Heart Vessels
DOI 10.1007/s00380-011-0220-7

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

Jun-ichi Oyama · Yoshihiro Kudo · Toyoki Maeda · Koichi Node · Naoki Makino

Received: 22 July 2011 / Accepted: 8 December 2011

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

J. Oyama (✉) · T. Maeda · N. Makino
Department of Cardiovascular, Respiratory and Geriatric Medicine, Kyushu University Hospital at Beppu and Medical Institute of Bioregulation, Kyushu University, 4546 Tsurumihara, Beppu, Oita 874-0838, Japan
e-mail: joyama@tsurumi.beppu.kyushu-u.ac.jp

J. Oyama · K. Node
Department of Cardiovascular Medicine, Saga University, 5-1-1 Nabeshima, Saga 849-8501, Japan

Y. Kudo
Department of Rehabilitation, Kyushu University Hospital at Beppu and Medical Institute of Bioregulation, Kyushu University, 4546 Tsurumihara, Beppu, Oita 874-0838, Japan

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

Published online: 11 January 2012

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.

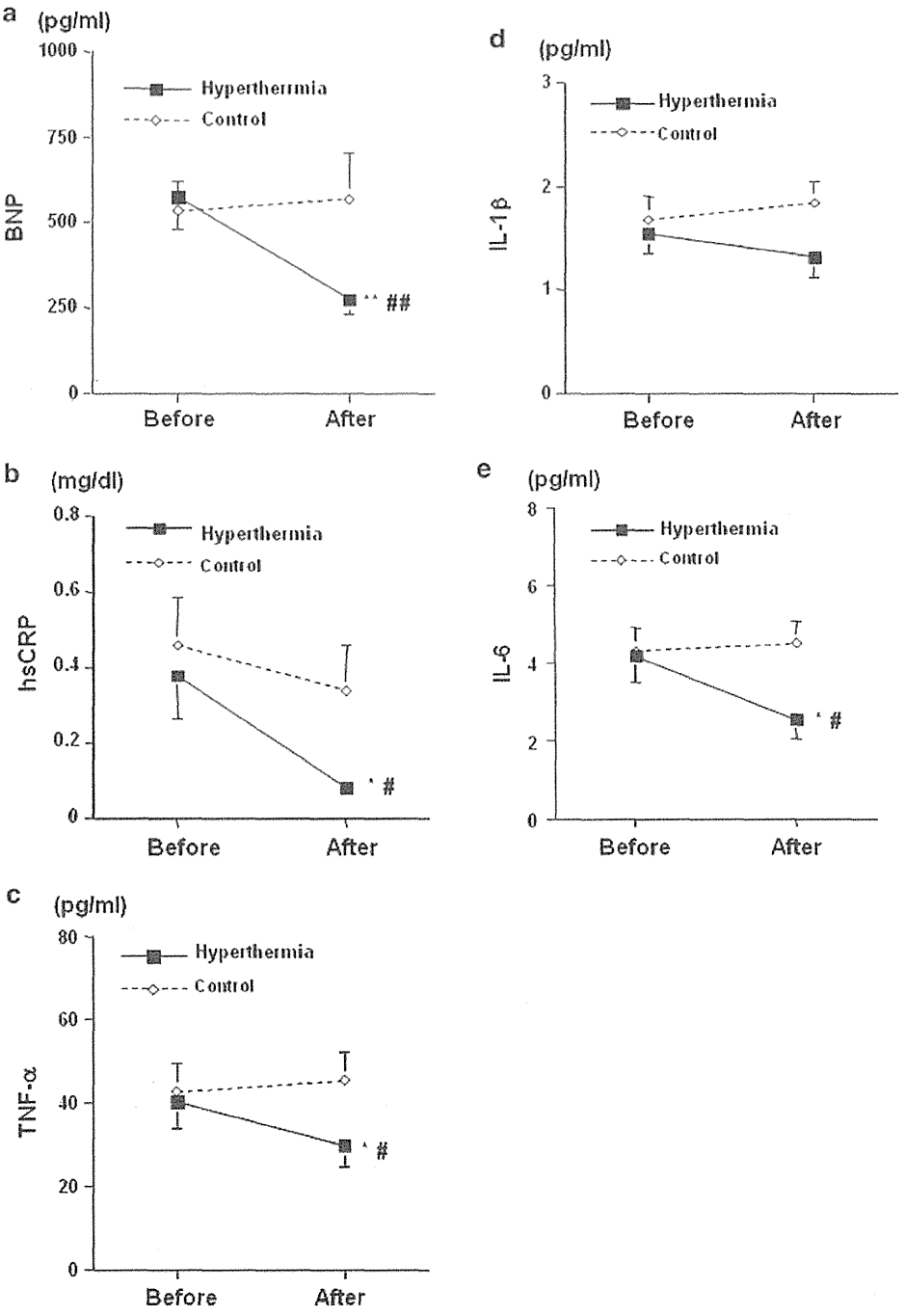


Fig. 1 Changes in the plasma level of BNP (**a**), hsCRP (**b**), TNF-α (**c**), IL-1β (**d**), and IL-6 (**e**) in each group. $^{*}p < 0.05$, $^{**}p < 0.01$ versus control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus before intervention

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

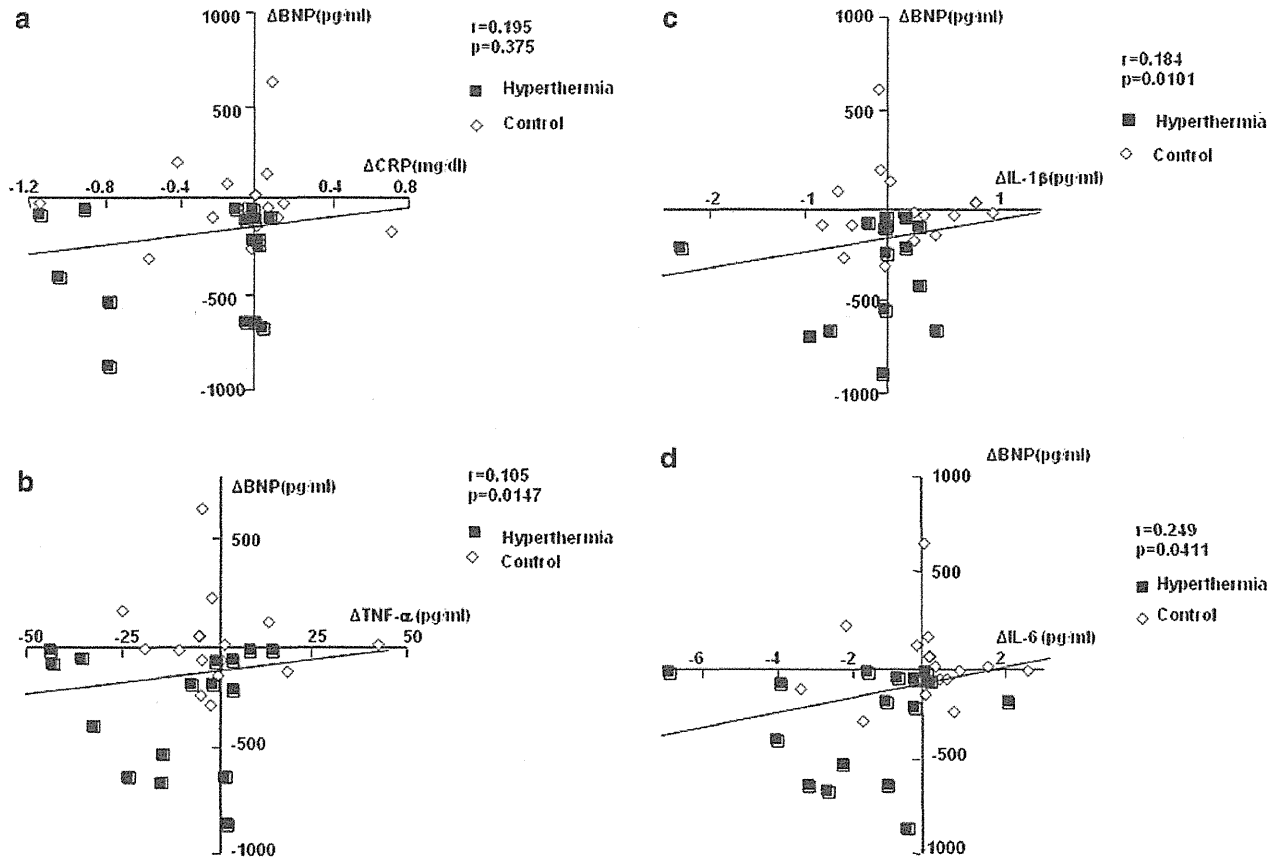


Fig. 2 The correlations between the changes in the level of BNP and the levels of hsCRP (**a**), TNF-α (**b**), IL-1β (**c**), and IL-6 (**d**) in all groups. Correlation coefficient and p value are indicated

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.

