such as mental stress, obesity, smoking, type 2 diabetes mellitus, ischemic heart diseases, Alzheimer's disease, sarcoidosis and Parkinson's disease [5–9]. These conditions may affect not only the telomeric structure itself but also surrounding genomic structures, including the epigenetic status, such as DNA methylation. Genomic DNA methylation is associated with conformations to allow (euchromatin) or suppress (heterochromatin) the expression of genes [10]. A recent report showed that the shortened telomere regions of embryonic fibroblasts tend to accompany subtelomeric hypomethylation in mice of the fifth generation of the telomerase activity-deficient *tert*$^{-}$/*tert*$^{-}$ mutant mouse [11]. In humans, subtelomeric DNA is hypomethylated in sperm and ova, and these regions are subjected to renewed methylation during development [12, 13]. In humans, this activity is carried out by DNMT3B (DNA methyltransferase 3B). Mutations in DNMT3B result in the autosomal-recessive ICF syndrome (immunodeficiency, centromeric region instability, facial anomalies) [14]. Subtelomeres, regions neighboring telomeres, in lymphoblastoid and fibroblast cells of ICF patients are hypomethylated to a similar extent, as seen in sperm. The telomeres in this syndrome are also abnormally short [12]. Short telomeres with hypomethylated subtelomeres accumulate in the peripheral leukocytes with aging. From this viewpoint, telomere attrition with aging may also be associated with increased subtelomeric hypomethylation in humans. In addition, telomere attrition, the decrease in long telomeres and the increasing hypermethylated subtelomeres of long telomeres are aging-related telomeric changes, and are facilitated by various disease conditions [4, 9].

Alzheimer's disease (AD) is a major neurodegenerative disorder. As the elderly population increases, the prevalence of AD and other age-related diseases increases. The involvement of oxidative stress (OS) in the pathophysiological condition of AD has been reported for more than 20 years [15, 16]. OS in AD patients has been demonstrated by elevated levels of lipid and protein in brain tissue [17, 18]. Recent reports show that somatic telomere lengths in AD patients tended to be altered in comparison with normal controls [6, 19]. These observations lead us to hypothesize that aging-associated telomere shortening is linked not only to altered subtelomeric methylation status in humans, but it is also affected by disease conditions such as AD. In the present study, telomere length distribution and subtelomeric methylation status were analyzed in AD patients.

Methods

Study population

Women with AD attending the outpatient clinic of the 309th Hospital of the Chinese People's Liberation Army, Beijing, China and the Chinese PLA General Hospital, Beijing, China, from May 2006 to March 2009, were enrolled. Some of their female family members and some of the hospital's healthy female workers, who never smoked and had undergone regular medical check-ups within a year of the enrollment, were also enrolled as healthy controls. To reduce the substantial confounding effects of gender and ethnicity on telomere length, all participants were Chinese women. DNA samples from the peripheral leukocytes of 29 female controls (aged 65–82) and 23 female AD patients (aged 67–80), meeting the criteria of the Diagnostic and Statistical Manual of Mental Disorders-IV diagnosis of the American Psychiatric Association with scores between 10 and 26 on the Folstein Mini-Mental Status Exam (MMSE), were subjected to the following analyses. PBMC samples were stored at −80 °C until use. All patients were examined by a psychiatrist and a neuropsychologist during the admission procedure and also underwent routine laboratory tests, standard neuropsychologic examinations, and cerebral perfusion study by single photon emission computed tomography. None of the AD subjects showed any complications or other neurologic or mental diseases, and no evidence of any developmental abnormalities or significant neurologic antecedents. Subjects were excluded from the study if they had any inflammatory disease, diabetes mellitus, were on estrogen replacement therapy, or had been treated with vitamins. All subjects were non-smokers. Some of their family members and some of our hospital's healthy workers, who had passed regular medical check-ups within a year of enrollment, were also enrolled as healthy controls. AD patients and controls were matched for age and dietary habits. The present study was approved by the local Human Ethics Committee, and written consent was obtained from all participants. Before treatment started, blood samples were drawn into 10-mL Vacutainer tubes with heparinized syringes. Over 20 times the volume of 10 mM Tris–HCl, 1 mM EDTA (pH 8.0) was added to the blood samples to remove erythrocytes by lowering osmotic pressure. Peripheral leukocytes were collected by centrifugation.

Telomeric length measurement

Telomere detection was performed as previously described, with a modification [4, 9]. Methylation-sensitive and methylation-insensitive isoschizomers *Hpa*II and *Msp*I were used, as in our previous studies [4, 9]. Briefly, genomic DNA was extracted from peripheral leukocyte specimens, and 0.1 μg of DNA was digested with the isoschizomers. The digests were subjected to Southern blot analysis with a long, hypersensitive digoxigenin-labeled telomeric probe containing the (TTAGGG)n sequence. The Southern blot smears of autoradiograms were captured on

an Image Master (Trioptics Japan, Shizuoka), and telomere length was quantitatively assessed.

If the mean difference between two experiments was >5 %, we intended to measure it again and use the mean value of three experiments, but the mean difference within was always within 5 %.

Terminal length analysis

The mean TRF (Terminal Restriction Fragment Length) was estimated with the formula Σ(ODi-background)/Σ(ODi-background/Li) [4, 9], where ODi is the chemiluminescent signal and Li is the length of the TRF fragment at position i. A loss of a few hundred base pairs from short telomeres may be important in cellular aging but cannot always be detected by traditional mean TRF analysis [20, 21]. We compared telomere length by telomere percentage analysis, with four intervals of length as defined by a molecular weight standard. In brief, the intensity of photostimulated luminescence was quantified as follows: each telomeric sample was divided into grid squares according to molecular size ranges >9.4, 9.4–4.4 and <4.4 kb. The percentage of intensity in each molecular weight range was measured (intensity of a defined region-background × 100/total lane intensity-background). Telomeric methylation was assessed by comparing *Msp*I and *Hpa*II telomere length distributions. The difference between the percentage of *Msp*I and *Hpa*II intensity in each molecular weight range was calculated. The proportion of the calculated difference (%*Hpa*II-TRF − %*Msp*I-TRF) in the >9.4 kb range to %*Hpa*II-TRF in the same range [(*Hpa*II–*Msp*I)/*Hpa*II (>9.4 kb)] was used to evaluate the methylation status of telomeres longer than 9.4 kb. Similarly, the proportion of the calculated difference (%*Msp*I-TRF − %*Hpa*II-TRF) in the <4.4 kb range to %*Msp*I-TRF in the same range [(*Msp*I–*Hpa*II)/*Msp*I (<4.4 kb)) was used to evaluate the methylation status of telomeres shorter than 4.4 kb.

Statistical analysis

Data normality was ascertained with the Kolmogorov–Smirnov test and the homogeneity of variance with the Levene Median test. If both normal distribution and equal variance tests were passed, differences in telomere length, including mean TRF length and telomere percentage analysis with age and condition (AD patients or age-matched healthy controls), were studied by a two-way ANOVA, followed by all pairwise multiple comparison procedures with Tukey's post hoc test. Data are expressed as means ± standard deviations, significance was set at $p < 0.05$. All analyses were carried out with the Sigma Statistical Analysis software package (Sigma 2.03, 2001; St. Louis, MO).

Results

The mean TRF of AD patients was similar to that of controls (Table 1). Although *Hpa*II-TRF appeared to be longer than *Msp*I-TRF, the mean difference between them in AD patients was not significantly different from that in controls (Table 1). Nor did telomere length or subtelomeric methylation status seem to be affected by AD. An analysis of telomere length distribution in AD patients showed that long telomeres (>9.4 kb) decreased (controls; 30.3 ± 7.9 %, AD; 24.4 ± 8.3 %, $p = 0.013$) and medium-sized ones (4.4–9.4 kb) increased (controls; 51.7 ± 3.3 %, AD 55.5 ± 6.4 %, $p = 0.015$) in comparison with controls (Fig. 1b). However, AD patients had similar proportional amounts of short telomeres (<4.4 kb) (controls; 18.0 ± 7.8, AD; 20.2 ± 8.9 %, $p = 0.371$), compared with controls (Fig. 1a). A similar trend was also found in the *Hpa*II TRF length distribution, although no significant difference in the longest range (>9.4 kb) between AD and control subjects was observed (Fig. 1b).

In the subtracted distribution (*Hpa*II-TRF distribution minus *Msp*I-TRF distribution), the area difference in the short range (<4.4 kb) was significantly larger in AD patients than in controls (controls; −3.62 ± 4.66 %, AD; −7.58 ± 4.42, $p = 0.003$) (Fig. 1c). The *Hpa*II–*Msp*I value may be affected by two factors: differences in telomere length and methylation status. In order to analyze the methylation specifically, methylation status was examined in long and short telomeres separately. Methylation-specific parameters, including the ratio of subtraction of *Msp*I-TRF lengths from *Hpa*II-TRF lengths (>9.4 kb and <4.4 kb) to *Hpa*II-TRF lengths (>9.4 kb) were then calculated (Fig. 2). The methylation levels in the longest and shortest telomere length ranges were reflected by [(*Hpa*II–*Msp*I)/*Hpa*II (>9.4 kb)] and [(*Msp*I–*Hpa*II)/*Msp*I (<4.4 kb)], respectively. The former was not significantly different between AD subjects and controls. However, the latter was significantly higher in AD patients (controls; 0.21 ± 0.23, AD; 0.41 ± 0.26, $p = 0.016$).

