Telomerase inhibiting activity *in vitro* from natural resources, marine algae extracts

Kazunori Kanegawa¹, Hideki Harada¹, Hisashi Myouga¹, Yoshinori Katakura², Sanetaka Shirahata² & Yuto Kamei^{1*}

¹ Marine and Highland Bioscience Center, Saga University, 152-1 Shonan-cho, Karatsu, Saga 847-0021, Japan;

² Graduate School of Genetic Resources Technology, Kyushu University 6-10-1, Hakozaki, Higashi-ku, Fukuoka 812, Japan

(* Author for correspondence; E-mail: kameiy@cc.saga-u.ac.jp)

Received 3 February 1999; accepted 30 March 2000

Key words: marine algae extract, telomerase inhibiting activity, telomere, TRAP

Abstract

We attempted to screen for telomerase inhibiting activity *in vitro* from a total of 304 marine algae samples which were collected from various Japan coasts by nonradioisotope telomeric repeat amplification protocol (Non-RT-TRAP) assay using human leukemia MOLT-4 cells. Ten of the MeOH extracts and two of the PBS extracts from those algal samples showed telomerase inhibiting activity. In particular, the MeOH extract from a green alga, *Caulerpa sertularioides* strongly inhibited telomerase activity when added to MOLT-4 cell culture at a level of 1.25% (v/v), suggesting that it may be possible to develop a novel anti-cancer agent in view of its specific anti-telomeric property.

Introduction

Telomeres, the ends of eukaryotic chromosomes, have a unique structure composed of tandem repeats of the sequence TTAGGG in humans and play several important roles essential for chromosome stability and replication. These roles include protection of chromosomes from degradation, end-to-end fusion, and recombination (Blackburn, 1991; Rhyu, 1995). However, in normal somatic cells the ends of chromosomes are replicated incompletely, what is known as the end-replication problem, thus telomere length shortens progressively with successive cell divisions (Harley et al., 1990; Levy et al., 1992; De Lange, 1994; Ohyashiki et al., 1997). When telomere length shortens to a certain critical level such that the telomeres can no longer stabilize chromosome ends, the cells exit from the cell cycle and finally die (Allcopp and Harley, 1995; Ogoshi et al., 1997). Thus, telomere shortening appears to act as a mitotic clock of the cell and finally leads to cell senescence (Harley, 1991; Harley et al., 1995). In cancer cells, on

the other hand, expression of telomerase results in acquisition of infinite proliferation ability. Telomerase is a fascinating enzyme containing an integral RNA with a short template element that directs the *de novo* synthesis of telomeric repeats at chromosome ends (Greider and Blackburn, 1989; Yu et al., 1990). Therefore, telomere length is constantly maintained at a size that is sufficient to stabilize chromosome ends. As a result, cells are immortalized. Though telomerase activity is not specific to cancer cells, but is also found in normal cells such as germ cells which need telomerase (Broccoli et al., 1995; Yasumoto et al., 1996), telomerase can be the target of novel anti-cancer agents. If we inactivate telomerase activity, cancer cells may no longer be able to maintain telomeres and eventually die. Moreover, if telomerase activity can be inhibited by some natural products, the effect of the anti-cancer agents will be more epoch-making, leading to a novel anti-cancer agent with little or no side-effect. Therefore, we focused on marine algae, which had been reported to contain various bio-

Figure 1. Sampling map of marine algae. A total of 304 marine algae were sampled from 57 points in Japan.

logical activities (Mahasneh et al., 1995; Wright et al., 1996).

In Japan, there are over 1500 species of marine algae. Some are reported to have antitumor activity (Kashiwagi et al., 1980; Itoh et al., 1993; Harada et

al., 1997), antibacterial activity (Sims et al., 1975; de Lara-Isassi, 1995), and antiviral activity (Ivanova et al., 1994), among others. At present, there are few reports on telomerase inhibiting activity in natural products including marine algae (Naasani et al., 1998).

Figure 2. Effect of PCR cycles on DNA amplification attributed to telomerase activity. Telomerase-containing cell lysate from untreated MOLT-4 cells was used for TRAP.

Thus we attempted to screen for telomerase inhibiting activity in marine algae from the Japan coasts using the PCR-based TRAP (Telomeric Repeat Amplification Protocol) assay (Piatyszek et al., 1995; Wright et al., 1995).

Materials and methods

Sample collection

A total of 304 species of marine algae were collected by skin-diving from 57 points along the Japan coastline from April 1994 to April 1997 (Figure 1). Collected algae were taken to the laboratory and classified, and then preserved in a deep freezer at -85°C until use.