References

1. Jessup M, Brozena S (2003) Heart failure. N Engl J Med 348:2007–2018
2. Tei C, Horikiri Y, Park JC, Jeong JW, Chang KS, Toyama Y, Tanaka N (1995) Acute hemodynamic improvement by thermal vasodilation in congestive heart failure. Circulation 91:2582–2590
3. Gabrielsen A, Sørensen VB, Pump B, Galatius S, Videbaek R, Bie P, Warberg J, Christensen NJ, Wroblewski H, Kastrup J, Norsk P (2000) Cardiovascular and neuroendocrine responses to water immersion in compensated heart failure. Am J Physiol Heart Circ Physiol 279:H1931–H1940
4. Miyamoto H, Kai H, Nakaura H, Osada K, Mizuta Y, Matsumoto A, Imaizumi T (2005) Safety and efficacy of repeated sauna bathing in patients with chronic systolic heart failure: a preliminary report. J Card Fail 6:432–436
5. Tei C (2001) Thermal therapy for congestive heart failure: estimation by TEI index. J Cardiol 37(Suppl 1):155–159
6. Kihara T, Biro S, Imamura M, Yoshifuku S, Takasaki K, Ikeda Y, Otuji Y, Minagoe S, Toyama Y, Tei C (2002) Repeated sauna treatment improves vascular endothelial and cardiac function in patients with chronic heart failure. J Am Coll Cardiol 39:754–759
7. Palazzuoli A, Antonelli G, Quatrini I, Nuti R (2011) Natriuretic peptides in heart failure: where we are where we are going. Intern Emerg Med 6:63–68
8. Kurt IH, Yavuzer K, Batur MK (2010) Short-term effect of levosimendan on free light chain kappa and lambda levels in patients with decompensated chronic heart failure. Heart Vessels 25:392–399
9. Ikeda N, Nakamura M, Yazaki Y, Ono T, Yamamoto M, Ito S, Yokouchi I, Yajima S, Iijima R, Hara H, Takagi T, Hara H, Asahara T, Sakai H, Sugi K (2011) A slightly elevated level of N-terminal pro-brain natriuretic peptide can predict coronary artery disease in a population with normal left ventricular function. Heart Vessels 26:473–479
10. Levine B, Kalman J, Mayer L, Fillit HM, Packer M (1990) Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. N Engl J Med 323:236–241
11. Matsumori A, Yamada T, Suzuki H, Matoba Y, Sasayama S (1994) Increased circulating cytokines in patients with myocarditis and cardiomyopathy. Br Heart J 72:561–566
12. Kubota T, McTiernan CF, Frye CS, Slawson SE, Lemster BH, Koretsky AP, Demetris AJ, Feldman AM (1997) Dilated cardiomyopathy in transgenic mice with cardiac-specific overexpression of tumor necrosis factor-α. Circ Res 81:627–635
13. Oyama J, Shimokawa H, Momii H, Cheng X, Fukuyama N, Arai Y, Egashira K, Nakazawa H, Takeshita A (1998) Role of nitric oxide and peroxynitrite in the cytokine-induced sustained myocardial dysfunction in dogs in vivo. J Clin Invest 101:2207–2214
14. Jug B, Salobir BG, Vene N, Sebestjen M, Sabovic M, Keber I (2009) Interleukin-6 is a stronger prognostic predictor than high-sensitive C-reactive protein in patients with chronic stable heart failure. Heart Vessels 24:271–276
15. Chrysohoou C, Pitsavos C, Barbetseas J, Kotroyiannis I, Brili S, Vasiliadou K, Papadimitriou L, Stefanadis C (2009) Chronic systemic inflammation accompanies impaired ventricular diastolic function, detected by Doppler imaging, in patients with newly diagnosed systolic heart failure (Hellenic Heart Failure Study). Heart Vessels 24:22–26
16. Yin WH, Chen JW, Jen HL, Chiang MC, Huang WP, Feng AN, Young MS, Lin SJ (2004) Independent prognostic value of elevated high-sensitivity C-reactive protein in chronic heart failure. Am Heart J 147:931–938
17. Araújo JP, Lourenço P, Azevedo A, Friões F, Rocha-Gonçalves F, Ferreira A, Bettencourt P (2009) Prognostic value of high-sensitivity C-reactive protein in heart failure: a systematic review. J Card Fail 15:256–266
18. Celik T, Iyisoy A, Celik M, Yuksel UC, Kardesoglu E (2009) C-reactive protein in chronic heart failure: a new predictor of survival. Int J Cardiol 135:396–397

Aging Clin Exp Res
DOI 10.1007/s40520-013-0045-6

ORIGINAL ARTICLE

Alterations in the telomere length distribution and the subtelomeric methylation status in human vascular endothelial cells under elevated temperature in culture condition

Toyoki Maeda · Guan Jing-Zhi · Masamichi Koyanagi · Naoki Makino

Received: 30 July 2012 / Accepted: 3 January 2013

Abstract Temperature-associated alteration in the telomere lengths of vascular endothelial cells has not been well investigated. Telomere length of human umbilical vein endothelial cells (HUVECs) cultured at a high temperature (42 °C) was analyzed. Here described are heat-associated phenotypical alterations of human vascular endothelial cell under prolonged heat stress in terms of telomere length, telomerase activity, and the expression of telomere associated proteins and heat shock proteins. The genomic DNA extracted from HUVECs cultured for 3 days under 42 °C was digested with methylation-sensitive and -insensitive isoschizomers and was subjected to genomic Southern blot probed with a telomere DNA fragment. Their telomere lengths and telomere length distributions were analyzed. Telomerase activity and the expressions of telomere-associated RNA, telomere-associated proteins (TERC, TERT, TRF1, and TRF2), and heat shock proteins (Hsp60, Hsp70, and Hsp90) were also analyzed. At 42 °C, cell growth was suppressed and the cell senescence rate was transiently elevated. A proportional decrease in the number of long telomeres was observed transiently at 42 °C. A trend of subtelomeric hypomethylation and lowered telomerase activity were observed at 42 °C after 3-day culture. The altered phenotypes on day 1 seemed reactive responses for cell protection to heat, and those on day 3 seemed exhausted reactions after 3-day culture. Maintained expression was observed in Hsps, TRF2, and TERC. These altered phenotypes might contribute to cell-survival under prolonged heat stress.

Keywords Heat stress · Vascular endothelial cell · Telomere · Subtelomere · DNA methylation

Introduction

Telomeres consist of repetitive DNA sequences with accessory protein components (TRF1, TRF2, and others) capping the terminals of chromosomes [1]. It is well known that telomere DNA shortening occurs during every cell cycle due to the duplication process that produces slightly shorter DNA strands. In addition, the DNA methylation status, one of the genomic epigenetic conditions, in telomeric region has been reported to alter in response to human telomere length changes [2–6]. Telomere length in somatic cells is negatively affected by stress factors [6]. Both pathological mental and physical stress accelerate telomere attrition [6]. Telomere shortening occurs in somatic cells with aging due to the occurrence of many rounds of the cell cycle and pathological stress [7]. On the other hand, a telomere-elongating cellular mechanism functions in limited cases. Telomerase consists of a protein component composed of reverse transcriptase (TERT) and an RNA component (TERC). Telomerase contributes to telomere elongation or telomere length maintenance in unique cell populations with active mitotic potential, such as cancer cells, stem cells, and reproductive cells. Generally, however, the telomere activity is suppressed to low levels in somatic cells and is not adequate to prevent the

T. Maeda and G. Jing-Zhi equally contributed to this article.

T. Maeda (✉) · M. Koyanagi · N. Makino
The Department of Cardiovascular, Respiratory and Geriatric Disease, Kyushu University Beppu Hospital, Beppu, Oita 874-0838, Japan
e-mail: maedat@beppu.kyushu-u.ac.jp

G. Jing-Zhi
The 309th Hospital of Chinese People's Liberation Army, Beijing, China

Journal : Large 40520 | Dispatch : 7-5-2013 | Pages : 8
Article No. : 45 | LE | TYPESET
MS Code : Original Article-Maeda | CP | DISK

telomere shortening that occurs during cell division. Telomerase activity is also affected by stress factors. However, the effects of heat stress on telomere length, subtelomeric methylation status, or telomerase activity have not been well studied. Human vascular endothelial cells have been used to analyze pathological stress-related changes in cell biology [8–10]. Yet there have been no reports that address the telomeric changes in vascular endothelial cells occurring under heat stress. Although the effects of transient heat stress on cells have been well investigated, no reports evaluating prolonged heat stress lasting more than 1 day have been published. Heat stress in vascular endothelial cells also induces the expression of various kinds of stress response genes, representatively, heat shock proteins (Hsps) [11, 12]. Hsps are ubiquitously synthesized in virtually all species and it is hypothesized that they might have beneficial health effects [13]. In response to stress stimuli, mammalian cells activate a signaling pathway leading to the transient expression of Hsp. Hsps are a family of proteins serving as molecular chaperones that prevent the formation of nonspecific protein aggregates and assist proteins in the acquisition of their native structures. Physiologically, Hsps play a protective role in the homeostasis of the vessel wall consisting of endothelial cells and smooth muscle cells [14]. We analyzed telomere DNA length, telomerase activity, and the expressions of telomere-associated components, and heat shock proteins under heat stress conditions using human vascular endothelial cells exposed for 1 day or longer to heat in culture.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics Corp. (San Diego, CA, USA). The cells were cultured in endothelial cell growth medium (Clonetics Corp.) at 37 or 42 °C and 5 % CO_2 in a gelatin-coated flask (Iwaki Glass, 2 Chiba, Japan). Culture media were refreshed every 24 h. On day 1 or 3, the cells were collected and were counted using a hemocytometer. Population doublings (PDs) were calculated using the formula: PD = [log (expansion)/log2], where expansion was the number of cells harvested divided by the initial number of cells seeded.

Senescence-associated β-galactosidase (SA-β-Gal) expression

The cells were washed in PBS, fixed for 10 min at room temperature in 2 % formaldehyde/0.2 % glutaraldehyde, and incubated at 37 °C (no CO_2) with fresh SA-β-Gal staining solution containing 1 mg/mL of X-gal, pH 6.0 for 12 h. One hundred cells were scored from each well (plate) using a light microscope.

Telomere detection

Telomere detection was performed as previously described [4]. Briefly, cell DNA (0.1 μg) was digested with methylation-insensitive or -sensitive isoschizomers, *Msp*I or *Hpa*II, at 37 °C for 2 h, and was subjected to Southern blot hybridization probed with telomere DNA $(TTAGGG)_n$. The autoradiogram was captured on an Image Master, and the telomere length was then assessed quantitatively (Fig. 1). Every sample was measured in triplicate.

