancers exhibit

long telomeres maintained by telomerase, which can extend 3' telomere overhangs (Blackburn, 1992). It could be a limiting factor in the cancer therapy mainly because several cell divisions may be required even after treatment with telomerase inhibitors. Tumor cells, however, maintain their telomere length with no telomerase activity (Bryan *et al.,* 1995). Thus, other possible mechanisms may exist that would stabilize chromosome termini in human cancer. Ku protein was also suggested to recognize the junction between double-stranded DNA and G-rich overhang, possibly G-quartets (Bianchi and Lange, 1999). The G-rich overhang of telomere sequence can adopt different intramolecular fold-backs and tetra-stranded DNA structures, *in vitro*, which inhibit telomerase activity (Zahler *et al.,* 1991). Drugs that would bind telomeric DNA need to be investigated whether agents may stabilize a G-G paired form preventing the activity of telomerase (Blackburn, 1991) and the machinery of transcription (Lee *et al.,* 2002). Many DNA binding compounds can be characterized for making the telomeric DNA as a target itself. In this study, novel therapeutic possibility to destabilize telomeric DNA sequences is focused on. The results may contribute to limit the lifespan of cancer cells. Drugs investigated in this report cause the interesting changes in the electrophoretic mobility and band intensity of DNA containing telomere sequence.

Materials and Methods

Materials

Ferric ammonium sulfate, magnesium chloride, potassium chloride, boric acid, trizma-base, and ammonium peroxydisulfate were purchased from Sigma Chemical Company (St. Louis, MO, USA). Urea, polyacrylamide, and bis-acrylamide were obtained from Fisher Scientific Co. (Fair Lawn, NJ, USA). Anthracycline antibiotics, ethidium bromide, 4',6'-diamidino-2-phenylindole (DAPI), Hoechst 33258, and other chemical compounds were commercially available (Sigma Co.). Pirarubicin was a generous gift of S. Kim (Dong-A Pharmaceutical Co.). DNA ladder (10-bp) was obtained from Promega Co. (Madison, WI, USA). The stock solutions of drugs were prepared in doubly distilled water at a concentration of 1 mg/ml and stored at 4°C. Experiments were performed in BPE buffer containing 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, and 1 mM EDTA, pH 7.0.

DNA substrate

Deoxyoligonucleotides containing human telomere sequence were designed and purchased from the Genemed Synthesis, Inc. (San Francisco, CA). The sequence of each strand is as follows:
G-strand: 5'-TTAGGGTTAGGGTTAGGG-3'
C-strand: 3'-AATCCCAATCCCAATCCC-5'

The concentration of DNA was estimated by measuring the absorbance at 260 nm. Each strands of human telomere DNA sequence were annealed by incubating at 90°C for 5 min, and then slowly cooling down to 25°C. The extinction coefficient has been obtained by calculating that of each deoxymononucleoside monophosphate (Fasman, 1975), and by measuring the percent of hyperchromicity of oligonucleotides. Then, the obtained extinction coefficient was used for the determination of the concentration of DNA substrates.

Spectral measurement

Absorbance was measured at 25°C, with a Kontron UVIKON 923 spectrophotometer. The cell path length of spectrophotometer was 1 cm. Concentrations of antitumor drugs were determined spectrophotometrically by measuring the absorbance and by using the following molar extinction coefficients, ε ($M^{-1}cm^{-1}$). Circular dichroism spectrum was recorded on Jasco J700A CD spectropolarimeter. The cell path length of CD measurements was 1 cm. DNA samples were allowed to equilibrate overnight at room temperature, and CD spectra were measured at 25°C.

Polyacrylamide gel electrophoresis

Oligonucleotide substrates treated with drugs were electrophoresed on 20% polyacrylamide nondenaturing gels in a buffer composed of 90 mM tris-borate, 2.5 mM EDTA, pH 8.3. Electrophoresis was performed at 100 V for 4 h. Bands were visualized under UV light after staining with ethidium bromide. Electrophoretic bands were detected as the shifts of electrophoretic mobility depending on the associated drugs. Bands were quantified by transferring the digital images of the gel from a Vilber Lourmat Bioprofil video system to the Bio-1D program. Drug concentrations were selected over the stoichiometric binding ratio between drug and DNA molecules.

