The effect of 2-deoxy-d-glucose on Werner syndrome RecQ helicase gene

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1. Introduction

Some of the protein factors that affect cellular senescence are associated with genome stability, especially telomere maintenance [1]. Telomeres are composed of short G-rich DNA repeat complexes with various proteins [2]. Telomerase, which extends the G-rich strand of telomeric DNA, is a ribonucleoprotein complex composed of telomerase reverse transcriptase (TERT) and telomerase RNA (TR) [3,4]. Shortened telomeres are known to limit proliferative life span, but the telomerase supplies enough genetic stability for cells to proliferate persistently.

DNA helicases play important roles in DNA replication, recombination, repair, and transcription, by unwinding the duplex DNA [5,6]. Among them, RecQ DNA helicases play important roles in maintenance of genome stability [7–9]. Several human diseases associated with defects in RecQ helicase genes are known and include Bloom's syndrome (BS), Werner's syndrome (WS) and Rothmund–Thomson syndrome (RTS). Each of these can lead to cancer or premature aging [10–12]. These premature aging syndromes are associated with shortened telomeres [13]. Importantly, Werner's syndrome RecQ (WRN) helicase regulate telomeres by affecting replication of G-rich telomeric DNA [10].

The compound 2-deoxy-d-glucose (2DG) is a potent inhibitor of glucose metabolism thought to mimic glucose deprivation in vivo that mimetic caloric restriction (CR) [14]. It is well known that the CR extends life spans of various animals [14]. In this study, tests are conducted to determine the effect of 2DG on telomere maintenance factors, telomerase and WRN helicase to elucidate whether 2DG is suitable for further research and development as a treatment to protect telomeres.
2.2. Cell viability assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay]

MTT assay was performed as described elsewhere [15]. In brief, cells were cultured in a well of microtiter plate. Ten micro litter of MTT/PBS solution (Wako Chemicals, Tokyo, Japan) was added to each well (containing 100 μl of cell culture) and incubated 4 h in a 37 °C, 5% CO2 humidified incubator. Then, 100 μl of 0.04 N HCl in 2-propanol was added, and the dark blue crystals were dissolved completely. The absorbance of 570 nm was measured by a microtiter plate reader (Thermo electron Corp., Vantaa, Finland) and normalized by the absorbance of 630 nm.

2.3. Construction of luciferase (Luc)-reporter plasmids

Luc reporter plasmids carrying the human WRN, BLM, HELB, TERT, and p21 promoter regions were constructed as previously described [16]. The 5'-flanking regions of these genes were obtained by PCR with PrimeStar Taq polymerase (Takara, Kyoto, Japan) and designated pGL4-BLM, pGL4-HELB, pGL4-WRN, pGL4-TERT, and pGL4-p21. The nucleotide sequences of the PCR products were determined by the Applied Biosystems, Foster City, CA) with Rv (5'-TAGCAAAATAGGCTGTCCCC-3') and GL (5'-CTTTATGTTTTGCCGTCTTC-3') primers.

2.4. Transient transfection and Luc assays

Plasmid DNAs were transfected into HeLa S3 cells by the DEAE-dextran method [16]. After 24 h of transfection, 2DG was added to the culture medium. After a further 24 h of incubation, cells were collected and lysed with 100 μl of 1× Cell culture lysis reagent, mixed, and centrifuged at 12000×g for 5 s. The supernatant was stored at −80 °C. Luciferase activity was measured with the Luc assay system and with the Dual-Luc assay system (Promega, Madison, WI), as described previously [16].

2.5. Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was carried out as described previously [17]. First-strand cDNAs were synthesized with Rever Tra Ace (Toyobo, Tokyo, Japan), random primers (Takara), and total RNAs extracted from HeLa S3 cells. Primer pairs to amplify human BLM, HELB, WRN, Sp1, and β-actin cDNAs are listed in Table 2. The conditions for PCR were as follows: 94°C 30 s, 55°C 30 s, and 72 °C 1 min, with 27 (BLM), 26 (WRN and HELB), 22 (Sp1), and 20 (β-actin) cycles.

2.6. Quantitative real-time PCR

Real time PCR analysis was carried out using the Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA) as described previously [18, 19]. For PCR amplification, cDNAs were amplified using SYBR Green Realtime PCR Master Mix (Toyobo) and 0.4 μM of each primer pair. Amplification was carried out starting with an initial step for 30 s at 94 °C, followed by 40 cycles (94°C 30 s, 55°C 30 s, and 72°C 1 min). Quantitative PCR analysis for each sample was carried out in triplicates. Relative gene expression values were obtained by normalizing Ct (threshold cycle) values of the target genes in comparison with Ct values of the BLM gene using the ΔΔCt method.