Preparation for algae extracts

Sample algae were washed three times with sterilized artificial seawater (ASW, Jamarin Laboratory)

and once with phosphate-buffered saline (PBS). Five g of each sample (wet weight) was mixed with 20 ml of PBS and homogenized at 8000 rpm for 5 min by a Polytron (Kinematica Inc.). After centrifugation at $1200 \times g$ for 20 min, supernatants were collected, filtered with a $0.2 \mu\text{m}$ -pore-sized filter (Millipore, filter type GV) to obtain the water-soluble extract, and the remaining pellets were further mixed with 20 ml of methanol, homogenized and centrifuged as described above. Supernatants were filtered through a $0.2 \mu\text{m}$ -pore-sized filter (Millipore, filter type GV) to obtain the lipid-soluble extracts.

Cells and culture conditions

T-cell acute lymphoblastic leukemia MOLT-4, chronic myelogenous leukemia K562, HeLa and KB cell were used as the experimental tumor cell. These cell lines were kindly provided by the Japanese Cancer Research Resources Bank (JCRB). Cells were cultured in E-RDF medium (Kyokuto) supplemented with 10%

fetal bovine serum (FBS) for 4 days at 37 °C in a 5% CO₂-incubator.

Cytotoxicity test

MOLT-4 cells were seeded into three 35 mm tissue-culture dishes (1×10^5 cells/2 ml medium), to which were added 12.5, 25 or 50 μ l of the algae extracts. The same volume of MeOH or PBS was added as a control. The cytotoxicity was directly monitored by microscopic observation after 4 days incubation.

TRAP assay

Telomerase was extracted from 1×10^6 cells in each dish that the cytotoxicity was not observed. These cells were suspended in ice-cold PBS and washed. After being washed, the pellets were homogenized in 200 μ l of ice-cold lysis buffer (10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM EGTA, 0.5% 3-[(3-cholamidopropyl)dimethylamino]-1-Propanesulfonate (CHAPS), 5 mM β -mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 10% glycerol). After incubation for 30 min on ice, the lysate was centrifuged at $16\,000 \times g$ for 30 min at 4 °C, and the supernatant was frozen and stored at -85 °C. One μ l of each extract was used for the TRAP assay. Each extract was added to 25 μ l of reaction mixture containing 1 $\times$ PCR buffer (20 mM Tris-HCl (pH 8.0), 1.5 mM MgCl₂, 68 mM KCl, 0.005% Tween 20, 1 mM EGTA), 50 μ M dNTPs, 344 nM TS primer (5'-AATCCGTCGAGCAGAGTT-3'), 0.5 μ M of T4 gene 32 protein (Boeringer Mannheim), and 2 units of Taq DNA polymerase (Boeringer Mannheim). After being incubated for 30 min at room temperature, the reaction mixture was heated at 90 °C for 3 min and then CX primer (5'-CCCTTACCCTTACCCTTACCCTTAA-3') was added to the mixture. PCR was carried out for 30 cycles at 94 °C for 45 s, at 50 °C for 45 s, and at 72 °C for 1 min. The PCR products were electrophoresed on a 12% polyacrylamide gel and stained with SYBR Green I Nucleic Acid Gel Stain (Molecular Probes, Inc.).

Evaluation of telomerase inhibiting activity

After electrophoresis and staining of the gel, ladder bands were detected and photographed by video graphic printer (Fas II, Toyobo Biochemicals). Ladder bands were analyzed by NIH Image software (free

software), and telomerase inhibiting activity was evaluated by comparing the light level of the detected ladders with the negative control. The criterion for telomerase inhibiting activity was according to the following formula: sample ladder/negative control ladder <0.3.

Results and discussion

When using the PCR-based TRAP assay, it is reported that PCR products do not always reflect the amount of templates at the first stage (Ehrlich et al., 1997; Nyren et al., 1997; Schnell and Mendoza, 1997). Therefore, we examined the numbers of PCR cycles ranging from 22 to 38 for a quantitative estimation of the levels of telomerase. Telomerase extracted from untreated MOLT-4 cells was prepared and used for the TRAP assay. Since each PCR product from 22 to 32 PCR cycles increased constantly and then reached a maximum at over 34 PCR cycles, we set 30 cycles as the appropriate number of PCR cycles for quantification in our assay (Figure 2).