Table 1 The mean TRF and the subtracted TRF of controls and AD patients

	Control	AD	*p* value
Age (years)	72.1 ± 4.9	71.6 ± 4.0	0.66
Mean *Msp*I TRF (kb)	6.4 ± 0.9	6.1 ± 0.8	0.13
*Hpa*II-*Msp*I TRF (kb)	0.7 ± 0.6	0.9 ± 0.5	0.17

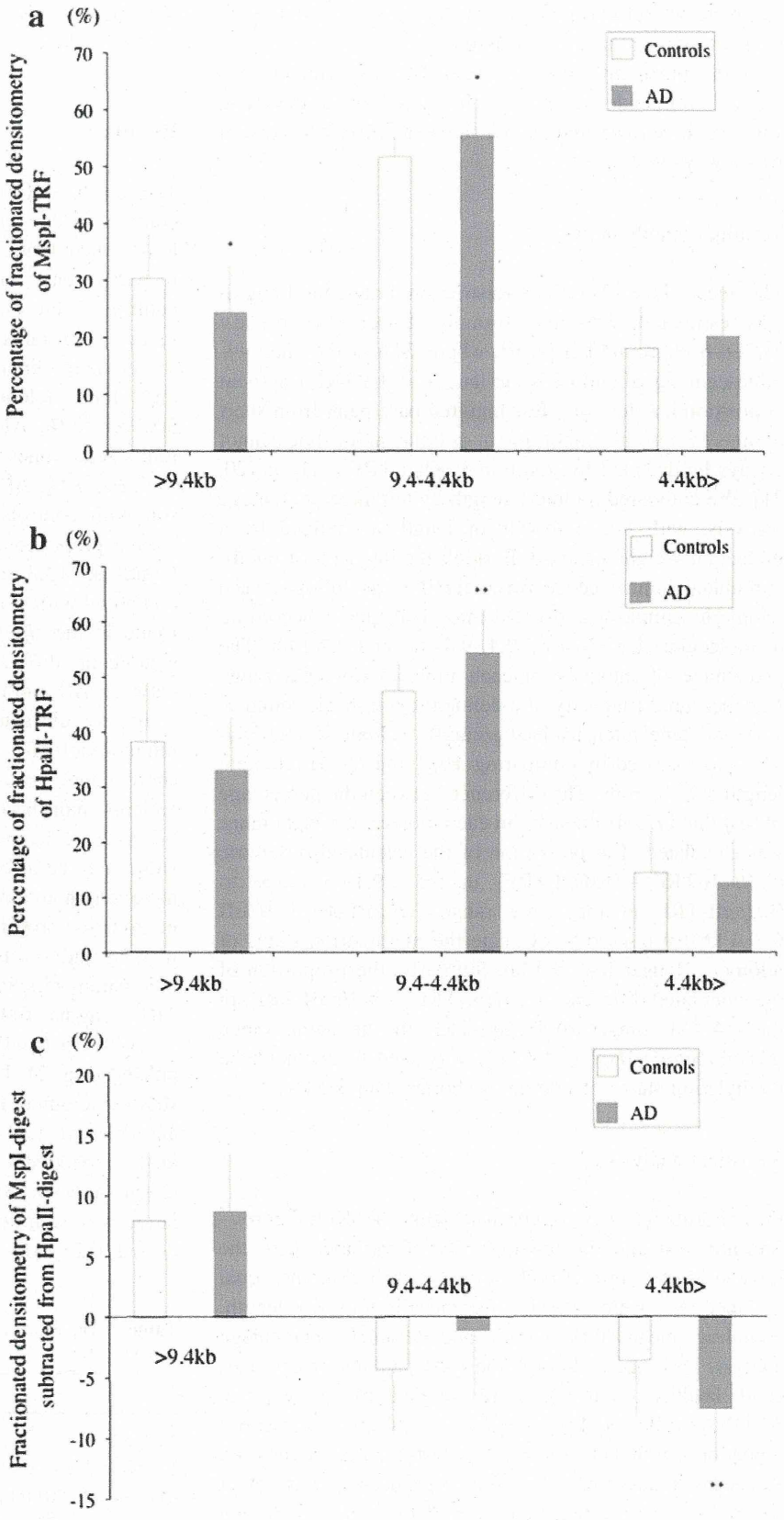

Fig. 1 Changes in subdivided *Msp*I-TRF and *Hpa*II-TRF distributions of telomere length in AD patients. **a**, **b** Changes in subdivided *Msp*I-TRF and *Hpa*II-TRF distributions in AD patients. Southern blots of *Msp*I-TRF (**a**) and *Hpa*II-TRF (**b**) divided into three portions (>9.4, 9.4–4.4, <4.4 kb). *Columns* Percentages of densitometry of each portion. *Vertical bars* standard deviations. **c** Subtracted *Hpa*II–*Msp*I TRF length distribution. *Columns* Subtracted values of *Msp*I-TRF from *Hpa*II-TRF densitometry in three subdivided parts. *Vertical bars* standard deviations. $*p < 0.05$, $**p < 0.01$ vs controls

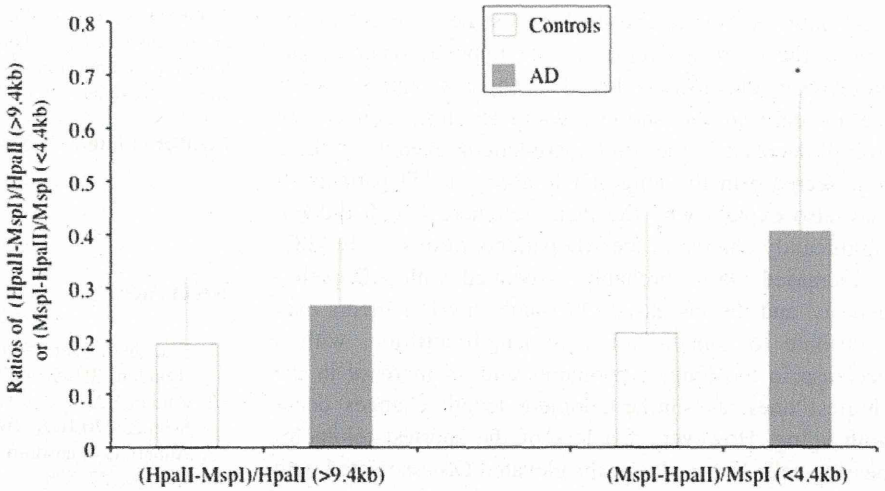

Fig. 2 Changes in relative methylation status of subtelomeres in AD patients. *Hpa*II–*Msp*I/*Hpa*II (>9.4 kb) and *Msp*I–*Hpa*II/*Msp*I (<4.4 kb) indicate subtelomeric methylation of longer (>9.4 kb) and shorter (<4.4 kb) telomeres, respectively. *Vertical bars* standard deviations. $*p < 0.05$ vs controls

Discussion

Alzheimer's disease (AD) is an aging-associated neurodegenerative disease, characterized by progressive cognitive decline and memory impairment [22]. Oxidative stress (OS) is involved in the pathogenesis of AD, and elevated OS in AD patients has been demonstrated by high levels of lipid and protein in brain tissue [15–18, 23]. Increased OS in AD patients contributes to telomere erosion and causes accelerated telomere shortening of local or systemic somatic cells [6]. It has also been reported that telomere length shortens abnormally in AD patients [6]. However, the level of cognitive dementia, including that due to Alzheimer's disease or vascular dementia is not always associated with the telomere length of leukocytes [24]. Tissue variations in telomere length have also been reported, as telomeres are shortened in leukocytes and buccal cells but elongated in the brain tissue of AD patients [19].

In our present analysis, the mean TRF of AD patients was not significantly different from that of controls, but the longest telomeres (>9.4 kb) were decreased and the medium-sized ones (4.4–9.4 kb) were increased. A decrease of the amounts of the longest telomeres has been reported to be an aging-associated telomere change [4]. Decreases in the longest telomeres lead to relative increases in all telomeres shorter than those of the longest range. However, the shortest telomeres did not increase in our AD patients. This result was in contrast with reported telomere changes in the healthy population, which shows an increase in the shortest telomeres with aging [3, 4]. Therefore, the observed telomere length changes in AD patients did not seem to be derived from a simple acceleration of the aging process. A disease-specific mechanism may therefore suppress the increase of the shortest telomeres in AD patients. Subtelomeric methylation was also proportionally enhanced in the shortest telomere length range. This is also in contrast with the aging-associated telomere changes that occur in the healthy population, in which there is a tendency for a decrease in subtelomeric methylation of the shortest telomeres [4].

There are two explanations for this observation. One is that more subtelomeres of the shortest telomeres became hypermethylated. The other is that cells bearing the shortest telomeres with hypomethylated telomeres are easily lost in AD patients. In mice, a loss of a few hundred base pairs from short telomeres had an important effect on cellular aging [20]. The increased oxidative stress associated with the pathogenesis of AD may promote the oxidation of telomere and subtelomere regions. OS can generate hydroxyl radicals, causing a wide range of DNA lesions, including base modifications, and yield products of DNA oxidation including 8-hydroxy- deoxyguanosine and O^6-methylguanine [25]. Such lesions have been shown to interfere with the ability of DNA to function as a substrate for DNA methyltransferases, resulting in global hypomethylation [26, 27]. As a result, genomic DNA, including the subtelomeric region, is hypomethylated. Increased subtelomeric hypomethylation of short telomeres would lead to further telomere attrition [12], resulting in increased cell loss. This hypothesis may also explain why the shortest telomeres do not increase in AD patients.

A decrease in the shortest telomeres has been observed in patients with another neurodegenerative disorder, Parkinson's disease (PD) [4]. OS is systemically elevated in PD patients, and cells containing short telomeres with less methylated subtelomeres may become senescent easily [4]. Via a similar mechanism, short telomere-bearing cells may be easily lost from the circulating leukocyte population in AD patients. This may explain the lack of change in the shortest telomeres in the peripheral leukocytes of AD patients and the relative increase in the subtelomeric

methylation of the shortest telomeres, because cells containing the shortest telomeres with hypomethylated subtelomeres would easily be lost. This apparent suppression of the increase in the shortest telomeres may conceal an overall decrease in mean telomere length, even when there is a decrease in the longest telomeres in AD patients. It may also explain why the mean telomere length did not significantly change in the AD patients in this study [28].

