厚生労働科学研究費補助金

循環器疾患・糖尿病等生活習慣病対策総合研究事業

温泉利用が健康づくりにもたらす総合的効果についてのエビデンスに関する研究

平成24年度　総括研究報告書

研究代表者　前田　豊樹

平成 25（2013）年　5 月

目　　次

厚生労働科学研究費補助金（循環器疾患・糖尿病等生活習慣病対策総合研究事業）
総括研究報告書

温泉利用が健康づくりにもたらす総合的効果についてのエビデンスに関する研究

研究代表者　前田　豊樹　九州大学病院別府病院内科准教授

研究要旨

本研究は、温泉療法の医学的効果を一般市民の疫学的調査と患者対象の治療効果、身体能力改善度、ゲノムレベルの老化マーカーであるテロメア構造変化の解析などで多面的に検証する目的で、平成２４年度より開始している。疫学研究、臨床研究、実験室レベルでの研究という３本柱で検証を進めつつある。疫学調査では、別府市の 65 歳以上の高齢者 2 万人を対象に「温泉と健康アンケート」を実施し、11,146 名の回答を得、統計解析により、温泉入浴頻度が高く、一回の入浴時間が長い方が、虚血性心疾患および脳卒中の既往が少ない傾向にあった。このことは、温泉入浴が生活習慣病予防に繋がる可能性を伺わせるものとして注目される。また、申請者らの施設では、慢性疼痛に苦しむ線維筋痛症患者に対して鉱泥浴治療を行っており、その前後での体細胞テロメア変化を調べた。実験室レベルでは、ヒト血管内皮培養細胞において、高温条件でのテロメア長の変化に関連する遺伝子群の発現を追跡することにより、生物学的な温度効果の確認を進めている。これまでのところ、アンケート調査からは、連日の温泉浴により虚血性心疾患や脳卒中減少に繋がる可能性を示す結果が得られている。臨床研究では、鉱泥浴により老化した体細胞の寿命を延長する可能性を示す結果が得られている。培養実験では、高温下では血管内皮細胞が、ストレスに抵抗してある種のアンチエイジング効果につながる一連の蛋白の発現増強を認めた。これらのことは、温泉の利用が、健康長寿に好ましい効果をもたらすことを示唆していると考えられる。

A. 研究目的

本研究は温泉療法の医学的効果を疫学、臨床効果、ゲノム変化と多面的に検証し、温泉治療の汎用性向上させ、生活習慣病の予防法あるいは補助的治療としての医療上の確固たる位置づけを目標とする。超高齢社会を迎えた我が国において、世界的温泉地に立地する当院での温泉の健康長寿への応用推進は大変意義深いことと考える。温泉治療効果には、抗炎症、抗うつ、免疫増強、抗がんなどの作用があるとされる（Rheumatol Int. 2011;31(1):1-8. J Epidemiol. 2006; 16:35-44. Cancer Nurs. 2005 ;28:390-8.）。本研究では、温泉治療患者に対して、原疾患の治療効果に加え、普遍的な尺度として身体活動度と末梢白血球のゲノム変化から温泉治療の医学的効果を検証する。ゲノム変化は末梢白血球のテロメア解析で行う。テロメアは染色体の末端構造で、加齢や生活習慣病、生活ストレスなどで短縮する。申請者らは、

現在、本研究と平行してテロメア長と疾病の関連についての研究も進めており、これに関連したものは、平成24年度期だけでも、すでに(1) Guan JZ, et al. Aging Clin Exp Res. 2012;24:213-7. (2) Maeda T et al. Int J Radiat Biol. 2013;89: 106-9. (3) Guan JZ, et al. Aging Dis. 2012;3:164-70. (4) Guan JZ, et al. Arch Med Res. 2012;43:15-20. (5) Maeda T, et al. J Neurogenet. 2012;26:245-51. (6) Guan JZ, et al. Gerontology. 2012;58:62-9. 等の論文として発表しており、我々がテロメア解析に習熟していることが示すものである。これを温泉の医学的効果の検証に応用する。温泉の温度効果について、臨床研究では、深部体温上昇効果の高い温泉浴治療、鉱泥浴に注目し、治療を受けた線維筋痛症患者を対象に体細胞テロメア解析を行った。培養細胞系で細胞への温度効果をテロメア解析などによって評価する。また、より広域での一般市民の温泉浴による健康増進効果やQOL向上効果の調査も同時に行い能率的に温泉の医学的効果の検討を進める。これについては、別府医師会、別府市の協力を得て、アンケートで地域の温泉入浴の実態と身体状況などの関連の調査も行う。このような一連の研究は、世界的にも類を見ない。本研究は、培養細胞におけるゲノムレベルの解析と、臨床的な温泉治療における治療効果とアンチエイジング効果の評価、ならびに市中の温泉利用と身体状態の調査によって温泉の医学的効果を多角的に分析し、これらの結果を合わせて総合的に検討して温泉の医学的効果を立証し、温泉治療の医療応用を推進することを目的とする。