2.7. Western blot analysis

Western blot analysis was carried out as described previously [16] with antibodies against WRN (Santa Cruz Biotechnology, Santa Cruz, CA), and BLM (Calbiochem, Darmstadt, Germany) followed by the addition of horseradish peroxidase (HRP)-conjugated secondary antibody (Calbiochem). Signal intensities were quantified with Image Ganguge Software (Fuji Film, Tokyo, Japan).

2.8. Telomerase amplification protocol (TRAP) assay

Cell pellets (3 × 10⁶) were treated with 200 μl of ice-cold 1× CHAPS lysis buffer (10 mM Tris–HCl [pH 7.5], 1 mM MgCl₂, 1 mM EGTA, 0.1 mM benzamidine, 1 mM β-mercaptoethanol, 0.5% CHAPS, and 10% glycerol), incubated on ice for 30 min, and centrifuged at 12000×g for 20 min at 4 °C. The supernatant was then stored at −80 °C. TRAP assays were performed with a TRAP assay kit (Chemicon, Temecula, CA) as described previously [20]. Signal

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Table 2

<table>
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<tr>
<th>Promoter</th>
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<td>ShBLM-88534</td>
<td>5'-GATACTGTAGATTCAGTCCTGAGTTC-3'</td>
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<tr>
<td></td>
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<td>AhBLM-89145</td>
<td>5'-GATACTGTAGATTCAGTCCTGAGTTC-3'</td>
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<tr>
<td>HELB</td>
<td>NW925393.1</td>
<td>ShHDHB-69026</td>
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<td></td>
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</tr>
<tr>
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intensities of the DNA ladders and internal controls were quantified with Image Gangue Software (Fuji Film).

3. Results

3.1. Proliferation of HeLa S3 cells after 2DG-treatment

Because 2DG is known as a potent inhibitor of glucose metabolism, we examined its effect on cell proliferation of HeLa S3 cells. The viability of HeLa S3 cells after 2DG treatment was thus examined using an MTT assay. As shown in Fig. 1, proliferation was suppressed by 2DG in a dose-dependent manner. In this experimental condition, apoptotic cells were not observed after 24 h treatment with 8 mM 2DG addition, but the growth of the cells was greatly attenuated.

3.2. WRN gene expression is augmented by 2DG

The WS phenotype shows the symptoms of premature aging with genomic instability, an elevated risk of malignancy and accelerated cellular senescence [21]. It has been shown that RecQ helicase, WRN is required for efficient lagging-strand replication of G-rich telomeric DNA [10]. To investigate WRN and other gene expressions after 2DG treatment, RT-PCR analysis was performed (Fig. 2A). Quantitative real time PCR indicated that the expression of the WRN gene increased gradually during treatment with 8 mM 2DG in a time dependent manner (Fig. 2B). On the other hand, Bloom’s syndrome RecQ helicase (BLM) gene expression was not changed (Fig. 2A). The human DNA helicase B, HELB (HDHB) is a RecD like helicase and has been suggested to be critical for the G1/S transition of the cell cycle and to participate in DNA damage response [22]. Similar to the WRN, HELB gene expression was shown to increase at 8–24 h after 2DG addition (Fig. 2B). Because WRN gene expression positively responded to 2DG, further examination was undertaken to determine whether WRN transcripts are accumulated by increasing the 2DG concentration in the culture medium. As shown in Fig. 2C, the WRN gene expression was up-regulated by 2DG when the cells were treated with 6–10 mM of 2DG.

3.3. Effect of 2DG on the WRN promoter

In order to examine if promoter activities of human WRN, BLM, and HELB genes are affected by the 2DG treatment, 5-flanking regions of these genes isolated by PCR were subcloned into a
pGL4-WRN, pGL4-HELB, pGL4-TERT, and pGL4-p21 transfected cells were compared with the Luc activity of the pGL4-BLM transfected cells (Fig. 3B). Interestingly, the WRN/BLM promoter activity ratio increased from 3 to 12 with the 2DG treatment. Similarly, HELB/BLM, TERT/BLM, and p21/BLM promoter activity ratios increased from 1 to 3, from 3 to 6, and from 0.5 to 1, respectively. As shown in Fig. 3C, the dose-dependent profile of the WRN/BLM promoter activity ratio is similar to that of the relative WRN gene expression (Fig. 2C).

3.4. Expression of WRN protein after 2DG treatment

The data above indicated that WRN gene expression and its promoter activity increased in response to treatment with 2DG. As a consequence, an examination of WRN protein expression by Western blotting with samples from cells treated with 8 mM of 2DG was undertaken. As shown in Fig. 4A and B, WRN/BLM expression ratio was augmented after 8–24 h 2DG treatment.