Increased OS is probably associated with AD pathogenesis, and the increased OS found in AD patients may contribute to somatic telomere length attrition, with a decrease in the longest telomeres and an increase in the shortest ones, as similar telomere length changes occur with aging. However, if a loss of the shortest telomere-bearing cells occurs due to the elevated OS associated with AD, the expected increase in the shortest telomeres may not occur, and only medium-sized ones would increase. Telomeres next to hypomethylated subtelomeres have been reported to undergo accelerated attrition, because cells containing fewer methylated subtelomeres are more impaired by elevated OS [4], which generates hydroxyl radicals, triggering various kinds of DNA damage [25]. Access of DNA methyltransferase to subtelomeres may be inhibited by DNA oxidation, resulting in subtelomeric hypomethylation [4]. DNA methylation is associated with chromatin modifications in mammals, regulating the accessibility of DNA-binding factors and controlling transcriptional activation of the region [29]. Subtelomeric hypomethylation is associated with increased accessibility of DNA-binding proteins for suppression of genes near telomeres [30]. Therefore, subtelomeric regions damaged by OS will probably become hypomethylated. In conditions associated with increased OS, including AD, genomic DNA containing telomeres and subtelomeres may become progressively hypomethylated and unstable, and cells containing short telomeres with hypomethylated subtelomeres may tend to enter the senescence stage. Cells with the shortest telomeres and hypermethylated subtelomeres are apparently increased in patients with AD.

These hypotheses will need to be confirmed, and the mechanism should be determined by other experiments, including ones with in vitro culture conditions. Alterations in telomere length distribution and subtelomeric methylation status can be detected, and may be useful as sensitive markers of the disease-associated effects of neurodegenerative disorders such as AD and PD on genomic DNA, even when the mean telomere length of somatic cells seems unchanged. To confirm the results of our study, further analysis will be necessary to clarify whether cells with increased short telomeres with subtelomeric hypomethylation in hyperoxidative conditions are prone to proceed to cell death in other types of chronic disease or in culture conditions.

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Conflict of interest None.

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ORIGINAL ARTICLE

Hyperthermia by bathing in a hot spring improves cardiovascular functions and reduces the production of inflammatory cytokines in patients with chronic heart failure

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Abstract Balneotherapy has been shown to reduce systemic blood pressure in healthy volunteers. Hyperthermia might ameliorate the inflammatory status in heart failure through improving cardiac function. The purpose of this study was to examine the beneficial effects of balneotherapy in patients with chronic heart failure (CHF). Thirty-two patients with systolic CHF classified as New York Heart Association functional status II or III were randomized to divide either a balneotherapy group or a control group. The patients in the balneotherapy group were immersed in a hot spring at 40°C for 10 min daily for 2 weeks; the control group patients took a shower daily. The left ventricular ejection fraction (EF) and cardiothoracic ratio (CTR) were evaluated and plasma brain natriuretic peptide (BNP), high-sensitivity C-reactive protein (hsCRP), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 levels were measured. The clinical symptoms improved after 2 weeks of hot spring therapy. Although the heart rate did not change, clinical symptoms, CTR, EF, and BNP were significantly improved. Moreover, the inflammatory responses, including hsCRP, TNF-α and IL-6 decreased significantly after balneotherapy. The improvement of BNP correlates with the changes in inflammatory biomarkers. Repeated hyperthermia by bathing in a hot spring is therefore considered to improve the cardiac and inflammatory status in patients with CHF.

Keywords Balneotherapy · Congestive heart failure · Inflammation · Cytokine

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Introduction

Chronic heart failure (CHF) has an extremely poor prognosis in spite of recently developed cardiovascular pharmacological therapeutics [1]. Therapeutic exercise has beneficial effects and has traditionally been used as an alternative non-pharmacological treatment for patients with CHF. However, it is sometimes difficult for elderly people to perform the appropriate therapeutic exercise due to problems associated with their extremities. Some investigators reported that bathing in a hot bath has acute potential benefits for patients with CHF through reducing systemic vascular resistance in CHF patients [2, 3]. However, whether balneotherapy by bathing in a hot spring improves the status of CHF remains unclear. Hyperthermia using a hot spring might improve cardiac function and clinical symptoms. The purpose of this study was to elucidate the chronic benefits of hyperthermia treatment using a hot spring in patients with compensated CHF.

Methods

Patient population

This study was a single-center, open-label, randomized trial. The Institutional Review Committee in Human

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Research of the Faculty of Medicine, Kyushu University, approved the study protocol. Written informed consent was obtained from each subject before the study.

Between April 2007 and March 2008, all consecutive patients who were referred to the Department of Rehabilitation in Kyushu University Hospital at Beppu because of heart failure were enrolled in this study. Finally, the study population included 32 patients (16 patients with dilated cardiomyopathy, 16 patients with ischemic cardiomyopathy) with chronic systolic heart failure classified as New York Heart Association (NYHA) functional status II or III, and a significant left ventricular dysfunction (EF $< 50\%$). The patients' clinical status was stabilized and they all underwent a detailed evaluation of physiological examination, including heart rate (HR), blood pressure (BP), left ventricular ejection fraction (EF) by the echocardiography, and cardiothoracic ratio (CTR) by chest X-ray. High-sensitivity C-reactive protein (hsCRP) quantified by utilizing latex-enhanced nephelometry with a Behring Nephelometer Analyzer System, interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α were measured by the ELISA method before and after the intervention (Biosource International, Inc, Camarillo, CA, USA). Plasma level of brain natriuretic peptide (BNP) was measured by chemiluminescent enzyme immunoassay (Shionogi, Osaka, Japan). All the determinant parameters, including physiological hemodynamics and blood sampling were performed at the timing of preprandial state in the morning 1 day before starting or 1 day after finishing the whole study protocol.

Balneotherapy

The patients were randomly divided into two groups. The patients in the balneotherapy group immersed their bodies to the xiphoid process in a hot spring at 40°C for 10 min. The half-body immersion in hot spring bath was performed to reduce hydrostatic fluid pressure and cardiac workload which is increased by full-body immersion increases [2]. The bathroom temperature was maintained at around 28°C to prevent heat loss from their bodies. After bathing, the patients rested for 1 h to keep their bodies warm with a blanket on the bed. Balneotherapy was performed daily (5 days a week) for 2 weeks. The patients maintained all of their other normal daily habits. Patients in the control group took a shower daily for 2 weeks.

Statistical analysis

All values are expressed as the mean ± SD. The results of the data were evaluated by two scientists who were not involved in the treatment in a blinded fashion. The comparisons of continuous variables between the two groups were performed by the two-sample t test or Wilcoxon rank-sum test, and those between before and after intervention by one-sample t tests or Wilcoxon sign-rank test according to their distributions. The comparisons of categorical values between the two groups were performed by Chi-square tests and Fisher exact tests. The relationship between the increases in BNP and the level of various inflammatory biomarkers were determined by a linear regression analysis. $p < 0.05$ was considered to be significant.

Results

Clinical characteristics

Table 1 lists the baseline characteristics of the study subjects. There were no significant differences between the control group and the balneotherapy group.

Effect of hot spring on deteriorated cardiovascular function

The HR did not change before and after treatment in either group. However, the mean BP and CTR decreased significantly after treatment in the balneotherapy group (Table 2). Moreover, the NYHA classification and EF drastically improved after hot spring immersion for 2 weeks. On the other hand, no significant differences were observed in hemodynamic variables of the control group before and after the 2-week period of daily showering. These results suggested that cardiac function and thereafter cardiac remodeling improved over those 2 weeks by balneotherapy.

Effect of the hot spring on imbalanced biophysiological and chemical parameters

Figure 1a shows the level of plasma BNP in each group before and after intervention. In the balneotherapy group,

Table 1 Baseline characteristics

	Control ($n = 16$)	Balneotherapy ($n = 16$)
Age	70.0 ± 4.2	68.7 ± 4.0
Gender (M/F)	8/8	8/8
ICM/DCM	8/8	8/8
Ht (cm)	158.1 ± 1.6	156.5 ± 2.5
BW (kg)	56.2 ± 2.1	55.7 ± 2.9
BMI	22.6 ± 0.8	22.7 ± 1.0
Drug		
β-Blocker (n)	9	8
ACEI/ARB (n)	11	10
Anti-platelet/anti-coagulation (n)	9	9
Diuretics (n)	11	10

Table 2 Improvement of physical parameters after balneotherapy

	Before intervention	After intervention
Control ($n = 16$)		
SBP (mmHg)	120.8 ± 7.0	118.1 ± 5.7
DBP (mmHg)	76.6 ± 3.7	77.4 ± 2.9
Mean BP (mmHg)	86.0 ± 3.4	87.5 ± 2.2
HR (bpm)	74.9 ± 4.2	76.4 ± 3.7
CTR (%)	59.4 ± 2.0	58.7 ± 1.7
EF (%)	36.4 ± 3.2	34.7 ± 3.3
NYHA (I/II/III/IV)	2.75 ± 0.11 (0/4/12/0)	2.75 ± 0.11 (0/4/12/0)
Balneotherapy ($n = 16$)		
SBP (mmHg)	107.1 ± 4.0	106.3 ± 3.8
DBP (mmHg)	70.1 ± 2.0	67.4 ± 2.4
Mean BP (mmHg)	83.5 ± 2.3	79.5 ± 2.5
HR (bpm)	75.4 ± 3.6	73.0 ± 3.8
CTR (%)	57.2 ± 1.2	52.1 ± 1.4*,#
EF (%)	35.5 ± 2.2	45.1 ± 1.6**,#
NYHA (I/II/III/IV)	2.81 ± 0.10 (0/4/12/0)	2.13 ± 0.13 (1/14/1/0**,##)

* $p < 0.05$
** $p < 0.01$ vs control group
# $p < 0.05$
$p < 0.01$ vs before intervention

BNP decreased significantly after the treatment. However, these parameters did not change statistically after intervention in the control group.