Terminal restriction fragment (TRF) length analysis

Telomere length distribution was analyzed by comparing the telomere length using a telomere percentage analysis with three intervals of length (>9.4, 9.4–4.4 and <4.4 kb) as defined by a molecular weight standard as previously described [4]. The percent of the stratified intensity in each molecular weight range of a Southern blot result smear was measured for each sample. The mean TRF was estimated using the formula $S(OD_i - \text{background})/S(OD_i - \text{background}/L_i)$, where OD_i

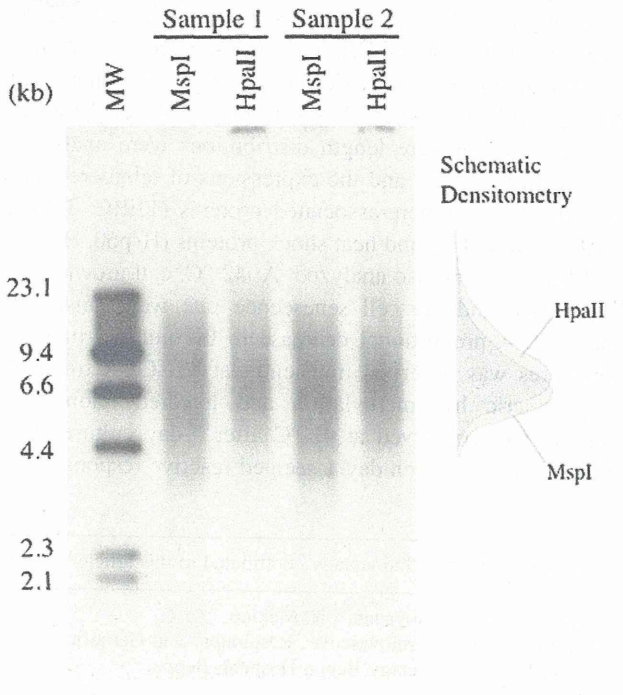


Fig. 1 Densitometric analysis of the isoschizomeric TRFs of *Msp*I- and *Hpa*II-digest. Representative Southern blot results of two samples digested with *Msp*I or *Hpa*II are shown. *Gray areas* on the *right* side depict *Hpa*II-densitometry and *Msp*I-densitometry, respectively

Journal : Large 40520 Dispatch : 7-5-2013 Pages : 8
Article No. : 45 LE TYPESET
MS Code : Original Article-Maeda CP DISK

is the chemiluminescent signal and L_i is the length of the TRF fragment at position i.

Semiquantitative RT-PCR for TERC RNA

Total RNA samples were extracted using RNAzol B (Tel-test). mRNA for human telomerase RNA component (TERC) was determined by RT-PCR using a DIG detection system (Roche Applied Science). Each human cDNA was produced by RT-PCR according to each human-derived sequence. For the amplification of β-actin cDNA, the forward primer β-actin (205 bp) 5′-CCTTCCTGGGCATGGAGTCCT-3′ and the reverse primer 5′-GGAGCAATGATCTTGATCTTC-3′ were used according to the published human TERC cDNA sequence [15]. And TERC forward primer 5′-TCTAACCCTAACTGAGAAGGGCGTAG-3′ and the reverse primer 5′-GTTTGCTCTAGAATGAACGGTGGAAG-3′ were used [16]. 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) and gene-specific primers according to the recommendations provided by the supplier. The PCR products were amplified through 15 cycles of chain reaction where the amplification is at an exponential phase. The PCR products were analyzed by agarose gel electrophoresis (1.3 %) followed by staining with ethidium bromide and scanning with Gel-Doc (Bio-Rad). For semiquantitative PCR, β-actin was used as an internal control to evaluate total RNA input, as described by our group [17].

Western blot and other analyses

Cells from a dish were homogenized with 100-μl lysis buffer (100 mM Tris pH 6.8, 4 % SDS, 20 % glycerol containing the protease inhibitor, M phenylmethanesulfonyl fluoride, 0.1 mM, leupeptin, 0.1 μl, and aprotinin, 0.1 μl). 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, Hercules, CA, USA) 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), TRF1 (Imgenex), TRF2 (Cell Signaling), Hsp60 (Assay designs), Hsp70 (Assay designs), Hsp90 (Enzo), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology). Detection was performed with secondary horseradish peroxidase-conjugated antibodies (Chemicon) and the ECL detection system as previously described [18]. The relative expression levels were determined compared to that of GAPDH.

Telomerase activity

Telomerase activity was examined by means of a modified telomerase repeat amplification protocol (TRAP) method with TeloChaser (Toyobo, Osaka, Japan), as previously described [19].

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 performed using a two-way analysis of variance (ANOVA) 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 is $p < 0.05$.

Results

Population doubling (PD) and cell senescence

The PD value of the HUVECs was assessed on day 1 and day 3 of culture. At 37 °C, the PD value increased to ~1.6 on day 1 and was found to be maintained at that level on day 3. At 42 °C, the PD value initially increased to ~1.2; however, it decreased steeply to ~0.2 on day 3 (Fig. 2a).

Senescence-associated β-galactosidase (SA-β-Gal) expression was observed in ~2 % of cells on day 1 and ~1 % of cells on day 3 at 37 °C and in ~4 % of cells on day 1 and ~2 % of cells on day 3 at 42 °C (Fig. 2b).

Such an initial increase and delayed decrease of cell senescence rate indicated that many cells entered cell senescence stage followed by cell death 3-day-cultured under heat. The proportion of senescent cells in the cell population which survived for 3-day heat of 42 °C was not less than that at 37 °C for 3 days. This indicates that the heat-sensible cells diminished on day 1 and day 3 at 42 °C, and the heat-tolerant cells remained on day 3 at 42 °C.

The mean TRF level and its distribution

The mean TL of the HUVECs was measured to assess the degree to which high-temperature conditions affect telomeric DNA (Fig. 3a). The mean *Msp*I-TRF values were 9.2 ± 1.0 kb at 37 °C for 1 day, 8.7 ± 0.3 kb at 37 °C for 3 days, 8.3 ± 1.0 kb for 1 day at 42 °C, and 8.9 ± 0.6 kb for 3 days at 42 °C. The mean *Hpa*II-TRF values were 10.1 ± 0.7 kb at 37 °C for 1 day, 9.6 ± 0.3 kb at 37 °C for 3 days, 9.3 ± 1.1 kb at 42 °C for 1 day, and 9.0 ± 1.0 kb

Journal : Large 40520 Dispatch : 7-5-2013 Pages : 8
Article No. : 45 LE TYPESET
MS Code : Original Article-Maeda CP DISK

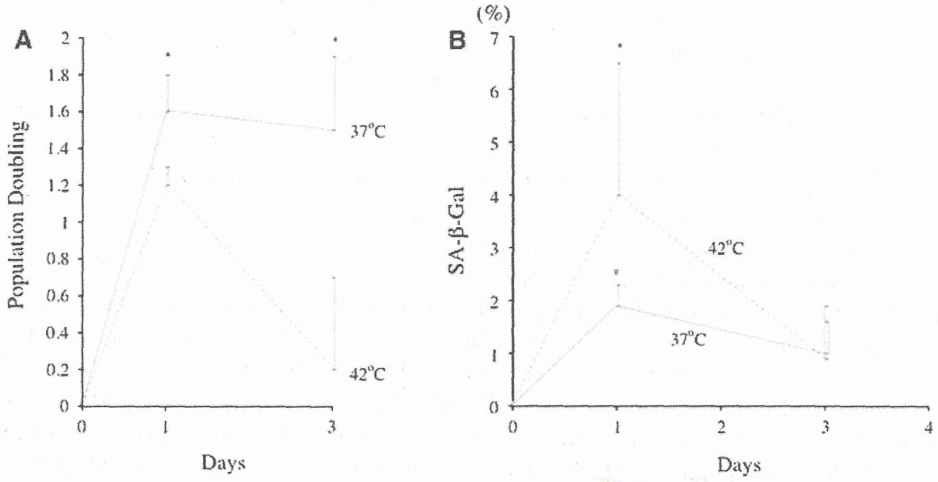


Fig. 2 The population doubling (PD) and the ratio of senescence-associated β-galactosidase (SA-β-Gal) staining of HUVECs cultured in the presence of H_2O_2. **a** The PD on day 1 and day 3 at 37 and 42 °C. The *horizontal bars* are standard deviations. $^*p < 0.05$, at 37 vs. 42 °C. **b** The percentages of SA-β-Gal-positive cells. $^*p < 0.05$, at 37 vs. 42 °C. $^{\#}p < 0.05$, on day 1 vs on day 3

at 42 °C for 3 days. The subtracted *Hpa*II–*Msp*I TRF values were 0.9 ± 1.0 kb at 37 °C for 1 day, 0.9 ± 0.4 kb at 37 °C for 3 days, 1.0 ± 0.9 kb at 42 °C for 1 day, and 0.1 ± 0.7 kb at 42 °C for 3 days. Thus, the mean TRFs did not altered at 42 °C, whereas the subtracted TRF *Hpa*II–*Msp*I was lower at 42 °C (0.1 ± 0.7 kb) than at 37 °C (0.9 ± 0.4 kb) on day 3 ($p = 0.04$). The % intensity of telomere length distribution (>9.4, 9.4–4.4, <4.4 kb) was as follows: 51 ± 8, 47 ± 6, 2 ± 3 % of *Msp*I at 37 °C for 1 day, 46 ± 1, 51 ± 2, 3 ± 2 % of *Msp*I at 37 °C for 3 days, 39 ± 11, 59 ± 10, 2 ± 1 % of *Msp*I at 42 °C for 1 day, 49 ± 5, 48 ± 3, 4 ± 2 % of *Msp*I at 42 °C for 3 days, 63 ± 5, 36 ± 5, 1 ± 1 % of *Hpa*II at 37 °C for 1 day, 60 ± 1, 38 ± 2, 2 ± 2 % of *Hpa*II at 37 °C for 3 days, 52 ± 14, 47 ± 14, 2 ± 2 % of *Hpa*II at 42 °C for 1 day, and 58 ± 5, 37 ± 3, 6 ± 4 % of *Hpa*II at 42 °C for 3 days. (Fig. 3b, c) The difference between the telomere length distribution between *Msp*I and *Hpa*II was as follows: 12 ± 6, −11 ± 4, −1 ± 4 % at 37 °C for 1 day, 14 ± 1, −13 ± 2, −1 ± 2 % at 37 °C for 3 days, 13 ± 5, −13 ± 5, 0 ± 3 % at 42 °C for 1 day, 9 ± 4, −11 ± 2, 2 ± 3 % at 42 °C for 3 days, >9.4, 9.4–4.4, <4.4 kb, respectively (Fig. 3d). The telomere length was affected significantly in *Msp*I-distribution and in *Hpa*II–*Msp*I-subtracted distribution. At 42 °C on day 1, long telomeres (>9.4 kb) decreased ($p = 0.02$) and middle-sized telomeres (9.4–4.4 kb) increased ($p = 0.03$). The amount of short telomeres (<4.4 kb) was not significantly affected. These changes in TL distribution disappeared on day 3, suggesting that cells bearing altered telomere length distribution diminished up to day 3. The alteration of subtelomeric methylation status appeared on day 3, which is a trend of subtelomeric hypomethylation of long telomeres ($p = 0.02$) (Fig. 3d).