Bleomycin treatment

Bleomycin was dissolved in distilled water at a concentration of 2 mM. The concentration was determined by measuring the absorbance A_{295nm} (ε_{295nm}= 14,100) (Suh and Povirk, 1997). Bleomycin-Fe (III) was prepared by adding an equal amount of 2 mM ferric ammonium sulfate in 0.5 mM HCl and stored at -20°C. Reaction mixtures contained 50 mM Hepes-NaOH (pH 8), 18 μM_{bp} telomere DNA, 25-100 μM Fe (III)-bleomycin, and 10 mM hydrogen peroxide, while bleomycin-Fe (III) was added last. The mixture was

incubated at 0°C for 1 h. Then, DNA samples were subjected to 20% polyacrylamide gel. The gels (10×10.6×0.2 cm) were run for 4 h at 100 V and subjected to quantitative gel image analysis.

Results and Discussion

Characteristics of human telomeric DNA sequence

Absorbance, circular dichroism measurements can characterize the physico-chemical properties of telomere DNA. The maximum absorbance of G-strand was observed at 257 nm and that of C-strand of telomere DNA sequence was at 265 nm shown in Figure 1 (A). The gel mobility of each strand is different on the denaturing polyacrylamide gel. The 19% denaturing polyacrylamide gel was running at 127 V for 4.5 hours. The electrophoretic band of C-strand of telomere DNA sequence moves faster than that of G-strand. This result shows that the secondary structure of G-strand and C-strand may be different, even though they have the same length of DNA sequence. For the double-stranded 18-bp telomere DNA, the characteristic of UV absorption spectra was demonstrated in Figure 2 (A). The solid line indicates the ordinary UV spectrum of DNA at 25°C. As the temperature increases, the UV spectrum of melted DNA is shown at 90°C as the dotted line. There is a dramatic change of absorbance at 260-270 nm by the denaturation of DNA. The percentage of hyperchromicity at 260 nm was measured: % H=[OD_{90} −OD_{25}]/OD_{25}, where OD denotes the optical density. The hyperchromicity was found to be 29%. Thus, this measured value can be used for obtaining the correct extinction coefficient of telomeric DNA at 260 nm, 25°C and neutral pH for double-stranded DNA (Fasman, 1975). The concentration of the telomeric DNA was determined using the molar extinction coefficient (at 260 nm)=15,039 ($M_{bp}^{-1}cm^{-1}$)(The details of calculation is not shown here).

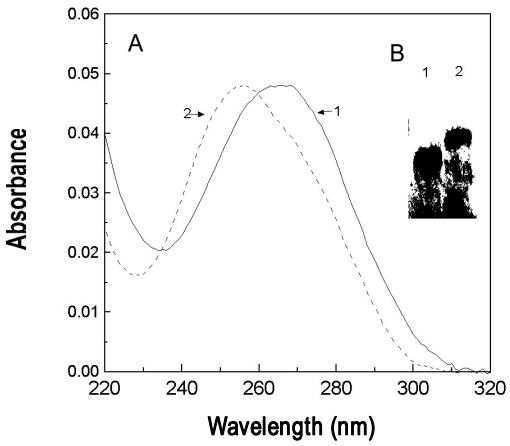

Figure 1. (A) UV spectrum of a single-stranded telomeric DNA sequence. The solid line: 5'-CCCTAA-3', the dotted line: 5'-TTAGGG-3'. (B) The electrophoretic properties of a single strand of human telomeric DNA on the denaturing 19% polyacrylamide gel: Lane 1 represents 5'-CCCTAA-3' and lane 2 is 5'-TTAGGG-3'.

The secondary structure of telomeric DNA sequence by CD measurement

DNA has been shown to adopt different conformations depending on its sequence and environment (Suh *et al.,* 1991; Suh, 2000). Telomeric DNA is composed of unusually repeating DNA sequence. Telomeres consist of 5'-TTAGGG-repeats in human. The secondary structure of the double-stranded telomeric DNA sequence can be monitored by circular dichroism (CD) measurement. The positive peak of CD spectrum appears at 265 nm and the negative peak appears at 240 nm, shown in Figure 2 (B). CD spectrum indicates that the double-stranded telomeric DNA is a B-form DNA in a right-handed conformation. The same result was observed by P^{31}-NMR measurement, even for 2-mM of DNA (in bp) in BPE at 4 M NaCl (data not shown).