B. 研究方法

アンケート調査：温泉と身体状況の調査アンケートは、その項目について、申請者、別府市医師会理事会、別府市役所健康づくり推進課の協同で検討して作成した。年齢、性別、温泉利用型健康増進施設の利用頻度、入浴時間などと既往歴、治療中の疾患、治療内容、身体活動度など、項目を極力絞り、できるだけ簡単なスケールで回答できるものとした。平成24年10月に九州大学医系地区部局臨床研究倫理審査委員会の承認を得た後、平成24年11月に、市民へ郵送にて配布、回収した。研究計画当初2000例の回収を目指す予定としていたが、65歳以上２万人に配布し、数千例の回収を目指すこととした。このアンケート結果解析により温泉の利用状況と疾病罹患率との相関を検討する。また、温泉治療効果については、特定の疾患の治療効果と身体能力改善の評価とテロメア解析を平行して行う。申請者はこれまでテロメア近傍のメチル化の変化が、疾病状態、身体機能低下、臨床血液検査値の比較的短期間の変動と相関することを見いだしており、この手法を温泉治療効果の判定にも用いる。具体的には、当院に受診する線維筋痛症患者を対象に、鉱泥浴温泉治療開始前と１～３ヶ月の治療後に末梢血採血を行う。そこから白血球分画を得、ゲノムDNAを抽出する。最終的には、アンケート調査結果と温泉治療効果を対比し

て、そこから温泉の医学的効果を多角的に評価する。疫学統計解析にあたっては、臨床疫学研究を専門とする名古屋市立大学大学院医学研究科公衆衛生学分野の鈴木貞夫教授の助言を得て行う。

鉱泥浴治療のテロメアへの影響：温泉浴の医学的効果のゲノムレベルでの検証は、鉱泥浴治療を受けた線維筋痛症患者を対象に各種臨床検査値の推移とテロメア解析を行った。鉱泥浴は、42℃に設定され、１日１回 10 分の入浴を行った。入院時と退院時の各種臨床検査値、テロメア関連パラメータの測定値を比較して、鉱泥浴の臨床的効果並びにゲノムレベルでの老化性変化への影響の検出を試みた。

培養実験による温熱効果の検証：細胞レベルでの温熱効果の検証を目的とした培養細胞実験は、ヒト臍帯静脈血管内皮細胞を用い、37℃条件下と 42℃条件下で培養して、テロメア長、テロメア関連蛋白、ヒートショック蛋白などの発現の追跡を行い、細胞レベルでの温熱効果を探索した。

テロメア解析については以下の方法を用いる。同じ DNA 配列を認識するが、メチル化の有無で切断能が異なる制限酵素２種類を別々に用いて、このゲノム DNA を消化してテロメアプローブでゲノムサザン法を行う。それぞれの制限酵素の切断パターン（デンシトメトリー）の差を見ることでテロメア周辺のメチル化の程度の差が検出できる。平均テロメア長ならびにその長さの分布は、これまで通り、メチル化非感受性制限酵素の切断によるサザン法で得られるスメアパターンをデンシトメトリーで取り込み、積算もしくは分割積算にて求めた。

培養実験における、蛋白発現の解析については、テロメア伸長酵素であるテロメラーゼについては、その活性と蛋白発現を調べ、その他の蛋白質については、ウエスタンブロットにて発現を調べた。

（倫理面への配慮）

アンケート調査は無記名であり、九州大学医系地区部局臨床研究倫理審査委員会により承認されている（許可番号 24-105）。個人の特定を防いでいる。テロメア解析は、九州大学大学院医学研究院ヒトゲノム・遺伝子解析倫理委員会より承認されている（承認番号第 412-00 号）．血液検体採取では文書同意を得た上、連結匿名化により個人情報を保護している。