Telomerase activity

The telomerase activity of the HUVECs was evaluated at 37 °C and 42 °C using TRAP assays (Fig. 4). The average value of TPG at 37 °C was set at 1 (1 ± 0.66 for 1 day and 1 ± 0.2 for 3 days). The relative levels of TPG at 42 °C were 1.32 ± 0.84 on day 1 and 0.57 ± 0.28 on day 3. Therefore, the relative level of telomerase activity in the HUVECs at 42 °C was maintained on day 1 ($p = 0.71$); however, it significantly decreased on day 3 ($p = 0.03$). The level of telomerase activity decreased under prolonged heat stress at 42 °C.

Expression of telomere-associated components and others

All telomere-associated components (TERT, TRF1 and TRF2) except TERC were upregulated on day 1 and downregulated on day 3. The expression of TERC did not seem to be affected by heat (Fig. 5; Table 1).

All analyzed heat shock proteins (Hsp60, Hsp70 and Hsp90) were upregulated on day 1 and downregulated on day 3 at 42 °C. However, only Hsp70 maintained a significantly higher expression level at 42 °C than at 37 °C.

Discussion

Biological stress has been reported to induce negative effects on the maintenance of telomere length in various cells [1, 20]. However, heat stress-associated telomeric changes have not been well investigated thus far. In the present study, vascular endothelial cells were used to analyze telomere-associated alterations induced at 42 °C.

Journal : Large 40520 Dispatch : 7-5-2013 Pages : 8
Article No. : 45 LE TYPESET
MS Code : Original Article-Maeda CP DISK

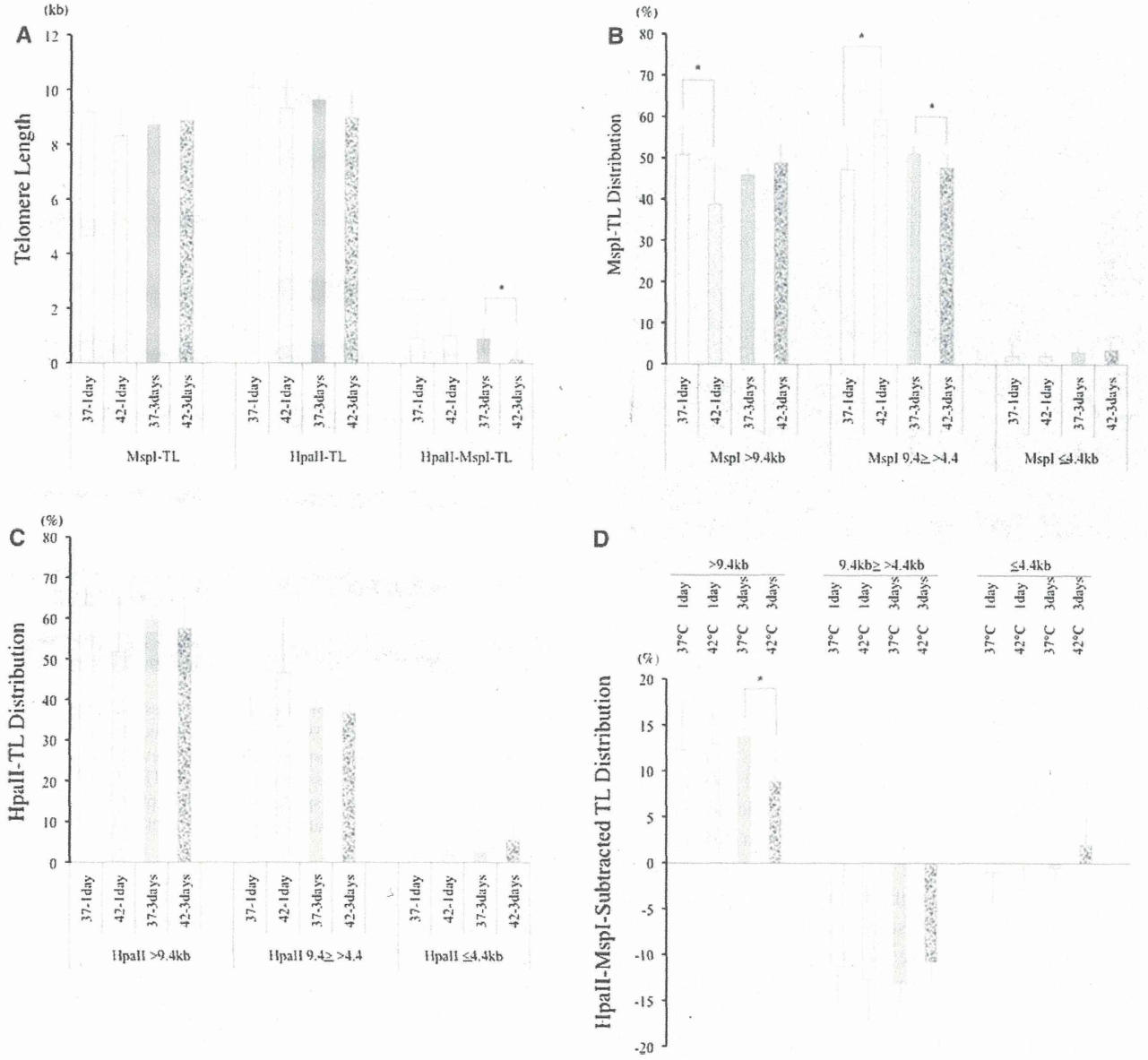


Fig. 3 The telomere length, the telomere length distribution of HUVECs exposed at different temperatures. The mean telomere lengths (**a**) and the telomere length distributions (**b–d**) are shown. The *Hpa*II–*Msp*I-subtracted percentages of telomere length range indicated (>9.4, 9.4–4.4, or <4.4 kb) are shown. Notice that only the longest telomere range (>9.4 kb) showed a significant difference between 37 and 42 °C (**d**). The *horizontal bars* represent the standard deviation. The 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 at 37 °C and those at higher temperatures. $^{*}p < 0.05$ vs at 37 °C

Cell growth was found to be suppressed at 42 °C, especially on day 3. The proportion of senescent cells increased on day 1 at 42 °C, then returned to a low level as observed at 37 °C on day 3. This observation indicates that the heat-sensitive cell population decreased in size during the 3-day exposure to 42 °C and the heat-resistant population survived beyond day 3. To the best of our knowledge, this is the first report to assess alterations in telomere length distribution under prolonged heat stress. Furthermore, the effects on cells of transient heat stress have been reported to occur within several hours; however, no reports have evaluated prolonged heat stress lasting more than 1 day.

In the present study, the telomere length and the subtelomeric methylation status were analyzed in heat-exposed cultured cell, to assess whether the heat stress suppresses or accelerates aging-associated phenotypes. Regarding telomere length distribution, the number of long telomeres decreased and the number of medium-sized telomeres (4.4–9.4 kb) increased at 42 °C on day 1. These telomeric changes disappeared by day 3. These results indicate that

Journal : Large 40520 Dispatch : 7-5-2013 Pages : 8
Article No. : 45 LE TYPESET
MS Code : Original Article-Maeda CP DISK

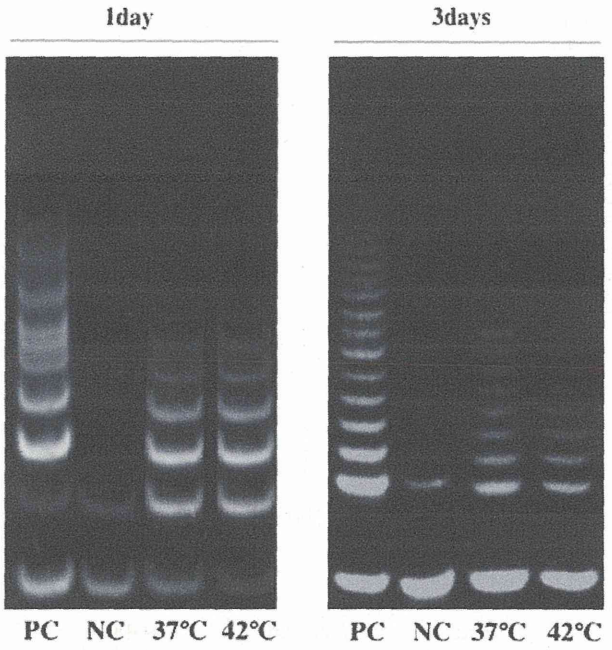


Fig. 4 The telomerase activity of endothelial cells at different temperatures. The relative telomerase activity was compared as a proportional ratio of the density the ladder of a sample to that of the mean value at 37 °C. The *panels* show photographs of representative TRAP assay results for HUVECs. The materials used for the positive control (pc) and negative control (nc) were provided with the kit