The levels of plasma hsCRP, TNF-α, IL-1β, and IL-6 were measured before and after intervention (Fig. 1b–e). The hsCRP, TNF-α, and IL-6 levels all significantly decreased after balneotherapy. On the other hand, the IL-1β level did not alter in any group after intervention.

Figure 2 showed the relationships between the change in the levels of BNP and various inflammatory biomarkers. There were slight but statistically significant correlations between improvement of BNP and cytokines including hsCRP.

Discussion

The novel findings of the present study are that immersion in a hot spring improved both the cardiac function as well as the patients' inflammatory status.

Balneotherapy

Conventionally, bathing has been recognized as harmful and even exhausting for severe CHF patients because thermal stress increases the cardiac workload and sympathetic activity, thus leading to decompensation of cardiac failure. However, the recent tendency has been to recognize that careful bathing and/or dry sauna is safer than it had previously been thought to be [2–6]. In our study, the deep-body temperature increased approximately 1.2°C after half-body bathing (data not shown), which coincided with the previous report [2]. The present study suggests that bathing at 40°C for 10 min can thus be safely performed even in CHF patients. It is not certain whether the beneficial effects of balneotherapy for patients with CHF are produced only by hot springs. Previously, investigators reported that the thermal effect of a sauna was better than that of hot water [2]. However, according to the mechanisms of hyperthermia, warming of the body either way might have similar beneficial effects for patients with CHF. Indeed, repeated dry sauna therapy has also been reported to be therapeutically effective for patients with CHF [4, 6]. The precise mechanism of balneotherapy for CHF remains uncertain. However, vasodilatation induced by hyperthermia may increase the vascular shear stress and production of endothelial nitric oxide. Therefore, repetitive hyperthermia may reduce afterload and improve cardiac dysfunction. The opportunities to bathe are sometimes limited for elderly patients with heart failure because of impaired activities of daily living and a reduced cardiopulmonary function, and/or disabilities of the extremities. Therefore, bathing has been hygienic as well as additional therapeutic benefits for such patients.

Brain natriuretic peptide is secreted mainly from the heart ventricle and this level in blood reflects cardiac dysfunction as a reaction to cardiac wall distension and stretching [7]. It is reported that improved cardiac EF is associated with a decrease in BNP level in patients with CHF [8] and elevated level of BNP is a predictor of ischemic heart disease in a population with normal cardiac function [9]. Therefore, BNP is a useful biomarker of CHF and the significant correlations of BNP and cytokines in Fig. 2 are thought to be therapeutical results of balneotherapy to improve cardiac function and decrease the inflammatory status in patients with CHF.

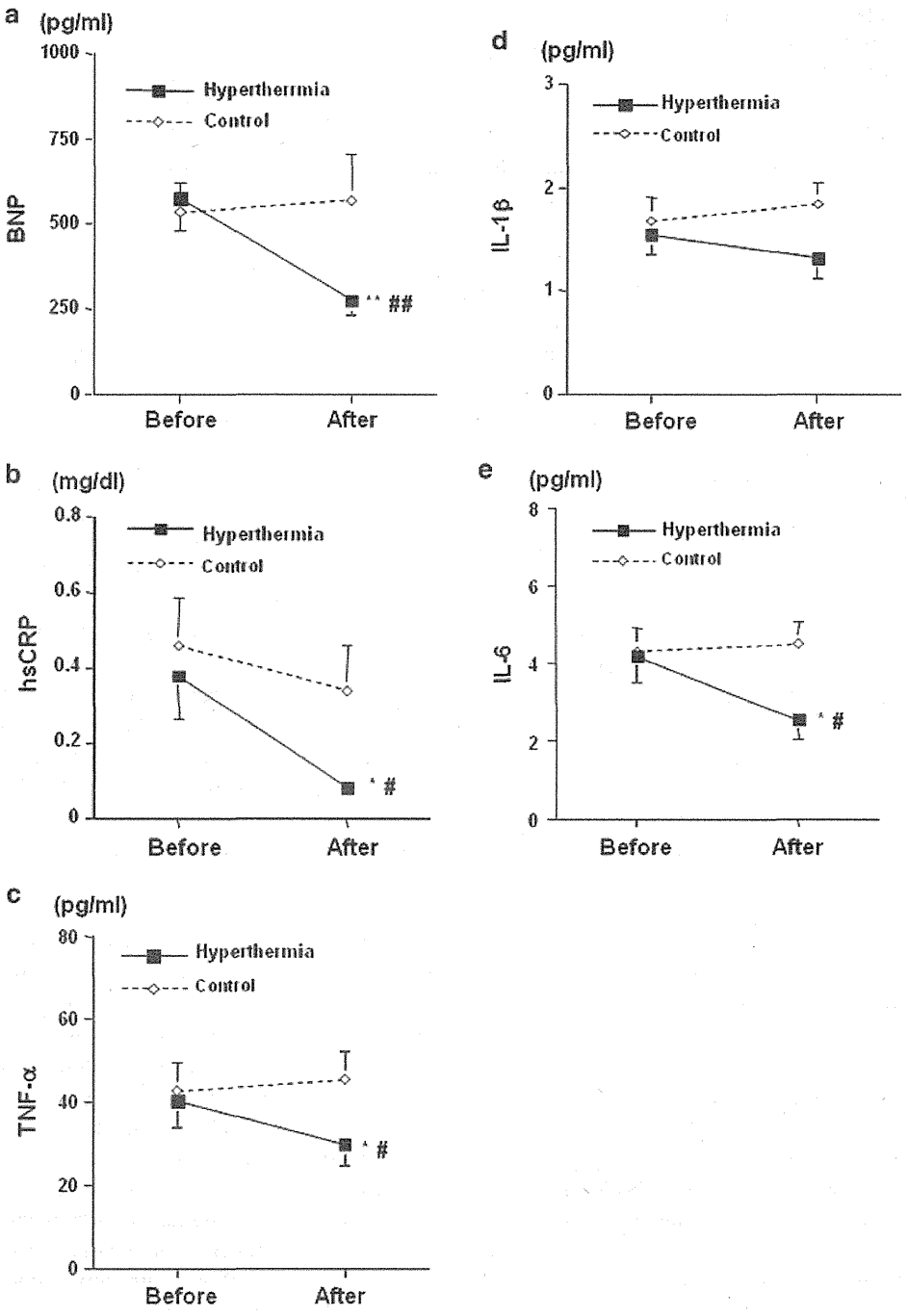

Fig. 1 Changes in the plasma level of BNP (**a**), hsCRP (**b**), TNF-α (**c**), IL-1β (**d**), and IL-6 (**e**) in each group. $^{*}p < 0.05$, $^{**}p < 0.01$ versus control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus before intervention

Cardiac function

Patients with CHF demonstrated oxygen consumption to slightly increase in a warm water bath, while the pulmonary arterial blood temperature increased by 1.2°C, and the heart rate increased by 20–25 beats per minute at the end of bathing. The systolic blood pressure showed no significant change, but the cardiac and stroke indexes increased and systemic vascular resistances decreased significantly during and after bathing. The mean pulmonary artery, mean pulmonary capillary wedge, and mean right atrial pressures increased significantly during bathing, but they were significantly lower than the control levels after bathing. The cardiac dimensions decreased and the left ventricular ejection fraction increased significantly after bathing [2, 3]. Moreover, repeated low-temperature dry sauna treatment improved the vascular endothelial and cardiac function (i.e., EF) in patients with chronic heart failure [4, 6]. Therefore, heating bodies

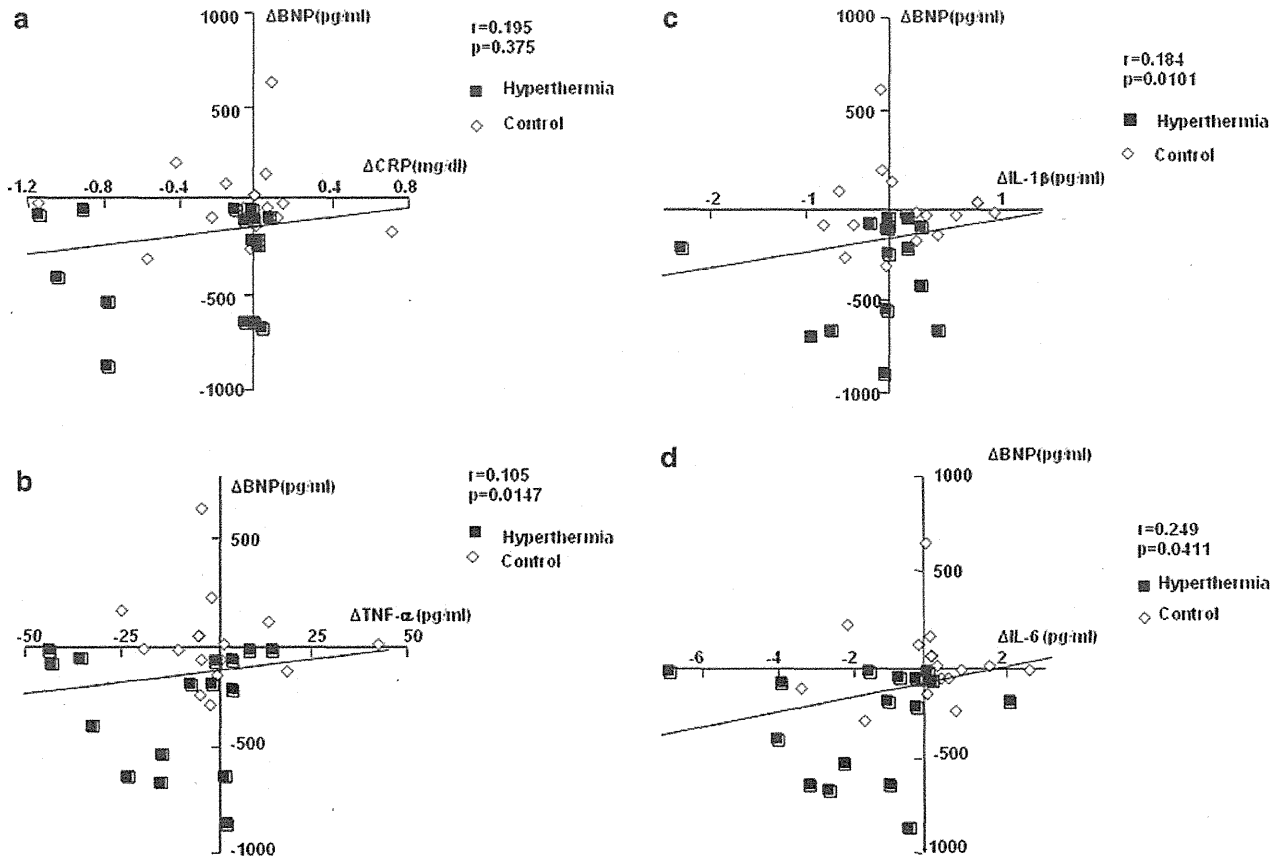

Fig. 2 The correlations between the changes in the level of BNP and the levels of hsCRP (**a**), TNF-α (**b**), IL-1β (**c**), and IL-6 (**d**) in all groups. Correlation coefficient and *p* value are indicated