C. 研究結果

アンケート調査結果：当初予想された 30~40%のアンケート回収率を多きく上回る 11,146 名(56%)から回答が寄せられた。男女別に見ると、男性 43%、女性 57%、年齢別では、65~69 歳 30%、70~74 歳 28%、75~79 歳 24%、80~84 歳 12%、85 歳以上 6%であった。温泉利用状況については、全回答者のうち毎日利用するのは、48%であった。入浴時間では、20 分以下が、67%に上った。利用年数では、10 年未満 33%、10 年以上 40 年未満が 36%、40 年以上 32%であった。なお現時点では、得られたデータのすべてが解析できているわけではないので、ここでは解析を進めているもののうち、

とくに三大疾病に関連したデータを示す。アンケート回答者においては、がん 12%、虚血性心疾患 7%、脳卒中 2.8%の既往率であった。男女別では、男性で、がん、虚血性心疾患、脳卒中について、それぞれ、14%、10%、4%、女性では、10%、5%、2%といずれも男性で高率であった。温泉の利用状況と３大疾患既往率について見ると、温泉を毎日利用する人とそうでない人では、がん、虚血性心疾患、脳卒中の既往率は、それぞれ 11%と 13%、6%と８%、2%と 3%であり、虚血性心疾患、脳卒中の既往については、有意に毎日温泉を利用する人で有意に少ないという結果であった（添付図１）。有意差のあった 2 疾患について、逆に既往歴の有無で毎日温泉を利用する人の割合を比較した場合、虚血性心疾患の既往がある人とない人では 73%と 74%、同様に脳卒中では 69%と 74%であったが有意な差は見られなかった（添付図 2)。入浴時間では、入浴に 20 分以上かける人と 20 分未満の人で、各疾患の既往率を比較すると、がんでは、11%と 12%、虚血性心疾患では 6%と 8%、脳卒中では 3%と 3%で、温泉入浴に 20 分以上かける人で虚血性心疾患の既往が有意に低かった。さらに虚血性心疾患の既往の有無で、温泉入浴時間を比較した場合、既往のある人で 20 分以上の人とそうでない人の割合は、それぞれ 29%と 34%で、虚血性心疾患の既往のある人では温泉入浴時間が 20 分以上の人が有意に少なかった（添付図３)。また温泉利用期間の差による３大疾患の既往率の違いは見られなかった（添付図４)。

鉱泥浴のテロメアへの影響：連日の入院鉱泥浴治療（平均入院治療日数 45 日）を行った線維筋痛症患者７名の解析結果を示す。線維筋痛症による全身各所の疼痛は、入院当初５点満点フェイススケールで平均 3.3 点であったものが、退院時には、0.8 点と有意に低下していた。臨床検査値の変化を見たところ、血清アルブミンの増加効果、貧血の改善効果が有意に認められた。これに対してテロメア変化では、入院時と退院時における平均テロメア長では有意差がなかった。しかし、アルブミン値と赤血球数の推移に対して、平均テロメア長が有意に短縮する傾向が検出された（添付図５)。

培養実験による温熱効果の検証：培養実験では、培養温度 42℃条件下では、ヒト血管内皮細胞のテロメアの平均長には影響がなかったが、初期(1 日)には、長いテロメアが一時的に減少し、長時間(３日)では、短いテロメアが減少した。さらに、ヒートショック蛋白の発現が上昇した。また、初期にはテロメア伸長酵素であるテロメラーゼのタンパク部分の発現が上昇した。(論文参照)

D. 考察

アンケート調査結果：申請当初 2000 人程度の解析を予定していたが、別府市と別府市医師会の協力の下で、1 万人を越える回答を得られた。当初の予定よりも、はるかに正確な統計解析ができるものと期待できる。温泉利用状況のうち、温泉利用頻度と一回の入浴時間が、３大疾病のうちの、

心臓病（虚血性心疾患）もしくは脳卒中の既往と関連している可能性が示されている。すなわち、毎日温泉入浴する群は、そうでない群に比べて、虚血性心疾患と脳卒中の既往が少ない。一方、虚血性心疾患ならびに脳卒中の既往群と非既往群では、温泉の毎日利用する率に有意差はなかった。これらのことは、疾患に罹患したから温泉を毎日利用しなくなったのではなく、温泉を毎日利用する方が、疾病罹患が減少することを示している可能性がある。温泉入浴時間では、20 分以上入る群の方が、虚血性心疾患の既往が少ないが、虚血性心疾患の既往のない群の方が、温泉入浴時間が 20 分以上であることが多かった。したがって虚血性心疾患になったから温泉の入浴時間が短くなったのか、入浴時間が長い方が、虚血性心疾患になりにくいのかはこの解析結果だけからは判別できない。