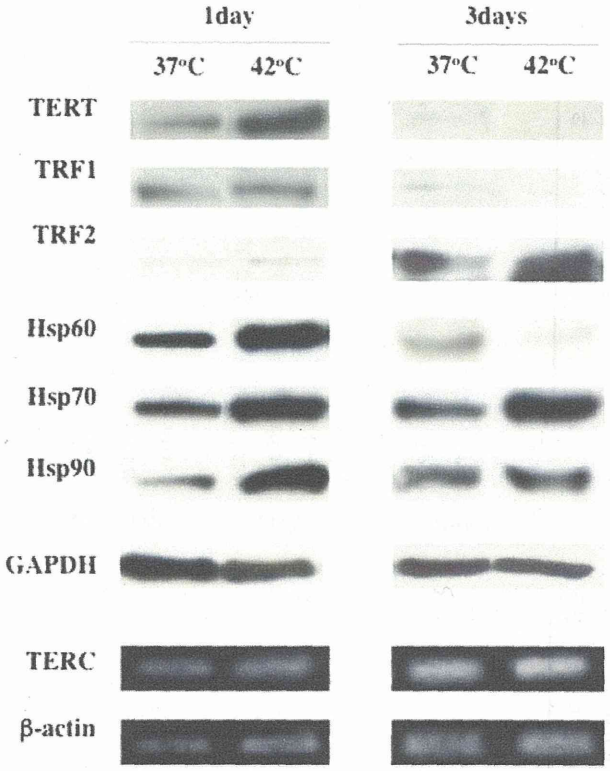


Fig. 5 The expression levels of TERC, telomere-associated proteins, and heat shock proteins of HUVECs cultured on day 1 and day 3 at different temperatures. The *panel* shows representative Western blot analysis results of telomere-associated proteins (TERT, TRF1, and TRF2), heat shock proteins (Hsp60, Hsp70, and Hsp90), and a TERC RNA RT-PCR result. The relative expression level of each component is shown in Table 1

telomere attrition is initially accelerated at 42 °C. Thereafter, the cells containing shortened telomeres, which seemed to be heat-labile, were lost from the total cell population by day 3 at 42 °C. At 42 °C, cells containing very short telomeres might be lost starting from early periods. This observation suggested that 3-day 42 °C heat accelerated cellular aging. Consequently, the cells that survived the 3-day exposure to 42 °C did not show clearly any significant features in telomere length distribution.

The alterations of subtelomeric methylated state have been observed along with aging-associated telomeric changes in human peripheral leukocytes. The decrease of long telomeres with hypomethylated subtelomere and the increase of short telomeres with hypomethylated subtelomere have been observed as a typical aging-associated telomeric change [4–6]. In this study, the observed heat-induced subtelomeric hypomethylation status on day 3 seemed to be a young pattern, suggesting that old cells, which were heat-labile, were eliminated during the 42 °C heat exposure for 3 days. Cells having survived after the heat-exposure showed a young pattern of subtelomeric methylation status. The heat exposure of 42 °C firstly accelerated aging-associated telomeric changes and finally eliminated the cells bearing the old pattern of telomeric status.

The difference of expression pattern between TRF1 and TRF2 can also be associated with the altered subtelomeric methylation status after 3-day exposure to heat. At 42 °C, TRF1 was down-regulated but TRF2 was not. The heat-induced subtelomeric hypomethylation might occur along with the lowered expression level of TRF1. TRF1 has been reported to negatively control the telomerase-associated

Table 1 The proportional values of the expressions of the telomere-associated components and heat shock proteins at 42 and 37 °C

	42/37 °C-relative expression level		
	1d	3d	*p* value (1d vs 3d)
TERT	1.80 ± 0.32*	0.25 ± 0.14*	<0.01
TERC	0.95 ± 0.27	1.86 ± 1.66	0.44
TRF1	1.43 ± 0.19	0.44 ± 0.25	<0.01
TRF2	3.93 ± 1.45*	1.19 ± 1.07	0.06
Hsp60	2.35 ± 0.58*	0.17 ± 0.11	0.02
Hsp70	2.73 ± 0.08*	1.46 ± 0.08	<0.01
Hsp90	3.35 ± 1.43	0.89 ± 1.46	0.08

1d 1 day, *3d* 3 days

* $p < 0.05$, at 42 vs 37 °C

Journal : Large 40520 Dispatch : 7-5-2013 Pages : 8
Article No. : 45 LE TYPESET
MS Code : Original Article-Maeda CP DISK

telomere length maintenance [21, 22], while TRF2 is associated with stabilizing the telomere structure [23, 24]. From this context, the lowered expression of TRF1 would be beneficial for maintaining telomere length under high-temperature conditions. In addition, the disequilibrium of the expression level between TRF1 and TRF2 might lead to structural change of telomere. This might induce unstable telomere structure accompanying demethylation of genomic DNA neighboring to telomere. In addition, it has been reported that heat shock can elicit a transient alteration of the higher-order structure of specific heterochromatic regions and induce the transcriptional activation of silent portions of the genome [25]. The same mechanism could be applied to the subtelomeric region under heat stress and consequently lead to hypomethylated status there. The prolonged high-temperature condition of 42 °C for 3 days seemed to impair various kinds of protein expression and damage cells. We showed here the phenotypical characteristics of the survived cells through a prolonged heat stress condition, i.e., maintained telomere length, subtelomeric hypomethylation of long telomeres, maintained TERC expression, and maintained TRF2 expression. TRF2 has been believed to play key roles in telomere maintenance [26, 27]. A recent report suggests roles for TRF2 protein in DNA repair in addition to chromatin reorganization and telomere maintenance [28]. TRF2 has also been suggested to protect young neurons against death induced by DNA-damaging agents [29]. Thus, TRF2 affects cell survival and differentiation by modulating DNA damage pathways, and gene expression, and the elevated expression of TRF2 could be beneficial for cell survival. Other than telomere-associated components, Hsps were also affected by heat exposure. The difference of protein expression levels drastically changed from day 1 at 42 °C. Some of these responses seemed to contribute to cell protection against heat stress. All analyzed proteins were upregulated on day 1 at 42 °C. This indicated an acute reactive response to heat stress. Protein expression activated at 42 °C on day 1 was preserved in TRF2 and Hsp70 on day 3. TERC expression also remained activated. Hsp90 expression was moderately preserved on day 3. Hsp70s function as molecular chaperones, assisting in protein synthesis, folding, assembly, trafficking between cellular compartments, and degradation [30, 31]. They are expressed constitutively and induced in response to various types of stress, including heat shock, ischemia, oxidative stress, glucose deprivation, and exposure to toxins [32]. Hsp70 protects cellular elements from injury by reducing oxidation, inflammation and apoptosis and by refolding damaged proteins. The results of the present study suggested that the expression of Hsp70 conferred survival advantages under prolonged heat exposure. Hsp70 increases also in response to heat shock in the cardiovascular system [33]. Hsp70 rapidly accumulates after heat shock and can increase as much as eightfold in rat hearts after whole animal heat shock [34, 35]. In fact, Amrani et al. [36] have suggested that the increase in rat hearts after whole animal heat shock occurs primarily in the vascular endothelium, which is associated with improved recovery of endothelial function from cardioplegic arrest. Leger et al. [37] indicated that the primary site of Hsp70 induction after whole animal heat shock is in the blood vessels. Hsp70 improves the viability of stressed vascular smooth muscle cells, possibly via its chaperone functions [38]. The beneficial effects of Hsp70 on cell viability demonstrated in the present study may also provide survival advantages for stressed vascular endothelial cells. Maintained upregulation of Hsp90 might also support cell survival. Heat shock protein 90 (Hsp90) is induced in response to cellular stress and stabilizes client proteins involved in cell cycle control and proliferative/anti-apoptotic signaling. Tanespimycin, an Hsp90 inhibitor, reduces tumour cell survival in vitro. In multiple myeloma, Hsp90 inhibition affects multiple client proteins that contribute to tumour cell survival, including elements of the PI3/Akt, STAT3, and MAPK signalling pathways. Hsp90 inhibition also abrogates the protective effect of bone marrow stromal cells and inhibits angiogenesis and osteoclastogenesis [39]. Thus, maintained expression of some proteins under prolonged heat observed in the present study are potentially able to support heat-tolerance. In summary, prolonged heat stress conditions such as those that occur at 42 °C for 3 days give rise to cell damage with transient aging-like alterations in length distribution and subtelomeric methylation. Cell survival under prolonged heat stress may be associated with the maintenance of upregulation of TRF2, Hsp70, and Hsp90. Further study is necessary to elucidate the relationship between these factors and the cell survival mechanism through prolonged heat shock.

Acknowledgments We would like to thank Ms. K. Tsuchida, Ms. S. Taguchi, and Ms. Y. Ueda for their expert technical assistance. This work was supported by grants from the Ministry of Education, Science, and Culture of Japan (#23590885), the National Natural Science Fund (NSFC) (81170329/H2501), and 2012 Health and Labour Sciences Research Grants Comprehensive Research on Life-Style Related Diseases including Cardiovascular Diseases and Diabetes Mellitus.

Conflict of interest None.

References

1. Blackburn EH (1991) Structure and function of telomeres. Nature (London) 350:569–573
2. Guan JZ, Guan WP, Maeda T, Makino N (2012) Alteration of telomere length and subtelomeric methylation in human endothelial cell under different levels of hypoxia. Arch Med Res 43:15–20

Journal : Large 40520 | Dispatch : 7-5-2013 | Pages : 8
Article No. : 45 | LE | TYPESET
MS Code : Original Article-Maeda | CP | DISK