passively with external stimuli might have some therapeutic benefits for patients with CHF through decreasing systemic vascular resistance and cardiac afterload.

Inflammatory responses

There is now considerable evidence that the plasma levels of inflammatory cytokines are elevated in patients with CHF [10, 11]. The expression of inflammatory cytokines not only results in immune responses to cardiac damage but it also results in a deterioration of the cardiac function and a progression of cardiac failure [12, 13]. Indeed, IL-6 is thought to be a strong prognostic predictor in patients with chronic heart failure [14] and the levels of IL-6 and TNF-α are associated with impaired LV function [15]. The level of hsCRP is also associated with adverse outcomes in cardiovascular diseases. The level of hsCRP increases with the severity of CHF in patients with diastolic HF as well as in systolic HF. This supports a potential role for CRP in immune activation in CHF. Therefore, the measurement of hsCRP levels thus has the potential role for risk assessment in patients with CHF [16–18]. The present study revealed that hsCRP and inflammatory cytokines decrease after balneotherapy, which could suggest that balneotherapy ameliorates the prognosis of CHF. Therefore, balneotherapy has the possibility to prevent cascading negative effects on the cardiac function.

Limitation of the study

Several limitations of this study should be mentioned. First, only a small number of patients were studied. Second, the cardiovascular hemodynamic variables were not measured during bathing. Patients with CHF have a lower cardiopulmonary function and the increased preload due to increased venous return during bathing must be the disadvantage of the balneotherapy. However, researchers have suggested that bathing at 40°C for 10 min is quite safe and favorable to bathing [2–6]. Indeed, the present study was conducted under the careful observation of medical doctors, and no patient enrolled felt any subjective worsening of heart failure symptoms, including fatigue, shortness of breath or fainting, or dropped out the study.

Conclusions

The present study demonstrates that repeated immersion in a hot spring improves both cardiac dysfunction and the inflammatory responses, thus leading to an improvement of the clinical activity and symptoms in patients with CHF. Balneotherapy may therefore be an excellent, alternative, non-pharmacological therapy for elderly patients suffering from heart failure, especially those who cannot undergo appropriate cardiac rehabilitation.

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ORIGINAL ARTICLE: BIOLOGY

Telomerase activity and telomere length distribution in vascular endothelial cells in a short-term culture under the presence of hydrogen peroxide

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Aim: The aim of this study was to assess the biological effects of oxidative stress on human vascular endothelial cells.

Methods: The telomeric changes and the alterations of the expression of telomere-associated proteins in human umbilical venous endothelial cells (HUVEC) cultured in the presence of hydrogen peroxide (H_2O_2) were analyzed.

Results: During the culture, the cell growth rate decreased, whereas the telomerase activity of the surviving cells increased. As the H_2O_2 level increased, long telomeres decreased proportionally, thus resulting in a telomere length distribution that was rich in short telomeres. These observations suggested that H_2O_2-affected endothelial cells bear telomeric features similar to those of aged cells. In contrast, the expression of telomere-associated proteins, TRF1 and TRF2, showed different changes. TRF1 increased in relation to H_2O_2 concentration, whereas TRF2 showed no significant change. The surviving cells exposed to H_2O_2 showed a H_2O_2-dose dependent increase in telomerase activity, whereas the telomere protein and RNA components were only elevated in low concentrations of H_2O_2.

Conclusions: The increase in telomerase activity and TRF1 protein expression of vascular endothelial cell might show an aspect of cellular protective reaction against oxygen stress. **Geriatr Gerontol Int 2013; 13: 774–782.**

Keywords: cell senescence, hydrogen peroxide, telomerase, telomere, telomeric proteins, vascular endothelial cell.

Introduction

A telomere is a structure consisting of thousands of repeats and accessory peptide factors located at the termini of chromosomes.[1,2] Telomeres become shortened little by little because of the inability of complete DNA duplication at the chromosome ends. A limitedly shortened telomere stops mitosis and induces "cell senescence". Such telomere shortening has been observed in peripheral blood nuclear cells with aging.[3–5] Older people have shorter telomeres in their somatic cells than young people. In addition, telomere shortening is accelerated by various pathological conditions including physical and mental stress caused by disease conditions including atherosclerotic disorders, such as ischemic heart diseases and cerebrovascular disease.[6–13]

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Reactive oxygen stress has been suggested to be involved in the cellular aging process with telomere attrition. For example, oxygen stress caused by H_2O_2 has been reported to induce telomere-associated cell senescence among different kinds of cells, such as fibroblasts, lens epithelial cells, endothelial cells, chondrocytes and keratinocytes.[14–18] Above all, the vascular endothelium seems to be a representative tissue that is exposed to high oxygen pressure. Atherosclerotic disorders are based on the degenerative changes of vascular endothelial cells caused by hyperoxidized lipids.[19] Therefore, vascular endothelial cells are exposed directly to hyperoxidized compounds present in the blood. Vascular endothelial cells have also been regarded as a representative somatic site exposed to hyperoxidative stress because of the higher oxygen content present in the circulating blood compared with other types of tissue fluid containing no oxygen carriers, such as hemoglobin. This speculation led us to hypothesize that vascular endothelial cells are more resistant to oxidative stress than other cells. The aim of the present study was to elucidate how the various levels of oxidative stress, including extremely hyperoxidative conditions induced

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by hydrogen peroxide, affect the telomeric features, telomere DNA and telomere-associated components (telomerase, TRF1 and TRF2) of vascular endothelial cells *in vitro*.

Methods

Cell culture

Human umbilical venous endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC) were purchased from Clonetics (San Diego, CA, USA). They were cultured in endothelial cell growth medium (Clonetics). The cells were cultured at 37°C and 5% CO_2 in a gelatin-coated flask (Iwaki Glass, Chiba, Japan), and routine subcultivation was carried out every 2 days with a split ratio of 1:4, and used at the third passage. Cells were counted at this stage. Then the culture medium was replaced by H_2O_2-containing medium and the cells were cultured for 3 days. H_2O_2-containing media were refreshed every 24 h. On day 3, the cells were collected and subjected to further analyses. Cells were counted using a hemocytometer. Population doublings (PD) were calculated using the formula: PD = (log [expansion] / log 2), where expansion was the number of cells harvested divided by the initial number of cells seeded.

Senescence-associated β-galactosidase expression

The cells were washed in PBS, fixed for 10 min at room temperature in 2% formaldehyde/0.2% glutaraldehyde, rinsed in PBS, and incubated at 37°C (no CO_2) with fresh senescence-associated β-galactosidase (SA-β-Gal) staining solution. The staining solution was made up as follows: 1 mg X-gal, per mL solution, 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, 2 mmol/L $MgCl_2$, 150 mmol/L NaCl, and 40 mmol/L citric acid and sodium phosphate at pH 6.0. This solution was left on the cells for 12 h to achieve the maximum staining. A total of 100 cells were scored from each well (plate) using a light microscope.

Telomere detection

Telomere detection was carried out as previously described.[4] Restriction enzyme *Msp*I was used. *Msp*I recognizes and cuts tetranucleotide CCGG. Briefly, blood cell DNA was extracted from samples and the DNA (0.1 μg) were digested at 37°C with 1 U *Msp*I for 2 h. The digests (10 μL) were resolved by agarose gel-electrophoresis, and transferred by Southern blotting to a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany). The blotted DNA fragments were hybridized to a hypersensitive probe of 500 bp long (TTAGGG)n labeled with digoxigenin. The membrane was then incubated with anti-digoxigenin-AP-specific antibody. The telomere probe was visualized by CSPD (disodium 3-(4-methoxy-spiro {1,2-dioxetane-3,2′-(5′-chloro) tri-cyclo[3.3.1.1] decan} –4-yl) phenyl phosphate; provided with the kit). The membrane was then exposed to Fuji XR film (Fuji Film, Tokyo, Japan) with an intensifying screen. The smears of the autoradiogram were captured on an Image Master (Trioptics Japan, Shizuoka, Japan), and the telomere length was then assessed quantitatively. Every sample was measured in triplicate.

Terminal restriction fragment length analysis

Telomere length distribution was analyzed by comparing the telomere length using a telomere percentage analysis with three intervals of length as defined by a molecular weight standard as previously described.[4] The intensity (photostimulated luminescence [PSL]) was quantified as follows: each telomeric sample was divided into grid squares as follows according to the molecular size ranges: >23.1, 23.1–9.4, 9.4–6.6, 6.6–4.4 and <4.4 kb. The percent of PSL in each molecular weight range was measured (%PSL = intensity of a defined region-background × 100 / total lane intensity-background). Peak terminal restriction fragment (TRF) lengths were used as representatives of TRF in the present study. The percentage of PSL in each molecular weight range was measured (%PSL = intensity of a defined region – background × 100 / total lane intensity – background). The mTRF was estimated using the formula S(ODi – background) / S(ODi – background / Li),[20] where ODi is the chemiluminescent signal and Li is the length of the TRF fragment at position i.