3.5. Telomerase is activated by 2DG treatment

In order to examine the effect of 2DG on telomerase activity, a TRAP assay was performed with the protein sample from HeLa S3 cells treated with various concentrations of 2DG. As shown in Fig. 4C and D, telomerase activity responded to 2DG dose-dependently. The highest telomerase activity was observed when cells were treated with 10 mM of 2DG (Fig. 4C, lane 6).

4. Discussion

Dysfunctions of the RecQ DNA helicase family are known to be responsible for tumorigenesis and premature aging [23]. The human RecQ DNA helicase gene family has five members, RecQ1, BLM, WRN, RecQ4, and RecQ5. Mutations in the WRN gene give rise to WS, which is associated with a relatively early age with many of the features of the normal aging process [24]. The BLM gene is mutated in BS, a rare disorder associated with pleiotropic phenotypes including immunodeficiency and impaired fertility [25]. In addition, WRN and BLM helicases are suggested to regulate mammalian telomeres [9]. In this study, the effects of 2DG on the gene/protein expressions of RecQ helicases and telomerase were investigated. Interestingly, telomerase was most notably activated by treatment with 10 mM of 2DG in accordance with the activation of the WRN promoter and its gene expression. Given that WRN helicase functions to resolve G-rich cap structures of telomeres during DNA replication [10], cooperative induction of the WRN promoter and its protein expression might be beneficial for cells to maintain telomeres properly.

2DG is a sugar analogue with a limited metabolism that reduces the glucose/energy flux without reducing food intake and is thought to elicit the same beneficial effects as CR [14]. Thus, 2DG is thought to mimic glucose deprivation in vivo or CR to reduce the amount of oxidative by products metabolizing fewer calories [26]. CR is the only intervention conclusively and reproducibly shown to slow aging and maintain health and vitality in mammals [14]. Furthermore, it was shown that CR reduces the cell proliferation rate in various rodent tissues [27]. Therefore, glucose deprivation by 2DG could reduce proliferation of cells.

The WRN promoter responded to 2DG treatment (Fig. 3), and the WRN gene/protein expressions were augmented by the 2DG treatment (Figs. 2 and 4). This might have come from GC-box/Sp1 elements in the WRN promoter (Fig. 3A). The magnitude of the response to 2DG increases according with the numbers of the GC-box/Sp1 elements in the 5′-upstream regions of these genes. Moreover, Sp1 gene expression was induced after 2DG-treatment (Fig. 2B), suggesting that WRN, HELB, TERT, and p21 promoters were
Fig. 4. 2DG treatment induces WRN protein expression and telomerase activity in HeLa S3 cells. (A) HeLa S3 cells were treated with 2DG (8 mM) for 0 (lanes 1 and 2), 4 (lanes 3–5), 8 (lanes 6–8), and 24 h (lanes 9–11). Proteins extracted from 2DG-treated cells (7.5×10⁶) were separated by a 7.5% SDS–polyacrylamide gel electrophoresis, and Western blotting was performed with anti-WRN and anti-BLM antibodies (upper and lower panels, respectively). (B) Each band was quantified and results show relative WRN/BLM protein expression ratio compared with that of the 2DG-non-treated cells. Significance of differences between control (0 h) and 2DG treated cells were analyzed by Student’s t-test (*P<0.005). (C) Effect of 2DG on telomerase activity in HeLa S3 cells. CHAPS lysis buffer extracts (20 ng) from HeLa S3 cells were analyzed by TRAP assay. HeLa S3 cells were treated with the indicated concentrations of 2DG (0–10 mM) for 24 h (lanes 1–14). Lanes 8–14 represent backgrounds with samples that were incubated at 85 °C for 10 min. 1× CHAPS (lane 15) and TSR8 (lane 16) represent negative and positive controls, respectively. (D) Signal intensities of TRAP-products and internal controls were quantified and telomerase activities were calculated as described in the manufacturer’s protocol. Histograms show relative telomerase activities compared with 2DG-non-treated cells. Results show the mean ± S.D. of three independent assays. Significance of differences between control (0 h) and 2DG treated cells were analyzed by Student’s t-test (*P<0.05).
activated by Sp1 expression. It has been reported that 2DG induces c-fos and dopamine-β-hydroxylase (DBH) genes in specific neurons in the brain of rats [28,29]. Therefore, it is possible that transcriptional conditions in the cells are altered by the 2DG treatment. This suggestion, however, requires further investigation to be confirmed.

As a CR mimetic drug, 2DG has a very narrow therapeutic range bordering on toxicity and thus eliminating it as a drug candidate for human beings [14]. If 2DG or its derivatives are further improved to have lower levels of toxicity, it will contribute to protect chromosomes from damages by keeping cells with longer telomeres by inducing telomere maintenance factors.

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References