3. Yehezkel S, Segev Y, Viegas-Péquignot E, Skorecki K, Selig S (2008) Hypomethylation of subtelomeric regions in ICF syndrome is associated with abnormally short telomeres and enhanced transcription from telomeric regions. Hum Mol Genet 17:2776–2789
4. Maeda T, Guan JZ, Oyama J, Higuchi Y, Makino N (2009) Age-related changes in subtelomeric methylation in the normal Japanese population. J Gerontol A Biol Sci Med Sci 64:426–434
5. Maeda T, Guan JZ, Higuchi Y, Oyama J, Makino N (2009) Age-related alterations in subtelomeric methylation in sarcoidosis patients. J Gerontol A Biol Sci Med Sci 64:752–760
6. Maeda T, Guan JZ, Oyama J, Higuchi Y, Makino N (2009) Aging-associated alteration in subtelomeric methylation in Parkinson's disease. J Gerontol A Biol Sci Med Sci 64:949–955
7. Aubert G, Lansdorp PM (2008) Telomeres and aging. Physiol Rev 88:557–579
8. Kellogg DL Jr, Crandall CG, Liu Y, Charkoudian N, Johnson JM (1998) Nitric oxide and cutaneous active vasodilation during heat stress in humans. J Appl Physiol 85:824–829
9. Ugurlucan M, Erer D, Kalko Y et al (2009) Aortic stiffness in diabetes mellitus–association with glutamine and heat shock protein 70 expression: a pilot study based on an experimental rodent model. Expert Opin Ther Targets 13:267–274
10. Golanski J, Michalska M, Polowinczak-Przybylek J, Krajewska U, Watała C (2011) Can we extrapolate the outcomes of in vitro studies on murine endothelium to studies of human platelet-endothelium interactions? A technical note. Arch Med Sci 7:34–37
11. Xu Q (2002) Role of heat shock proteins in atherosclerosis. Arterioscler Thromb Vasc Biol 22:1547–1559
12. Pohlman TH, Harlan JM (2000) Adaptive responses of the endothelium to stress. J Surg Res 89:85–119
13. Njemini R, Bautmans I, Onyema OO, Van Puyvelde K, Demanet C, Mets T (2011) Circulating heat shock protein 70 in health, aging and disease. BMC Immunol 12:24
14. Xu Q, Metzler B, Jahangiri M, Mandal K (2012) Molecular chaperones and heat shock proteins in atherosclerosis. Am J Physiol Heart Circ Physiol 302:H506–H514
15. Tao Q, Lv B, Qiao B, Zheng CQ, Chen ZF (2009) Immortalization of ameloblastoma cells via reactivation of telomerase function: phenotypic and molecular characteristics. Oral Oncol 45:e239–e244
16. Fuiiwara M, Kamma H, Wu W, Yano Y, Homma S, Satoh H (2006) Alternative lengthening of telomeres in the human adrenocortical carcinoma cell line H295R. Int J Oncol 29:445–451
17. Makino N, Maeda T, Oyama J-I, Higuchi Y, Mimori K (2009) Improving insulin sensitivity via activation of PPAR-γ increases telomerase activity in the heart of OLETF rats. Am J Physiol Heart Circ Physiol 297:H2188–H2195
18. Sugano M, Tsuchida K, Maeda T, Makino N (2007) siRNA targeting SHP-1 accelerates angiogenesis in a rat model of hindlimb ischemia. Atherosclerosis 191:33–39
19. Maeda T, Kurita R, Yokoo T, Tani K, Makino N (2011) Telomerase inhibition promotes an initial step of cell differentiation of primate embryonic stem cell. Biochem Biophys Res Commun 407:491–494
20. Zakian VA (1995) Telomeres: beginning to understand the end. Science 270:1601–1607
21. van Steensel B, de Lange T (1997) Control of telomere length by the human telomeric protein TRF1. Nature (London) 385:740–743
22. Soohoo CY, Shi R, Lee TH, Huang P, Lu KP, Zhou XZ (2011) Telomerase inhibitor PinX1 provides a link between TRF1 and telomerase to prevent telomere elongation. J Biol Chem 286:3894–3906
23. Smogorzewska A, van Steensel B, Bianchi A et al (2000) Control of human telomere length by TRF1 and TRF2. Mol Cell Biol 20:1659–1668
24. van Steensel B, Smogorzewska A, de Lange T (1998) TRF2 protects human telomeres from end-to-end fusions. Cell 92:401–413
25. Rizzi N, Denegri M, Chiodi I et al (2004) Transcriptional activation of a constitutive heterochromatic domain of the human genome in response to heat shock. Mol Biol Cell 15:543–551
26. Biroccio A, Rizzo A, Elli R et al (2006) TRF2 inhibition triggers apoptosis and reduces tumorigenicity of human melanoma cells. Eur J Cancer 42:1881–1888
27. Smogorzewska A, de Lange T (2002) Different telomere damage signaling pathways in human and mouse cells. EMBO J 21: 4338–4348
28. Zhang P, Dilley C, Mattson MP (2007) DNA damage responses in neural cells: focus on the telomere. Neuroscience 145: 1439–1448
29. Cheng A, Shin-ya K, Wan R et al (2007) Telomere protection mechanisms change during neurogenesis and neuronal maturation: newly generated neurons are hypersensitive to telomere and DNA damage. J Neurosci 27:3722–3733
30. Bukau B, Horwich AL (1998) The Hsp70 and Hsp60 chaperone machines. Cell 92:351–366
31. Hartl FU, Hayer-Hartl M (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 295: 1852–1858
32. Kiang JG, Tsokos GC (1998) Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. Pharmacol Ther 80:183–201
33. Benjamin IJ, McMillan DR (1998) Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. Circ Res 83:117–132
34. Taylor RP, Harris MB, Starnes JW (1999) Acute exercise can improve cardioprotection without increasing heat-shock protein. Am J Physiol Heart Circ Physiol 276:H1098–H1102
35. Oyama J, Maeda T, Sasaki M, Higuchi Y, Node K, Makino N (2012) Repetitive hyperthermia attenuates progression of left ventricular hypertrophy and increases telomerase activity in hypertensive rats. Am J Physiol Heart Circ Physiol 302:H2092–H2101
36. Amrani M, Latif N, Morrison K et al (1998) Relative induction of heat shock protein in coronary endothelial cells and cardiomyocytes: implications for myocardial protection. J Thorac Cardiovasc Surg 115:200–209
37. Leger JP, Smith FM, Currie RW (2000) Confocal microscopic localization of constitutive and heat shock-induced proteins HSP70 and HSP27 in the rat heart. Circulation 102:1703–1709
38. Bielecka-Dabrowa A, Barylski M, Mikhailidis DP, Rysz J, Banach M (2009) HSP 70 and atherosclerosis—protector or activator? Expert Opin Ther Targets 13:307–317
39. Richardson PG, Mitsiades CS, Laubach JP, Lonial S, Chanan-Khan AA, Anderson KC (2011) Inhibition of heat shock protein 90 (HSP90) as a therapeutic strategy for the treatment of myeloma and other cancers. Br J Haematol 152:367–379

Journal : Large 40520 | Dispatch : 7-5-2013 | Pages : 8
Article No. : 45 | LE | TYPESET
MS Code : Original Article-Maeda | CP | DISK

ISSN: 2320-1983 Print ISSN: 2320-1991 Online Volume 3 Issue 2

Trade Science Inc.

Applied Cell Biology

Regular Paper

ACB, 3(2), 2014 [48-54]

Vascular endothelial cell surviving through under prolonged elevated temperature shows persistent or transient up-regulation of telomerase and stress-associated proteins

Toyoki Maeda[1]*, Guan Jing-Zhi[2], Masamichi Koyanagi[1], Naoki Makino[1]

[1]The Department of Cardiovascular, Respiratory and Geriatric Disease, Kyushu University Beppu Hospital, Beppu, Oita, 874-0838, (JAPAN)

[2]The 309th Hospital of Chinese People's Liberation Army, Beijing, (CHINA)

E-mail : maedat@beppu.kyushu-u.ac.jp

Received: 05th January, 2014 ; Accepted: 15th February, 2014

ABSTRACT

The effects of heat on vascular endothelial cells are studied. Telomere of human umbilical venous endothelial cells (HUVECs) cultured at 42°C was studied. The expression of several factors concerning to telomere maintenance and vascular endothelial physiology was analyzed.

HUVECs were cultured for 1 day or for 3 days under 42°C. Their telomere lengths and telomere length distributions were analyzed and compared with those at 37°C. The telomerase activity and the expression of telomere-associated RNA, telomere-associated proteins (TERC, TERT, TRF1, and TRF2), heat shock proteins (Hsp60, Hsp70, and Hsp90) and endothelial nitric oxide synthase were also analyzed.

The cell growth was suppressed both on day1 and on day3 at 42°C. Unexpectedly, however, the cell senescence rate was elevated only on day1 at 42°C, compared to that at 37°C. The mean telomere length was not different between at 37°C and at 42°C, whereas in the telomere length distribution long telomere decreased and middle-sized telomere increased only on day1 at 42°C. Telomere-associated proteins, heat shock proteins, and NOS were up-regulated at day1, and the up-regulation disappeared at day3.

The results suggested that long telomeres were affected and various genes are up-regulated in reaction to elevated temperature, and that the cells surviving through the prolonged exposure to heat lose the early responses. The cell subpopulation bearing long telomere seemed more sensitive to heat stress. The observed initial up-regulation of telomere-associated proteins and others showed an aspect of heat responses of vascular endothelial cells. Among the responses, some seemed to be favor for the biological function and the survival of vascular endothelial cell, for example, the upregulation of telomerase activity and the elevated expression of TERT, heat shock protein, and NOS. In addition, an upregulation was maintained uniquely in Hsp70 at a later stage of heat exposure. This observation implicates that the upregulation of Hsp70 play an important roll for cell survival under prolonged heat stress.

KEYWORDS

Vascular endothelial cell;
Telomere;
Heat shock protein.

Regular Paper

INTRODUCTION

Telomere consists of repetitive DNA sequence with accessory protein components (TRF1, TRF2, and others) capping the terminals of chromosome[1]. It is well known that telomere DNA shortening proceeds at every cell cycle due to a duplication process yielding a little shorter DNA strand. The telomere length of somatic cells is negatively affected by stress factors including oxidative stress[2]. Pathological mental and physical stress accelerates telomere attrition[2]. Telomere shortening is considered to occur in somatic cells with aging through many rounds of cell cycle and pathological stress[3]. On the other hand, there is a telomere-elongating cellular mechanism, which functions in limited cases. Telomerase consists of a protein component of reverse transcriptase (TERT) and a RNA part (TERC). Telomerase contributes the telomere elongation or telomere length maintenance in unique cell population with active mitotic potential, such as cancer cells, stem cells, and reproductive cells. Generally, the telomere activity is, however, suppressed to a low level in the somatic cells, which is not enough to prevent the telomere shortening with cell divisions. Telomerase activity is also affected by stress factors. However, the effect of heat stress on telomere length or telomerase has not been well studied. Heat shock proteins (Hsp) are ubiquitously synthesized in virtually all species and it is hypothesized that they might have beneficial health effects[4]. In response to stress stimuli, mammalian cells activate a signaling pathway leading to the transient expression of heat shock proteins (Hsp). Hsp's are a family of proteins serving as molecular chaperones that prevent the formation of nonspecific protein aggregates and assist proteins in the acquisition of their native structures. Physiologically, Hsp's play a protective role in the homeostasis of the vessel wall consisting of endothelial cells and smooth muscle cells[5]. Previous reports have shown that heat-associated vasodilatation is associated with vascular NO synthesis[6]. Vascular endothelial cells express endothelial nitric oxide synthase (eNOS). eNOS mediates NO synthesis in vascular endothelial cells, which relaxes the surrounding smooth muscle cells of vascular wall to vasodilatation. NO synthesis has been regarded as a functional marker for vascular endothelial cells. We pursued the heat-associated alteration in the vascular reactivity for vasodilatation of cultured HUVECs by assessing the eNOS expression level of HUVECs under heat-exposure. Heat-associated effects on genomic DNA including telomere erosion, have not been well investigated. Here we analyzed telomere DNA, and the expressions of telomere-associated components, heat shock proteins and nitric oxide synthase in reaction to heat-stress by using vascular endothelial cells in culture exposed to heat.