Semiquntitative reverse transcription polymerase chain reaction for telomerase RNA component

Reverse transcription polymerase chain reaction analysis. Total RNA samples were extracted using RNAzol B (Tel-Test, Friendswood, TX, USA). mRNA for human telomerase RNA component (TERC) was determined by reverse transcription polymerase chain reaction (RT–PCR) using a DIG detection system (Roche Applied Science, Rotkreuz, Switzerland). Each rat cDNA was produced by RT–PCR according to each human-derived sequence. For the amplification of TERC cDNA, the forward primer β-actin (205 bp) 5′-CCTTCCTGGGCATGGA GTCCT-3′ and the reverse primer 5′-GGAGCAATGA TCTTGATCTTC-3′ were used according to the published human TERC cDNA sequence.[21] And TERC forward primer 5′-TCTAACCCTAACTGAGAAGGGC GTAG-3′ reverse primer 5′-GTTTGCTCTAG AATG AACGGTGGAAG-3′ were used.[22] The values for TERC mRNA levels were normalized to the β-actin mRNA level in the same sample. The PCR products were directly

synthesized from 2 μg of total RNA isolated from each sample using the Superscript One-step RT–PCR System with Platinum *Taq* (Invitrogen, Carlsbad, CA, USA) and gene-specific primers according to the recommendations provided by the supplier. The PCR products were analyzed by agarose gel electrophoresis (1.3%) followed by staining with ethidium bromide and scanning with Gel-Doc (Bio-Rad Laboratories, Hercules, CA, USA). For semiquantitative PCR, β-actin was used as an internal control to evaluate total RNA input, as described by our group.[23]

Western blot and other analyses

Cells from a dish were homogenized with 100 μL lysis buffer (100 mmol/L Tris pH 6.8, 4% sodium dodecyl sulfate [SDS], 20% glycerol containing the protease inhibitor, 0.1 mmol/L M phenylmethanesulfonyl fluoride, 0.1 μL leupeptin and 0.1 μL aprotinin). Gel electrophoresis was used to separate 10 μg protein on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (162-0112; Bio-Rad Laboratories) blocked with 5% dry milk or blocking solution for western blot (Roche). Membranes were blocked and incubated with antibodies against telomerase reverse transcriptase (TERT) (Rockland, Gilbertville, PA, USA), TRF1 (Imgenex, San Diego, CA, USA), TRF2 (Cell Signaling, Danvers, MA, USA) or beta-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-Ser 824 TERT (Assay Bio Tech, Sunnyvale, CA, USA).[24,25] Detection was carried out with secondary horseradish peroxidase-conjugated antibodies (Chemicon, Temecula, CA, USA) and the ECL detection system as previously described.[26]

Telomerase activity

Telomerase activity was examined by means of a modified telomerase repeat amplification protocol (TRAP) method[27] with TRAPeze Telomerase Detection Kit S7700 (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Briefly, the substrate oligonucleotide is added to 0.5 mg protein extract. If telomerase is present and active, telomeric repeats (GGTTAG) are added to the 3′ end of the oligonucleotide. After amplification, the PCR products were resolved on a 12% polyacrylamide gel, stained with ethidium bromide and detected using a FLA 5000 system (Fuji Film). The intensities of the bands were quantified with Image J (NIH, Bethesda, MD, USA). According to the manufacturer's instructions, the telomerase activities were calculated and presented as total product generated. Telomerase activity in the presence of hydrogen peroxide was assessed by carrying out TRAP assay with a reaction mixture containing hydrogen peroxide.

Statistical analysis

Assays were repeated three times and analyzed statistically. The normality of the data was examined with the Kolmogorov–Smirnov test and the homogeneity of variance with the Levene Median test. If both the normal distribution and equal variance tests were passed, intergroup comparisons were carried out using a two-way analysis of variance test followed by all pairwise multiple comparison procedures using Tukey's post-hoc test. The data are expressed as the mean ± standard deviation. The criterion for the significance was $P < 0.05$. All analyses were carried out using a Sigma Statistical Analysis software package (Sigma 2.03, 2001; St. Louis, MO, USA).

Results

Population doubling in the presence of H_2O_2

The PD of the HUVEC was assessed on day 3 of culture. The PD appeared to decrease proportionally with the increasing concentration of H_2O_2 (Fig. 1a). The decreased PD showed that cell growth was suppressed by the presence of H_2O_2 in a concentration-dependent manner.

Cell senescence induced by the presence of H_2O_2

SA-β-Gal expression was highest at 200 μmol/L H_2O_2 (Fig. 1b). The oxygen stress produced by H_2O_2 seemed to increase the number of senescent cells concentration-dependently until 200 μmol/L, whereas the 500-μmol/L concentration of H_2O_2 seemed to greatly damage cells and to increase cell death rather than induce cell senescence (Fig. 1).

Mean TRF level and its distribution in the presence of H_2O_2

The mean TRF of the cultured HUVEC on day 3 was measured to see how much H_2O_2 affected the telomeric DNA. The mean TRF level became shorter in the presence of H_2O_2 in a concentration-dependent manner (Fig. 2a). The telomeric attrition was accompanied by an alteration in the telomere length distribution (Fig. 2b). A tendency for there to be a decrease in the longer telomeres and an increase in the shorter telomeres was observed (Fig. 2b). However, the longest telomere region (>23.1 kb) was not affected by the incubation with H_2O_2.

Telomerase activity in the presence of H_2O_2

To determine how the telomerase activity was affected by H_2O_2, the activity of the cultured HUVEC was evaluated by a TRAP assay. Unexpectedly, the

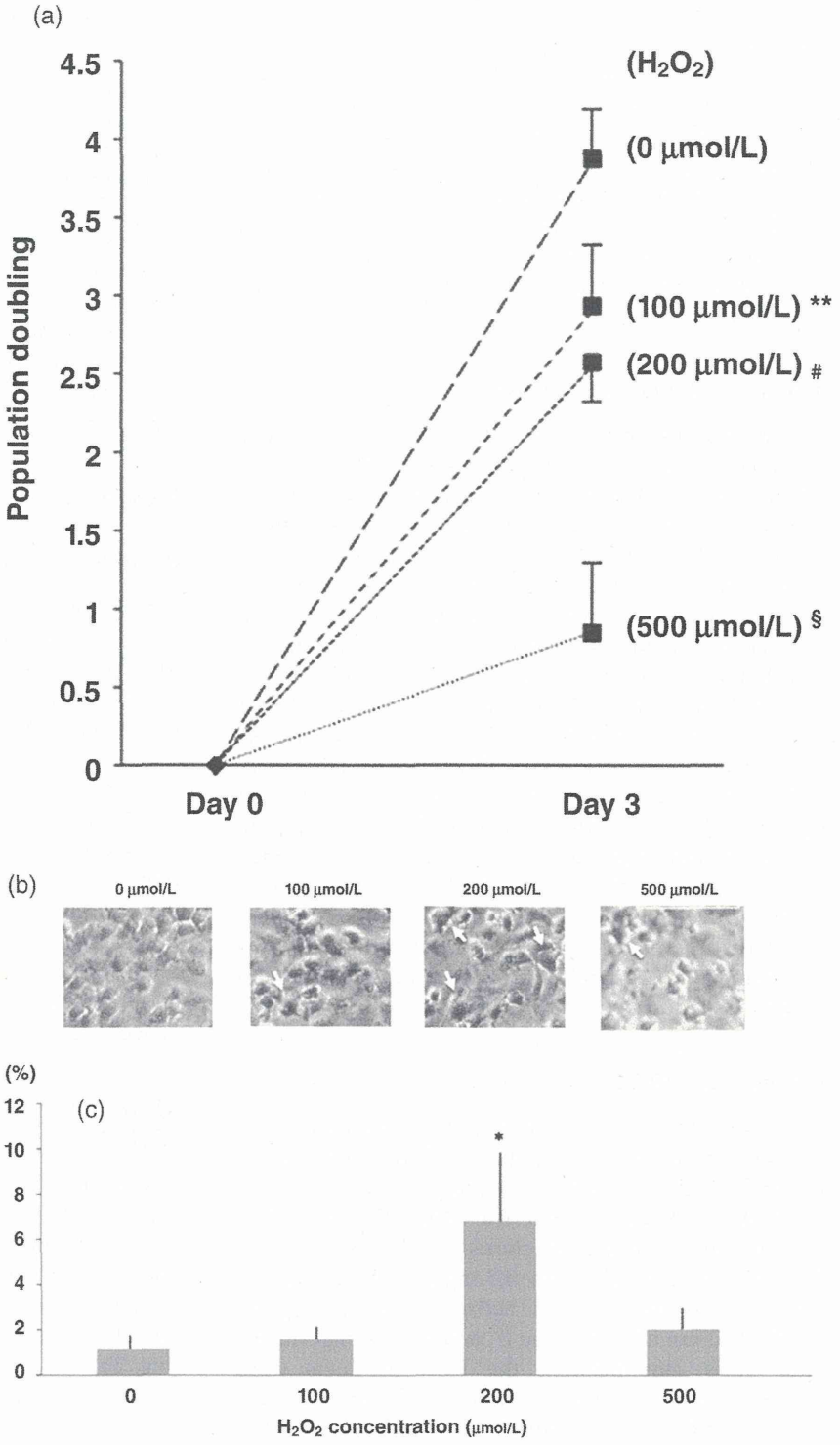

Figure 1 The population doubling (PD) and the ratio of senescence-associated β-galactosidase (SA-β-Gal) staining of human umbilical venous endothelial cells (HUVEC) cultured in the presence of H_2O_2. (a) The PD after 3 days in culture. The horizontal bars are standard deviations. $^{**}P < 0.01$, 0 μmol/L versus 100 μmol/L. $^{\#}P < 0.05$, 100 μmol/L versus 200 μmol/L. $^{\S}P < 0.05$, 200 μmol/L versus 500 μmol/L. (b) The SA-β-Gal-stained cells (white arrows) at different H_2O_2 concentrations. (c) The percentages of the cells that were stained. $^{*}P < 0.05$ versus control (at 0 μmol/L of H_2O_2).