Human telomere DNA sequence interacts with antitumor antibiotics

The chemical structures of anticancer drugs, which were used in this study, are shown in Figure 3. The

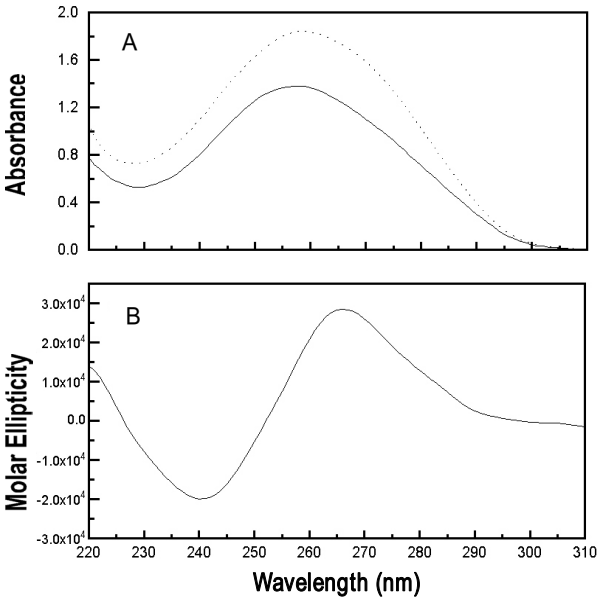

Figure 2. (A) UV spectrum of the telomeric DNA sequence at 25°C in represented as the solid line and at 90°C as the dotted line. (B) CD spectrum of 18-bp telomeric DNA at 25°C is shown.

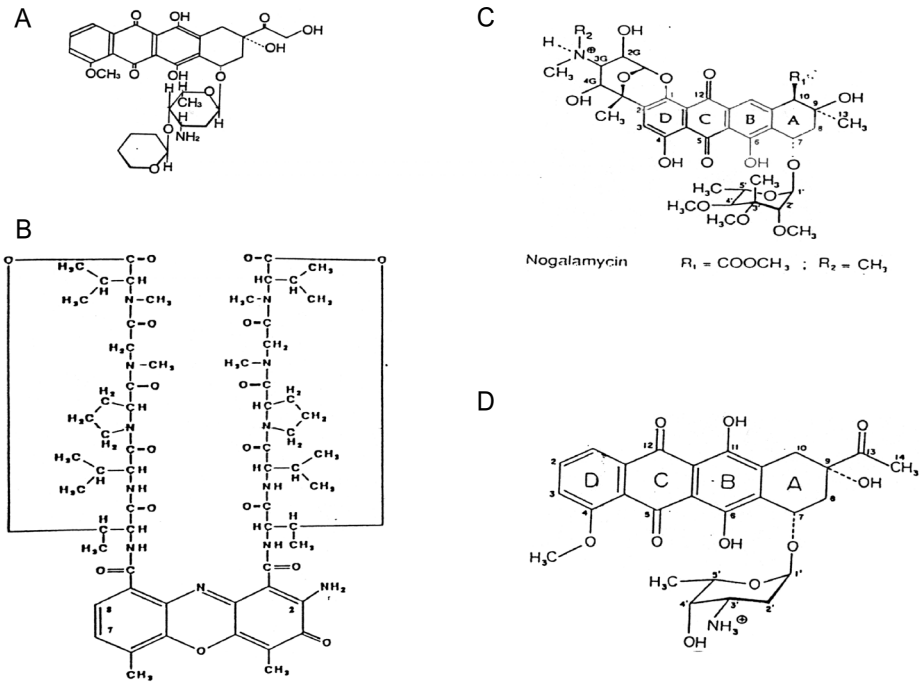

Figure 3. Molecular formula of chemical compounds used in this study: (A) pirarubicin; (B) actinomycin D; (C) nogalamycin; (D) daunomycin.

concentration of these compounds was determined by using the extinction coefficients (Bailly *et al.,* 1998; Suh *et al.,* 2001): ε_{440nm} of actinomycin D=24,400 and ε_{474nm} of nogalamycin =11,200; ε_{480nm} of daunomycin, and pirarubicin=11,500; and ε_{290nm} of bleomycin= 14,100; ε_{340nm} of DAPI=27,000; and ε_{338nm} of Hoechst 33258=42,000. The effects of various anthracycline antibiotics binding to the telomere sequence are shown in Figure 4. As the retarded mobility of the oligonucleotides containing telomere sequence with nogalamycin is observed. Nogalamycin may affect to slow down the migration of telomeric DNA sequence. In comparison, actinomycin D seems to have no effect on the mobility of telomere sequence at excess amount of it shown in Figure 4. There was not much changes of band intensity by increasing actinomycin D concentration, presumably because these two compounds intercalate into 5'-GT of telomere sequence by causing the helical distortion of DNA for the favorable association of ethidium (White *et al.,* 2000). Unlike the effects of nogalamycin and actinomycin D, the association of other compounds with telomere sequence seems to be different shown in Figure 4. By increasing the amount of other compounds, the band intensity is decreased. Though DAPI and Hoechst bound to DNA as groove binders, intercalating drugs such as daunomycin and pirarubicin showed similar characteristics. Because of the side groups of groove binders, their association with DNA slows