鉱泥浴患者におけるテロメア解析：鉱泥浴は、一般的な温水浴に比較して、深部体温の上昇効果が著しい。このことが、全身の血行改善を促し、疼痛物質やストレス物質の洗い流しや希釈効果を生み、鎮痛効果やリラックス効果に繋がるとされる。今回の観察でも、鉱泥浴は、線維筋痛症患者に対して、良好な鎮痛効果を示した。さらに、低タンパク血症や貧血の改善効果も認められた。痛みが和らいだことが栄養改善に繋がった可能性がある。一方、テロメア長は、これらの好ましい臨床検査の改善傾向とは逆に、短縮する傾向を認めた。これは、一見矛盾して見えるが、一つにはテロメアの短くなった老化細胞が延命されて、末梢血中により長くとどまれることによる可能性がある。すなわち、白血球の寿命を延長している可能性がある。

高温条件下培養細胞のゲノム老化の変容：42℃環境への暴露は、長時間に及ぶ場合、血管内皮細胞に障害を与えるストレスになると考えられる。しかし、短時間であれば、そのストレスに対抗しようとするヒートショック蛋白やテロメラーゼの発現を上昇させるなど、温熱による細胞障害を防ぐ作用が誘導されていた。テロメラーゼの発現上昇は、ゲノム老化に抑制性に作用すると考えられた。このことは、温熱刺激は、生体にダメージを与える可能性があるが、短時間であれば、温熱に対抗するホルミシス効果としてのアンチエイジング効果につながる生体作用を誘導する可能性があることを示していると考えられる。

E. 結論

温泉アンケート調査：温泉の連日入浴は、虚血性疾患と脳卒中の予防に繋がる可能性がある。今後、温泉利用頻度、温泉入浴時間、温泉利用期間、各年齢層などのパラメータを細分した上で、さらに統計解析を行い、今回の結果を検証し直すとともに、最も好適な条件を見つけ出していく必要があると思われる。

鉱泥浴の老化への影響：鉱泥浴には、鎮痛効果、栄養改善効果に加え、それと平行して細胞レベルでの延命効果がある可能性がある。ただし、今回の結果を確認するた

めに、症例の蓄積が必要である。

血管内皮細胞の高温培養：高温への暴露は、血管内皮細胞において、ストレスに対抗する一種のホルミシス効果としてのアンチエイジング作用を誘導できる可能性が示された。今後、さらに高温暴露の時間や、温度条件を複数設定して再実験を行うことなどにより今回の仮説を検証していく必要がある。

F. 健康危険情報

目下のところ、温泉利用が、ある種の疾病の罹患、増悪などの危険を増大させることを示す結果は得られていない。

G. 研究発表

1. 論文発表

Maeda, T., et al. Alterations in the telomere length distribution and the subtelomeric methylation status in human vascular endothelial cells under elevated temperature in culture condition. Aging Clinical and Experimental Research （印刷中）