telomerase activity in the H_2O_2-treated HUVEC did not decrease in a concentration-dependent manner or even tend to show an inverse correlation with the H_2O_2 concentration, despite the shortening of the TRF. Indeed, little telomerase activity was detected at 0 μmol/L, and elevated activity was detected only in the H_2O_2-treated cells, although the elevation was not statistically significant (Fig. 3). A similar analysis was carried out using BAEC to confirm this result. The H_2O_2-treated BAEC did not show a significant increase either, yet a similar tendency toward an inverse correlation was also observed (Fig. 3). The variation in the telomerase activity

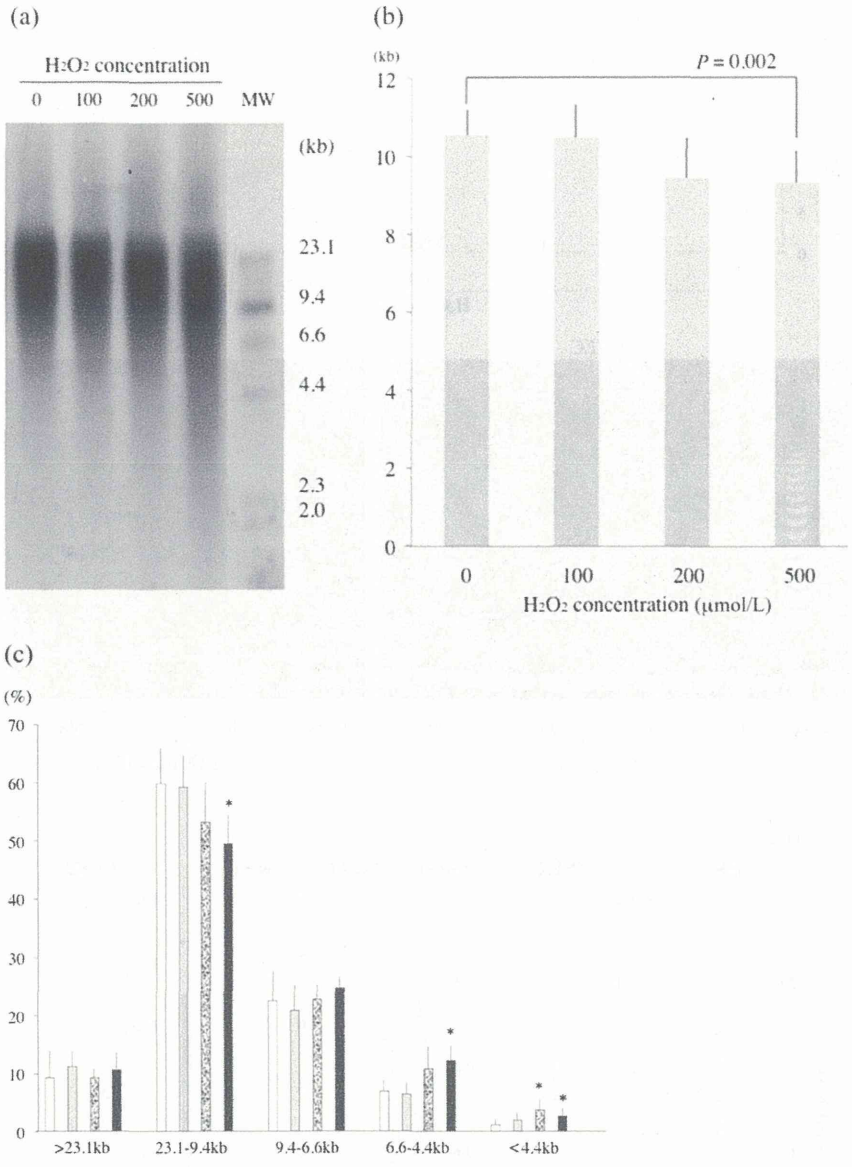

Figure 2 The mean telomere length and the telomere length distribution of human umbilical venous endothelial cells cultured in the presence of H_2O_2. (a) A representative genomic Southern blot result with telomere DNA probe is shown. (b) The mean telomere lengths are shown. The *Msp*I-terminal restriction fragment lengths are presented as the mean values ± standard deviation. The horizontal bars represent the standard deviation. A significant difference was observed between the control cells and those treated with 500 μmol/L. (c) The telomere length distribution. Notice that the longest telomere range (>23.1 kb) was not changed in the cells exposed to different concentrations of H_2O_2. The horizontal bars represent the standard deviation.

detected at higher H_2O_2 concentrations implied a variation in the telomerase activity among cells exposed to H_2O_2. Some cell subpopulations are hypothesized to sporadically express elevated telomerase activity and others are not. The telomerase activity did not change with a positive control cell lysate supplied by the manufacturer in the presence of 500 μmol/L of H_2O_2 (data not shown).

Expression of TERT and TERC

The expressions of the protein component of telomerase (TERT) and the RNA component of telomerase (TERC) were assessed. The expression of TERT increased at 100 μmol/L, and decreased at higher concentrations of H_2O_2. The expression of TERC was elevated at 100 μmol/L, and returned to the original level at higher concentrations. The level of phosphorylated TERT (p-TERT), of which the phosphorylation site is Ser^{824} located at motif 4 in a conservative region, was high at 0 μmol/L and 100 μmol/L of H_2O_2, decreased at 200 μmol/L, and was almost undetectable at 500 μmol/L. This p-TERT expression pattern seemed a reversed pattern of telomerase activity, whereas neither of the expression patterns of TERT nor that of TERC corresponded to the levels of telomerase activity at different concentrations of H_2O_2 (Fig. 4).

Expression of telomere-associated proteins

The observation of telomere-shortening and elevated telomerase activity in the cells led us to examine

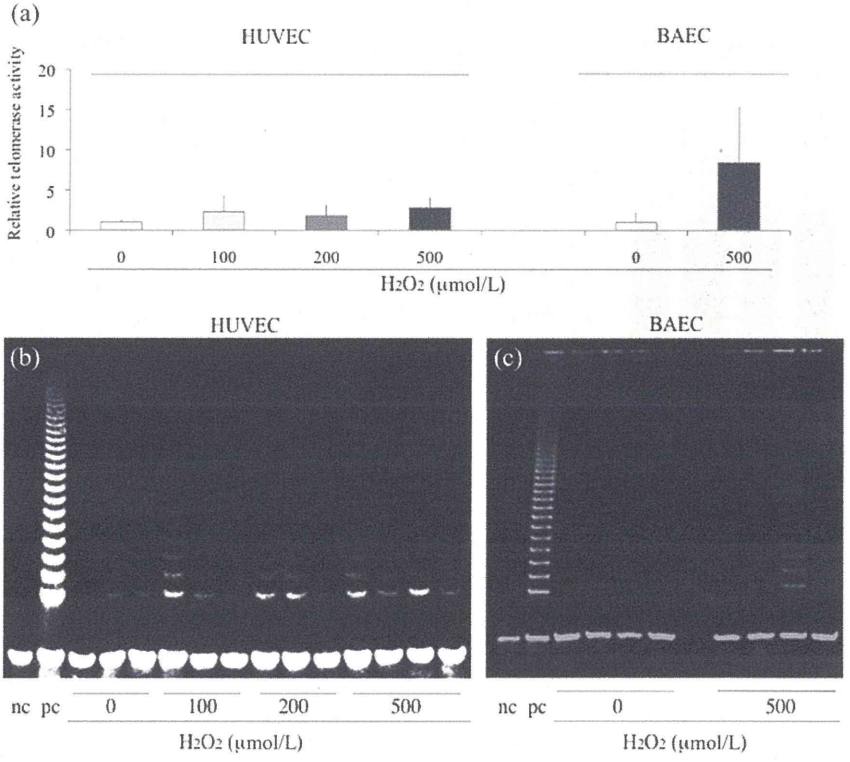

Figure 3 The telomerase activity of endothelial cells in the presence of H_2O_2. (a) The relative telomerase activity at different H_2O_2 concentrations (μmol/L). The relative telomerase activity is presented as a proportional ratio of the density of the ladder of a sample to that of the mean value at 0 μmol/L of H_2O_2. (b,c) Photographs of representative telomerase repeat amplification protocol assay results for (b) human umbilical venous endothelial cells (HUVEC) and (c) bovine aortic endothelial cells (BAEC) at different H_2O_2 concentrations. The materials used for the positive control (pc) and negative control (nc) were provided with the kit.