Then we screened telomerase inhibiting activity from the marine algae extracts (Harada and Kamei, 1997), using MOLT-4 cells. From this screening, we noted that the strength of telomerase activity in MOLT-4 cells was a very important factor, because low levels of telomerase activity might cause a false negative, and an uncertain detection of telomerase activity made our study inefficient. Therefore, we investigated for such activity when the cells showed the highest level of telomerase activity during incubation (Figure 3). After 1×10^5 of untreated cells were seeded into a 35 mm dish with 2 ml medium, the numbers of cells gradually increased until day 7. Thus we extracted the telomerase from the cells after incubation for 4 days because the cells showed the highest-level of telomerase activity. Though it is not clear why telomerase activity decreased remarkably at day-5, it might be caused by some cytokine signals of MOLT-4 cells that have terminated log phase-growth.

Though a few MeOH- and PBS-extracts of marine algae showed cytotoxicity, the telomerase inhibiting activity was found in some seaweeds. The MeOH-extracts from 3 green algae, 6 red algae, and 1 species of brown algae and the PBS-extracts from 1 red alga and 1 species of an other seaweed exhibited telomerase inhibiting activity (Table 1). In particular, the MeOH extract from *Caulerpa sertularioides*, which inhabits Ishigaki Island in Okinawa Prefecture, showed

Figure 3. Telomerase activity in culturing MOLT-4 cells. Cells were seeded into 35 mm tissue-culture dishes with 2 ml medium. NIH image software was used for measuring telomerase activity. Telomerase activity was expressed by the relative ratio to the strongest one.

Table 1. Marine algae extracts with telomerase inhibiting activity. MeOH extract from a green alga, *Caulerpa sertularioides* showed the highest telomerase inhibiting activity

Algae type	Scientific name	Inhibiting value
Green	<i>Caulerpa sertularioides</i>	0.29
	<i>Halicoryne wrightii</i>	0.41
	<i>Cladophoropsis zollingeri</i>	0.40
Red	<i>Laurencia papillosa</i>	0.35
	<i>Laurencia yamadae</i>	0.36
	<i>Ahnfeltia paradox</i>	0.45
	<i>Galaxaura robusta</i>	0.40
	<i>Galaxaura falcata</i>	0.38
	<i>Meristotheca coacta</i>	0.37
	<i>Acrosorium flabellatum</i> ^a	0.44
Brown	<i>Isige sinicola</i>	0.33
Others (Seaweed)	<i>Zostera marina</i> ^a	0.40

^a PBS extract.

the strongest telomerase inhibiting activity (Figure 4). When we assayed for telomerase inhibiting activity of the MeOH extract from *C. sertularioides* by adding 25 μ l of the MeOH extract to 2 ml of medium, the ladder bands were faintly detected. Though the mechanism is not clear, it may act on telomerase expression or inactivation after active compounds from *C. sertularioides* penetrated and influenced MOLT-4 during culturing for 4 days. To examine whether this telomerase inhibiting activity of *C. sertularioides* is specific to MOLT-4 or not, K562, HeLa and KB cells were also assayed. However little or no telomerase inhibiting activity was found in these cell lines (data not shown). It appears that their individual levels of telomerase activity may be stronger than that of MOLT-4 cell, or the concentration of the alga extract might not be sufficient to inhibit telomerase activity in K562, HeLa and KB cells. These possibilities should be investigated more in detail. Whereas the MeOH extract of *C. Sertularioides* showed telomerase inhibiting activity, some other algal MeOH extracts, for example, *Corallina pilulifera* showed rather telomerase promoting activity. When 50 μ l of the MeOH extract of *C. pilulifera* was added to the culture, telomerase activity

Figure 4. Telomerase inhibiting activities of the MeOH extracts from marine algae.

was 1.42 times higher than negative control. Though 25 µl of MeOH only was represented as negative control in Figure 4, neither 12.5 nor 50 µl of MeOH showed any differences on telomerase activity.

In conclusion, our findings indicated that the MeOH extract from marine green alga, *C. sertularioides* might contain the biologically active compounds which inhibit telomerase activity, may have a possibility to be applied to a novel anti-cancer agent. We believe that these are the first findings of telomerase inhibiting activity from marine algae.