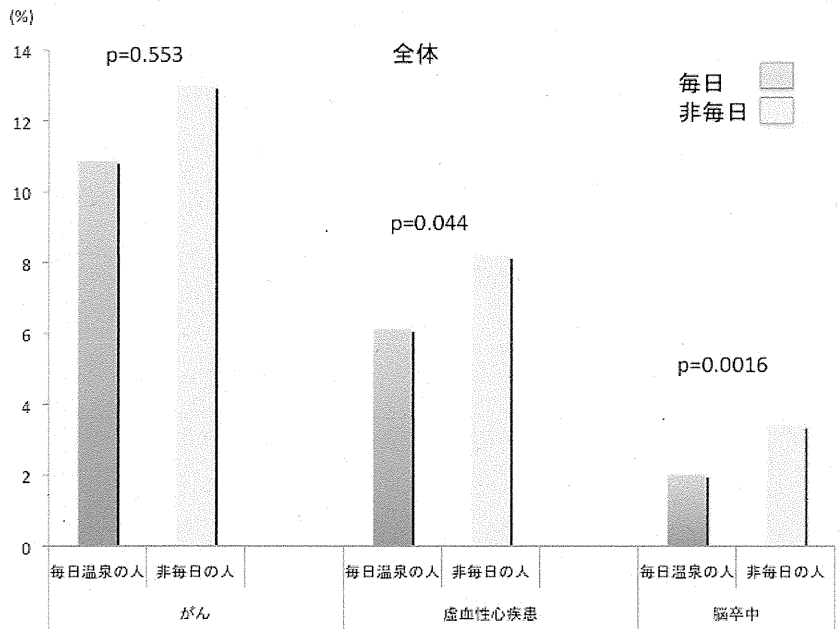
H. 知的財産権の出願・登録状況

なし

II. 研究成果の刊行に関する一覧表

Toyoki Maeda, Jing-Zhi Guan, Masamichi Koyanagi, Naoki Makino. Alterations in the telomere length distribution and the subtelomeric methylation status in human vascular endothelial cells under elevated temperature in culture condition. Aging Clinical and Experimental Research DOI 10.1007/s-40520-013-0045-6 (印刷中)

添付図


(%)
14
12
10
8
6
4
2
0
p=0.553
全体
毎日
非毎日
p=0.044
p=0.0016
毎日温泉の人
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非毎日の人
がん
虚血性心疾患
脳卒中