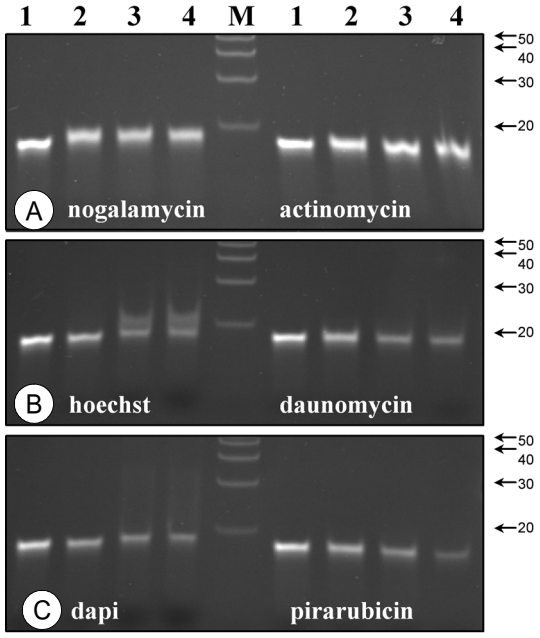

Figure 4. Telomeric DNA samples were run on the nondenaturing 20% polyacrylamide gel after the treatment of various drugs. Electrophoretic mobility of the 18 mer telomeric DNA is shown in the absence (lane 1) and presence (lane 2-4: by increasing the concentration of drugs from lane 2 to lane 4) of: (A) nogalamycin, and actinomycin; (B) hoechst 33258, and daunomycin; (C) DAPI and pirarubicin, respectively. Lane-M indicates the 10 bp DNA ladder.

down the electrophoretic mobility on the gel. Daunomycin and pirarubicin whose structures were shown in Figure 3 seems not to demonstrate the sequence specificity for telomere sequence. Thus, they don't seem to cause the unwinding or lengthening of DNA. Interestingly, nogalamycin is an anthracycline antibiotic but its binding to DNA is similar to the binding of actinomycin D rather than other anthracycline antibiotics. This difference of the electrophoretic characteristics of drug treatment implied to cause the conformational changes of telomeric DNA by noncovalent association. The characteristics of DNA binding drugs was recently reported (Suh *et al.,* 2002).

Bleomycin cleavage of telomere DNA

As the structure of bleomycin is demonstrated before, bithiazole is known to fit into a G-py sequence to induce the double-strand breaks of DNA molecules (Suh and Povirk 1997). The efficiency of cleavage was estimated by the quantification of free and cleaved DNA in bleomycin-treated samples. The cleaved telomere DNA fragments are produced by cleaving a repeating unit, 5'-TTAGGG-3' of both ends of each strand, since bleomycin was known to cleave the potential binding site 5'-GT. The efficiency of cleavage was calculated and demonstrated as the disappearance of the band intensity of initial bands on the gel shown in Figure 5. By increasing the addition of total bleomycin, more cleavage has been occurred. Under this condition of DNA cleavage by bleomycin, about 1% cleavage of initial band had occurred by 1 μM treatment of bleomycin-Fe (III). At the ratio of 5:1 bleomycin-telomere DNA, almost 80% of the initial band intensity is observed to disappear. The further cleaved DNA fragments, however, could not be detected on the gel.

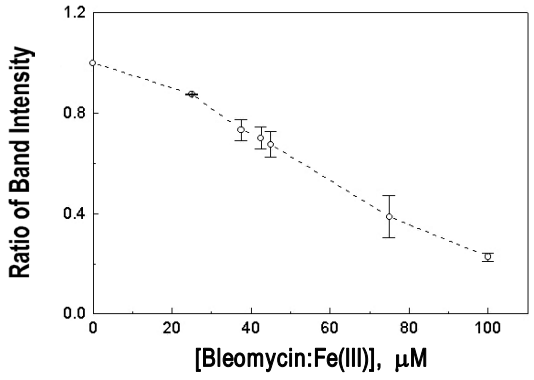

Figure 5. Ratio of the initial band intensity is plotted as a function of the concentration of added bleomycin-Fe (III). Results are the mean of two experiments (mean value ± standard error).

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