References

- Allcopp RC and Harley CB (1995) Evidence for a critical telomere length in senescent human fibroblasts. *Exp Cell Res* 219: 130.
- Blackburn EH (1991) Structure and function of telomeres. *Nature* 350: 569–573.
- Broccoli D, Young JW and de Lange T (1995) Telomerase activity in normal and malignant hepatopoietic cells. *Proc Natl Acad Sci USA* 92: 9082–9086.
- De Lange T (1994) Activation of telomerase in a human tumor. *Proc Natl Acad Sci USA* 91: 2882–2885.
- De Lara-Isassi G (1995) Screening for toxic activity of some marine benthic algae. *Food Addit Contam* 12: 485–490.
- Ehrlich R, Ellinger T and McCaskill JS (1997) Cooperative amplification of templates by cross-hybridization (CATCH). *Eur J Biochem* 15: 358–364.
- Greider CW and Blackburn EH (1989) A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. *Nature* 337: 331–337.
- Harada H and Kamei Y (1997) Selective cytotoxicity of marine algae extracts to several human leukemic cell lines. *Cytotechnology* 25: 213–219.
- Harada H, Noro T and Kamei Y (1997) Selective antitumor activity *in vitro* from marine algae from Japan coasts. *Biol Pharm Bull* 20: 541–546.

- Harley CB (1991) Telomere loss: Mitotic clock or genetic time bomb? *Mutat Res* 256: 271–282.
- Harley CB, Futcher AB and Greider CW (1990) Telomeres shorten during aging of human fibroblasts. *Nature* 345: 458–460.
- Harley CB, Vaziri H, Counter CM and Allsopp RC (1995) The telomere hypothesis of cellular aging. *Exp Cell Res* 220: 194–200.
- Itoh H, Noda H, Amano H, Zhuang C, Mizuno T and Ito H (1993) Antitumor activity and immunological properties of marine algal polysaccharides, especially fucoidan, prepared from *Sargassum thunbergii* of Phaeophyceae. *Anticancer Res* 13: 2045–2052.
- Ivanova V, Rouseva R, Kolarova M, Serkedjieva J, Rachev R and Manolova N (1994) Isolation of a polysaccharide with antiviral effect from *Ulva lactuca*. *Prep Biochem* 24: 83–97.
- Kashiwagi M, Mynderse JS, Moore RE and Norton TR (1980) Antineoplastic evaluation of Pacific basin marine algae. *J Pharm Sci* 69: 735–738.
- Levy MZ, Allsopp RC, Futcher AB, Greider CW and Harley CB (1992) Telomere end-replication problem and cell aging. *J Mol Biol* 225: 951–960.
- Mahasneh I, Jamal M, Kashashneh M and Zibdeh M (1995) Antibiotic activity of marine algae against multi-antibiotic resistant bacteria. *Microbios* 83: 23–26.
- Naasani I, Seimiya H and Tsuruo T (1998) Telomerase inhibition, telomere shortening, and senescence of cancer cells by tea catechins. *Biochem Biophys Res Commun* 249: 391–396.
- Nyren P, Karamohamed S and Ronaghi M (1997) Detection of single-base changes using a bioluminometric primer extension assay. *Anal Biochem* 15: 367–373.
- Ogoshi M, Takashima A and Taylor RS (1997) Mechanisms regulating telomerase activity in murine T cells. *J Immunol* 158: 622–628.
- Ohyashiki K, Ohyashiki JH, Iwama H, Hayashi S, Shay JW and Toyama K (1997) Telomerase activity and cytogenetic changes in chronic myeloid leukemia with disease progression. *Leukemia* 11: 190–194.
- Piatyszek MA, Kim NW, Weinrich SL, Hiyama K, Hiyama E, Wright WE and Shay JW (1995) Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). *Methods Cell Sci* 17: 1–5.
- Rhyu MS (1995) Telomere, telomerase and immortality. *J Natl Cancer Inst* 87: 884–894.
- Schnell S and Mecdoza C (1997) Enzymological considerations for a theoretical description of the quantitative competitive polymerase chain reaction (QC-PCR). *J Theor Biol* 21: 144–440.
- Sims JJ, Donnell MS, Leary JV and Lacy GH (1975) Antimicrobial agents from marine algae. *Antimicrob Agents Chemother* 7: 320–321.
- Wright AD, Konig GM, Angerhofer CK, Greenidge P, Linden A and Desqueyroux-Faundez (1996) Antimalarial activity: the search for marine-derived natural products with selective antimalarial activity. *J Nat Prod* 59: 710–716.
- Wright WE, Shay JW and Piatyszek MA (1995) Modifications of a telomeric repeat amplification protocol (TRAP) results in increased reliability, linearity and sensitivity. *Nucleic Acids Res* 23: 3794–3795.
- Yasumoto S, Kunitura C, Kikuchi K, Tahara H, Ohji H, Yamamoto H, Ide T and Utakoji T (1996) Telomerase activity in normal human epithelial cells. *Oncogene* 13: 433–439.
- Yu GL, Bradley JD and Blackburn EH (1990) *In vivo* alteration of telomerase sequences and senescence caused by mutated *Tetrahymena* telomerase RNAs. *Nature* 344: 126–132.