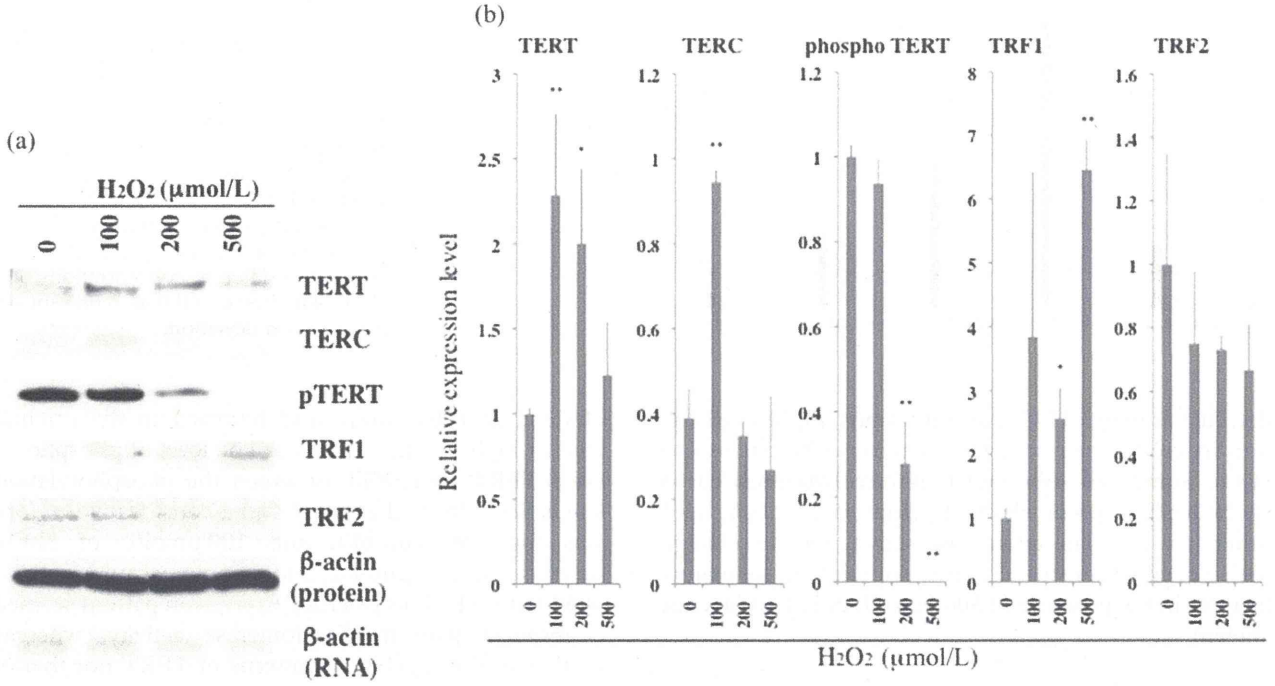

Figure 4 The expression levels of telomerase RNA component (TERC), and telomere-associated proteins. (a) A TERC RNA reverse transcription polymerase chain reaction (RT–PCR) result and a representative western blot analysis result showing the expression of TERC RNA and telomere-associated proteins (TERT; phosphorylated TERT, TRF1 and TRF2) at different H_2O_2 concentrations. The photographs of the RT–PCR result of TERC and β-actin are provided as inversed positive images. (b) Relative expression levels. The relative expression levels were determined in comparison with that of β-actin (set at 1), β-actin (RNA) for TERC and β-actin (protein) for western results. Horizontal bars represent standard deviations. $n = 6$. $^*P < 0.05$, $^{**}P < 0.01$ versus control (at 0 μmol/L of H_2O_2).

whether the expression of stress-induced proteins associated with maintaining the telomere structure or controlling cell senescence were altered in the H_2O_2-treated HUVEC. As the TRF1 expression was elevated, the H_2O_2 concentration increased. The expression level was highest at 500 μmol/L, similar to the telomerase activity. The TRF2 expression showed a mild trend to be lowered in relation to H_2O_2 concentration, but it was not significant.

Discussion

Although previous studies have shown that reactive oxygen species (ROS) have negative effects on the maintenance of telomere length and the control of telomerase activity in various cells, the ROS-associated telomere changes have been described well in cells with high telomerase activity, such as cancer cells, tissue stem cells or cells transduced with a TERT expression vector.[28–32] In the present study, HUVEC, somatic cells with little telomerase activity, were used to analyze the telomere-associated alterations induced by H_2O_2. Vascular endothelial cells are one of the main sites involved in the process of atherosclerosis, which is highly exposed to ROS species even under physiological conditions, because the circulating blood contains high oxygen pressure brought by hemoglobin. Thus, vascular endothelial cells can be exposed to more ROS compared with the other tissues contacting tissue fluids containing lower oxygen pressure.

We chose H_2O_2 as a ROS donor in the present study to analyze the ROS-associated telomeric changes in vascular endothelial cells. In the presence of H_2O_2, cell growth was suppressed, and the telomere length was shortened. The H_2O_2-associated changes in the telomere length distribution were similar to the aging-associated telomeric changes in peripheral blood leukocytes of aged subjects, where the number of long telomeres decreases and short telomeres increases.[4] This confirms that our experimental system using HUVEC culture in the presence of H_2O_2 provides a useful model of the aging-associated features of somatic telomere biology. Therefore, this culture condition can be applicable to an assay of the anti-aging effect of candidate chemicals. However, the present study showed some unexpected results. The amount of the longest telomere was stable at all of the H_2O_2 concentrations examined. Second, the telomerase activity was not suppressed in relation to the concentration of H_2O_2. In addition, the telomerase activity was not associated with the expression level of telomerase mRNA or protein. The longest telomeres (>23.1 kb) were maintained at a constant level, even at the highest H_2O_2 concentration (500 μmol/L). Endothelial cells rich in the longest telomeres, which can be regarded as young cells, might therefore be resistant to oxygen stress from H_2O_2, whereas the cells bearing shorter telomeres might be impaired more easily, and telomere attrition might be accelerated in these cells with a lower efficiency of telomere length maintenance. The present study found that telomerase activity was not suppressed by H_2O_2 in HUVEC; in contrast, some cell populations seemed to show elevated telomerase activity. This phenomenon was observed not only in HUVEC, but also in BAEC, thus suggesting that such sporadic apparent H_2O_2-associated telomerase activation is not human-specific, but might be common in mammalian vascular endothelial cells. However, previous studies have shown that telomerase activity in different cells was reduced in culture with H_2O_2.[18,33] This discrepancy seems to be partly because of difference in culture period. In the present study, cells were exposed continuously for 3 days. This was much longer compared with the conditions used in previous reports, where the culture period was at most several hours. During the 3 days, cells resistant to oxidative condition with elevated telomerase activity could grow selectively. A long period of culture in the presence of H_2O_2 can be applied to select cells, which have the potential to induce telomerase activity. However, the elevated telomerase activity of a limited number of cells could not completely prevent telomere shortening of the whole cell population. Cells bearing higher telomerase activity might contribute to maintaining cells with the longest telomeres, and might give cells a survival advantage. This elevation in telomerase activity might be a result of cellular protective effects against exogenous oxygen stress. For example, it is known that keratinocytes exposed to a low concentration of H_2O_2 have a prolonged lifespan.[34] These findings implicate the possible existence of a hormesis mechanism suppressing ROS-induced cell senescence. In the present study, the telomerase activation did not result in telomere elongation. Although the longest telomeres were maintained by telomerase activation, the mean telomere length was shortened in the presence of H_2O_2. Telomerase seemed to elongate and maintain the telomere length less efficiently under an elevated ROS condition for the shorter telomeres. The partly elevated telomerase activity might function not only for the maintenance of the longest telomere length, but also for protecting cells against H_2O_2-mediated genotoxic effects.[18,35] Or a small subpopulation of cells with the longest telomeres might have a higher level of telomerase, whereas the larger subpopulation; that is, the subpopulation with shorter telomeres, might have low telomerase activity, resulting in the average of all the telomere lengths appearing to be shorter under the higher H_2O_2 concentration.

The vascular endothelium is exposed to ROS by the bloodstream, which contains a higher oxygen concentration than the tissue fluids.[36] A protective mechanism

against ROS-mediated cell damage therefore seems more necessary for vascular endothelial cells than for other cell types located in tissues exposed to less oxidative conditions. The observed fluctuation in the telomerase activity in the surviving HUVEC exposed to high H_2O_2 concentrations might be partially beneficial for cell survival. Telomerase activation in some cells might therefore be mediated by a post-translational modification, such as the phosphorylation of an amino acid residue essential for the telomerase activation.[37,38] We assessed a candidate phosphorylation of telomerase TERT, Ser^{824}-phosphorylation. Contrary to our expectancy, the expression level of Ser^{824}-phosphorylated TERT decreased as H_2O_2 concentration increased. It might be unlikely, but still possible that the Ser^{824} phosphorylation of TERT might be negatively associated with telomerase activity. It is not clear whether the phosphorylation at Ser824 controls telomerase activity, or telomerase activity might be controlled by the phosphorylation of different sites, or by other kinds of modification. Further study is therefore required to elucidate the mechanism for the maintenance of the telomerase activity or of the partial telomerase activation observed in the presence of H_2O_2. We also analyzed telomere-associated proteins, TRF1 and TRF2, which might affect telomerase activity. TRF1 has been reported to negatively control the telomerase-associated telomere length maintenance,[39] whereas TRF2 is associated with stabilizing the telomere structure.[40,41] However, TRF2 is also associated with telomere shortening.[42] And under telomere-erosive conditions, TRF 1 can contribute to telomere stability.[43,44] The TRF1 expression of HUVEC increased in association with the elevation of H_2O_2 concentration, whereas the TRF2 expression did not change significantly, but only with a weak decreasing trend in reaction to elevated concentrations of H_2O_2. The elevation of TRF1 expression might rather be a reaction to H_2O_2-associated enhancement of telomerase activity to stabilize telomere structure than an accelerator of telomerase activity.

Our observation of the maintenance or fluctuating enhancement of the telomerase activity might be one of the aspects of the hormesis effect, in which telomerase activity and the expression of TRF1 are enhanced, possibly contributing to cell survival under hyperoxidative condition by H_2O_2. Somatic cells with highly elevated telomerase activity, which have survived through such severe hyperoxidative conditions, might lead to tumorigenesis.[41,45] The cellular mechanism(s) responsible for the protective enhancement in the telomerase activity of somatic cells injured by high oxygen stress will need to be elucidated in further studies. Alterations in the behaviors of the telomere structure-associated components in vascular endothelial cells exposed to elevated oxygen stress conditions also warrant further investigation.

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Disclosure statement

No conflict of interest.

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