MATERIALS AND METHODS

Cell culture

Human umbilical venous endothelial cells (HUVEC) were purchased from Clonetics Corp. (San Diego, CA). The cells were cultured in endothelial cell growth medium (Clonetics Corp.) at 37°C or 42°C and 5% CO_2 in a gelatin-coated flask (Iwaki Glass, 2 Chiba, Japan). Culture media were refreshed every 24 h. On day 1 or 3, the cells were collected and were counted using a hemocytometer. Population doublings (PDs) were calculated using the formula: PD=[log (expansion)/log2], where expansion was the number of cells harvested divided by the initial number of cells seeded.

Senescence-associated β-galactosidase (SA-β-Gal) expression

The cells were washed in PBS, fixed for 10 min at room temperature in 2% formaldehyde/0.2% glutaraldehyde, and incubated at 37°C (no CO_2) with fresh SA-β-Gal staining solution containing 1mg/mL of X-gal, pH 6.0 for 12h. One hundred cells were scored from each well (plate) using a light microscope.

Telomere detection

Telomere detection was performed as previously described[7]. Blood cell DNA was extracted from samples and the DNA (0.1ug) were digested with *Msp*I. The digests (10ul) were subjected to Southern blot hybridization with a telomere DNA probe. The autoradiogram was captured on an Image Master, and the telomere length was then assessed quantitatively. Every sample was measured in triplicate.

Terminal length (TL) analysis

Telomere length distribution was analyzed by com-

Regular Paper

paring the telomere length using a telomere percentage analysis with three intervals of length (>9.4, 9.4≥≥4.4 and <4.4kb) as defined by a molecular weight standard as previously described[7]. The percent of the stratified intensity in each molecular weight range was measured. The mean TL was estimated using the formula S(ODi - background)/ S(ODi – background /Li), where ODi is the chemiluminescent signal and Li is the length of the TRF fragment at position i.

Semiquntitative RT-PCR for TERC RNA

Total RNA samples were extracted using RNAzol B (Teltest). mRNA for human telomerase RNA component (TERC) was determined by RT-PCR using a DIG detection system (Roche Applied Science). Each human cDNA was produced by RT-PCR according to each human-derived sequence. For the amplification of β-actin cDNA, the forward primer β-actin (205bp) 5' -CCTTCCTGGGCATGGAGTCCT-3' and the reverse primer 5'-GGAGCAATGATCTTGATCTTC-3' were used according to the published human TERC cDNA sequence[8]. And TERC forward primer 5'-TCTAACCCTAACTGAGAAGGGCGTAG-3' reverse primer 5'-GTTTGCTCTAG AATGAACGGTGGAAG-3' were used[9]. 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) 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). For semiquantitative PCR, β-actin was used as an internal control to evaluate total RNA input, as described by our group[10].

Western blot and other analyses

Cells from a dish were homogenized with 100μl lysis buffer (100mM Tris pH 6.8, 4% SDS, 20% glycerol containing the protease inhibitor, M phenylmethanesulfonyl fluoride, 0.1mM, leupeptin, 0.1μl, and aprotinin, 0.1μl). Gel electrophoresis was used to separate 10μg protein on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (162-0112, Bio-Rad Labo-ratories, Hercules, California) 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), TRF1 (Imgenex), TRF2 (Cell Signaling), Hsp60 (Assay designs), Hsp70 (Assay designs), Hsp90 (Enzo), eNOS (BD Biosciences), phospho-eNOS (Cell Signaling), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology). Detection was performed with secondary horseradish peroxidase-conjugated antibodies (Chemicon) and the ECL detection system as previously described[11]. The relative expression levels were determined compared to that of GAPDH.

Telomerase activity

Telomerase activity was examined by means of a modified telomerase repeat amplification protocol (TRAP) method with TeloChaser (Toyobo, Osaka, Japan), as previously described[12].

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 performed using a two-way analysis of variance (ANOVA) 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 is $p<0.05$.

RESULTS

Population doubling (PD) and cell senescence

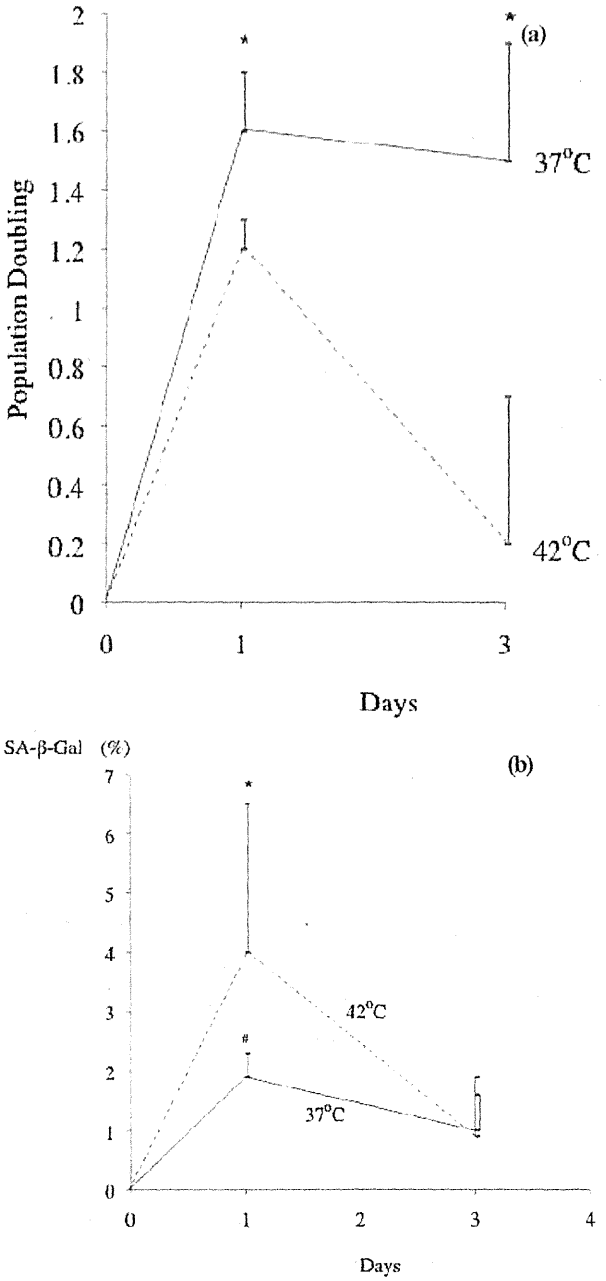
The PD of the HUVECs was assessed on day 1 and day 3 of culture. At 37°C, the PD was elevated to ~1.6 on day1 and the PD value was maintained on day3. At 42°C, initially the PD was elevated to ~1.2, but decreased steeply to ~0.2 on day3 (Figure 1a).

Senescence-associated β-galactosidase (SA-β-Gal) expression was observed in ~2% of cells on day1 and ~1% on day3 at 37°C, and ~4% on day1 and

Regular Paper

~2% on day3 at 42°C (Figure 1b).

The PD value did not increased at 37°C, and decreased at 42°C during day1 and day3. This apparent suppress of cell growth might be derived partly from damaged subpopulation during cell preparation before starting culture being left. The elevated cell senescence on day1 both at 37°C and at 42°C supported this idea. The difference between PD and cell senescence were derived from heat stress, and cells were suppressed in growth potential at 42°C. Cell population survived through the heat condition of 42°C for 3 days bore a similar proportion of senescent cells as at 37°C. This indicated that heat-sensible cells were completely eradicated during day1 and day3 at 42°C. As a result, heat-durable cells survived on day3 at 42°C.



a. The PD on day1 and day3 at 37°C and 42°C. The horizontal bars are standard deviations. *p<0.05, at 37°C vs at 42°C on day1. b. The percentages of SA-b-Gal-positive cells. *p<0.05, at 37°C vs at 42°C. #p<0.05, on day1 vs on day3.

Figure 1 : The population doubling (PD) and the ratio of senescence-associated b-galactosidase (SA-β-Gal) staining of HUVECs cultured in the presence of H_2O_2

The mean TRF level and its distribution

The mean TL's of HUVECs were measured to assess how much high temperature condition affected the telomeric DNA (TABLE 1). At 42°C, long telomere (>9.4kb) decreased on day1 and middle-sized telomere (9.4kb-4.4kb) increased. These differences between 37°C and 42°C disappeared on day3. The proportions between values at 37°C and those at 42°C were also compared to pursue the heat-specific changes from day1 to day3 (TABLE 2). The decrease of long telomere and the increase of middle-sized telomere were observed only on day1 and the short telomere (<4.4kb) was not significantly affected. These changes of TL distribution disappeared on day3. Unexpectedly, cells with longer telomere seemed more easily affected by heat stress, compared to those with shorter telomere.

TABLE 1 : The telomere length and the telomere length distribution of HUVECs exposed to heat

	37°C		42°C	
	Day1	Day3	Day1	Day3
MspI-TL(kb)	9.2±1.0	8.7±0.3	8.3±1.0	8.9±0.6
>9.4kb(%)	51±8	46±1	39±11*	49±5
9.4-4.4kb(%)	47±6	51±2	59±10*	48±3
<4.4kb(%)	2±3	3±2	2±1	4±2

*37°C vs42°C p<0.05

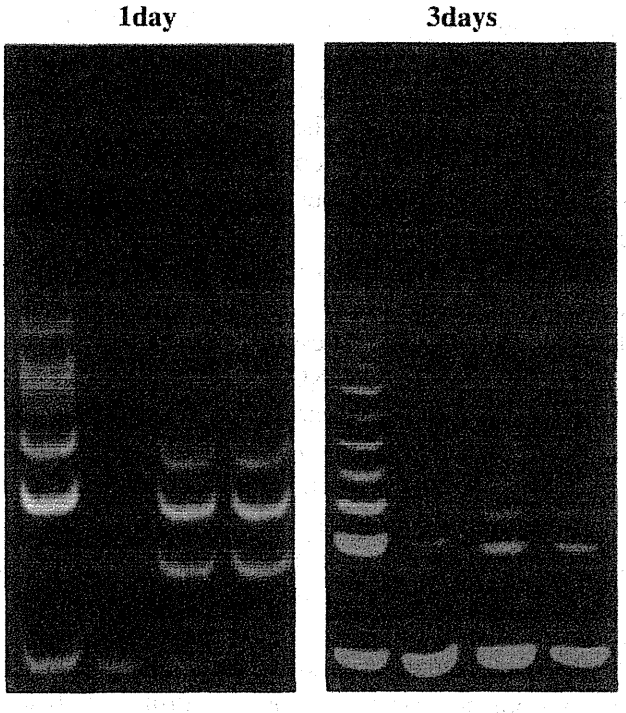
TABLE 2 : The proportional values of telomere length parameters at 42°C compared to those at 37°C

	Day1	Day3	p-value
MspI-TL	0.90±0.11	1.02±0.07	0.06
>9.4kb	0.76±0.21	1.06±0.10	0.01
9.4-4.4kb	1.26±0.20	0.93±0.06	0.01
<4.4kb	1.01+±0.74	1.21±0.69	0.63

Regular Paper

Telomerase activity

The telomerase activity of HUVECs at 37°C and 42°C was evaluated by TRAP assay (Figure 2). Average value of TPG at 37°C was put as 1(1±0.66 for 1day, 1±0.2 for 3days). The relative TPG7s at 42°C were 1.32±0.84 on day1 and 0.57±0.28 on day3. Thus, the relative telomerase activity of HUVECs at 42°C was maintained on day1 ($p = 0.71$) but significantly decreased on day3 ($p = 0.03$). Telomerase activity decreased under prolonged heat stress at 42°C.



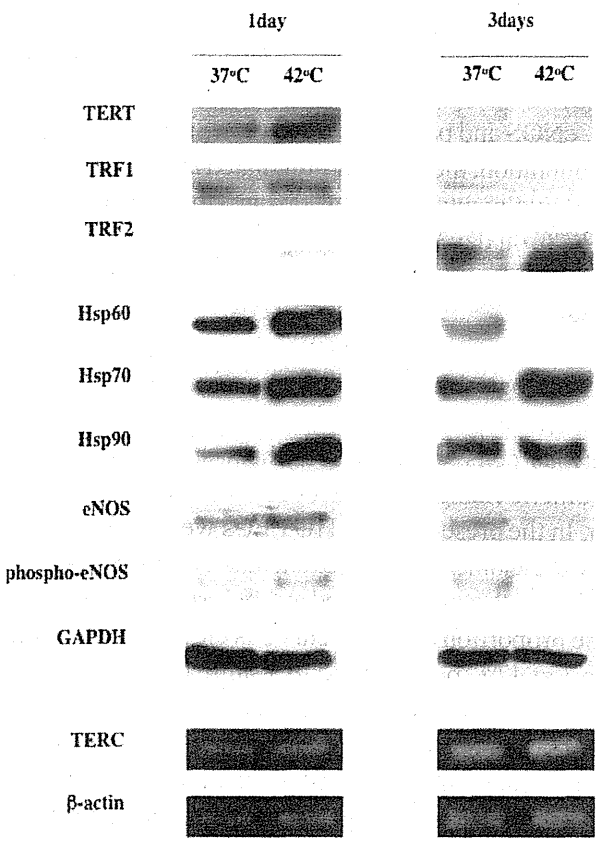
The photographs of representative TRAP assay results are shown. The materials used for the positive control (pc) and negative control (nc) were provided with the kit

Figure 2 : The telomerase activity of endothelial cells in the presence of H_2O_2

Expression of telomere-associated components and others

All the three telomere-associated components (TERT, TRF1, and TRF2) except TERC were up-regulated on day1, thereafter were down-regulated on day3. TERC did not seem to be affected by heat in the expression. (Figure 3, TABLE 3).

All the analyzed heat shock proteins (Hsp60, hsp70, and hsp90) were up-regulated at 42°C on day1 and



The representative Western blot results of telomere-associated proteins (TERT, TRF1, and TRF2), heat shock proteins (Hsp60, Hsp70, and Hsp90) and a TERC RNA RT-PCR result are shown. The relative expression level of each component is shown in TABLE 3

Figure 3 : The expression levels of TERC, telomere-associated proteins, heat shock proteins, and NOS of HUVECs cultured on day1 and day3 at different temperatures

TABLE 3 : The proportional values of the expressions of the telomere-associated components, heat shock proteins and eNOS at 42°C compared to those at 37°C

	Day1	Day3	p-value
TERT	1.80±0.32	0.25±0.14	0.01>
TRF1	1.43±0.19	0.44±0.25	0.01>
TRF2	3.93±1.45	1.19±1.07	0.06
Hsp60	2.35±0.58	0.17±0.11	0.02
Hsp70	2.73±0.08	1.46±0.08	0.00
Hsp90	3.35±1.43	0.89±1.46	0.08
eNOS	2.43±0.52	0.25±0.04	0.02
TERC	0.95±0.27	1.86±1.66	0.44

The photo results are shown in Figure 3. The relative expression levels were determined by the proportion of each band density to that of β-actin or GAPDH (set at 1 each), β-actin (RNA) for TERC and GAPDH (protein) for western results

Regular Paper

down-regulated day3. However, only hsp70 still kept a significantly higher expression level at 42°C compared to at 37°C.

eNOS and phosphorylated eNOS showed a similar alteration, they were up-regulated at 42°C on day1, but down-regulated at 42°C on day3 to below the level at 37°C on day3.

DISCUSSION

Biological stress has been reported to induce negative effects on the maintenance of telomere length in various cells[1,13]. However, heat stress-associated telomeric changes have not been well investigated, so far. In the present study, HUVECs were used to analyze the telomere-associated alterations induced at 42°C. Cell growth was suppressed at 42°C especially on day3. The proportion of senescent cells elevated indeed on day1 at 42°C but returned to a same level as at 37°C on day3. This observation indicated that a heat-sensitive cell population diminished during 3-day-exposure to 42°C, and a heat-resistant population survived until day3. In the telomere length distribution, apparently long telomere decreased and telomere of medium size (4.4-9.4kb) increased at 42°C on day1. This indicated that growth rate was suppressed more in cells with long telomere than those with short telomere under exposure to heat-stress in an early stage of heat exposure. The shortest range of telomere (<4.4kb) showed an increasing trend of increase at 42°C on day3, although it was not significant. A part of cell population bearing short telomeres might be damaged and diminished at 42°C, resulting in non-clear increase of them on day3. The grown subpopulation at 42°C by day1 seemed to diminish by day3, because the TL distribution of the survived cell population at 42°C on day3 returned to a similar pattern at 37°C on day3.

This suggested that a subpopulation of cells with short telomere grew initially in heat-exposure but lost the heat-endurance during a prolonged heat exposure. The mechanism of the apparent heat-associated growth suppression of cells with long telomere is not clear, so far. Cells containing long telomeres, i.e., young cells, may be equipped with an unknown mechanism suppressing cell growth under a heat-stressed condition, possibly being a favor for the cell survival.

All the analyzed proteins up-regulated on day1 at 42°C. The survived cells for 3 days at 42°C revealed an elevated expression of Hsp70, and maintained the expression of TERC, TRF2, and Hsp90. All the others, TERT, TRF1, Hsp60, and NOS were suppressed in expression at 42°C. The expression of Hsp70 might contribute to survival advantage under a prolonged heat-exposure. We here show that an up-regulation after 3-day-culture at 42°C is maintained only in Hsp70 among analyzed heat shock proteins. Heat shock protein70 (Hsp70) protects cellular elements from injury by reducing oxidation, inflammation and apoptosis and by refolding damaged proteins. Hsp70 improves viability of stressed vascular smooth muscle cells, possibly via its chaperone functions[14]. The beneficial effects on cell viability are here shown to contribute not only to vascular smooth muscle cells but also possibly to vascular endothelial cells.

The expression of NOS was up-regulated in an early stage of heat-exposure and was down-regulated later. This suggested that heat-stress had an inducible potential for NOS expression. NOS has been reported to contribute to vasodilatation, and so heat exposure would result in vasodilatation[6]. However the vasodilatation by NOS would diminish after prolonged heat exposure. The heat-associated alterations of NOS expression seemed rather to result in changing vasodilatation potential than contribute to survival advantage for heat stress.

Longer telomeres seemed transiently affected under exposure to heat stress. However, prolonged heat exposure did not leave a persistent altered pattern of telomere length distribution. In contrast to the growth suppression and the down-regulation of various proteins including telomere-associated proteins, other heat shock proteins, and NOS, only Hsp70 up-regulated persistently under heat stress. Hsp70 may be a strong survival factor for heat-damaged cells. Further study is necessary to confirm the vascular cellular heat-resistance associated with Hsp70.

ACKNOWLEDGMENT

We would like to thank Ms. K. Tsuchida, Ms. S. Taguchi, and Ms. Y. Ueda for their expert technical assistance. This work was supported by grants from the