図１

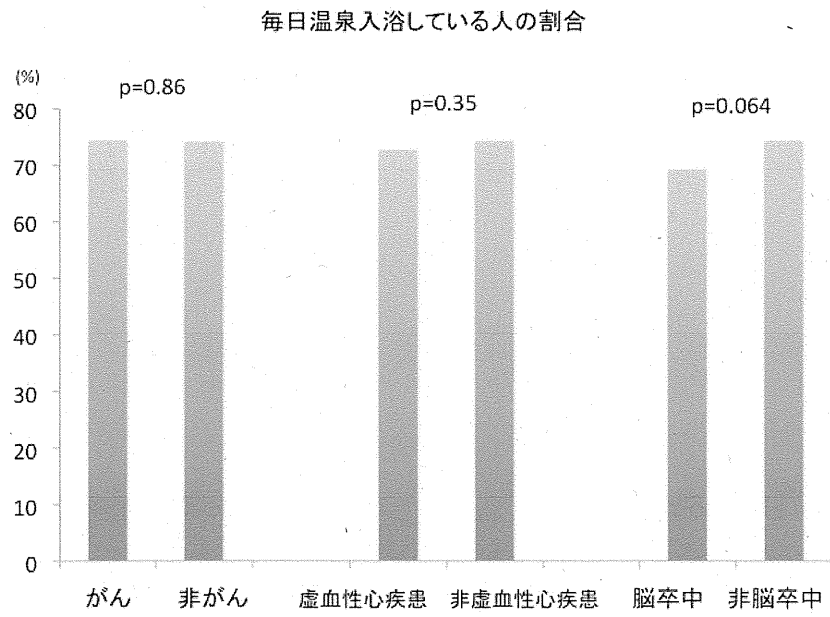
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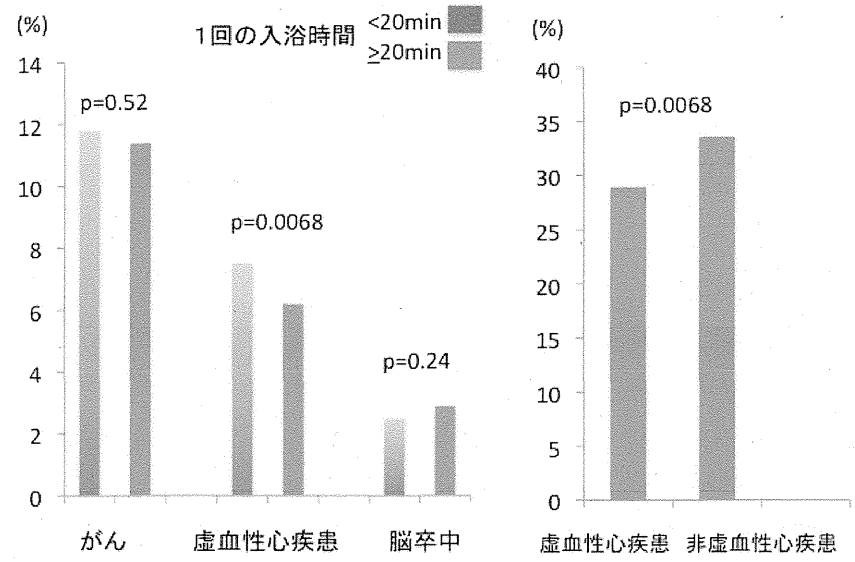
図２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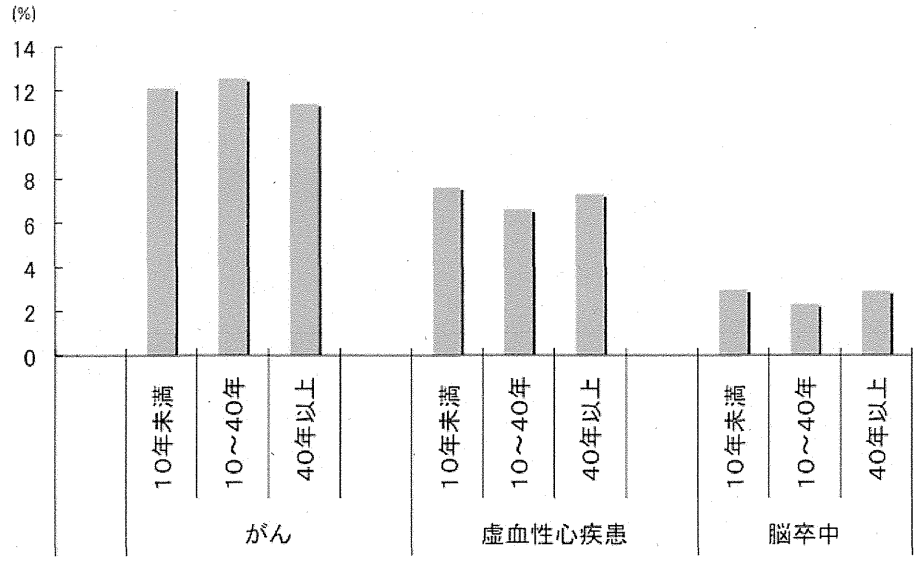
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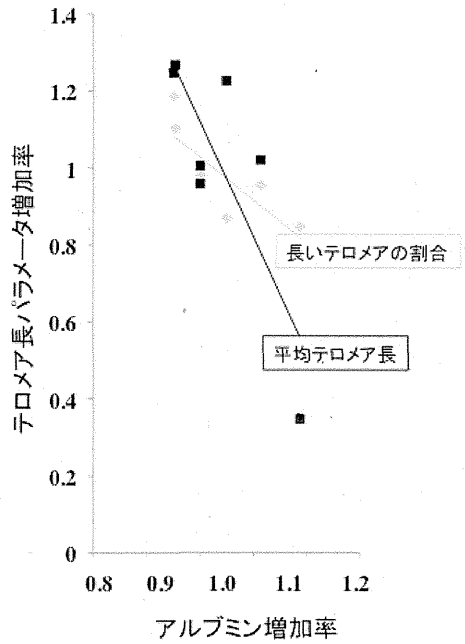
図４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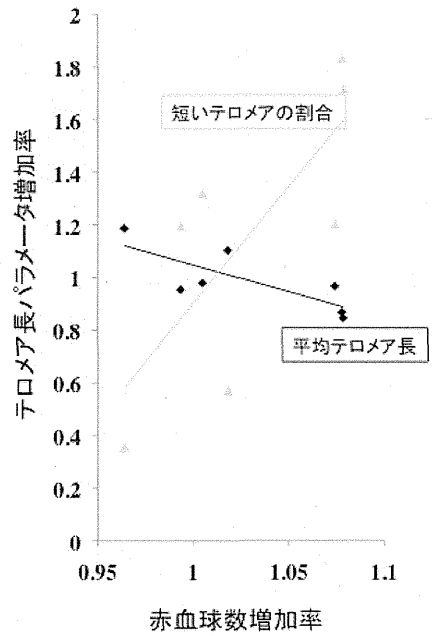
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赤血球数増加率
短いテロメアの割合
平均テロメア長

図５

以上

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

A1 T. Maeda and G. Jing-Zhi equally contributed to this article.

A2 T. Maeda (✉) · M. Koyanagi · N. Makino
A3 The Department of Cardiovascular, Respiratory and Geriatric
A4 Disease, Kyushu University Beppu Hospital, Beppu,
A5 Oita 874-0838, Japan
A6 e-mail: maedat@beppu.kyushu-u.ac.jp

A7 G. Jing-Zhi
A8 The 309th Hospital of Chinese People's Liberation Army,
A9 Beijing, China

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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

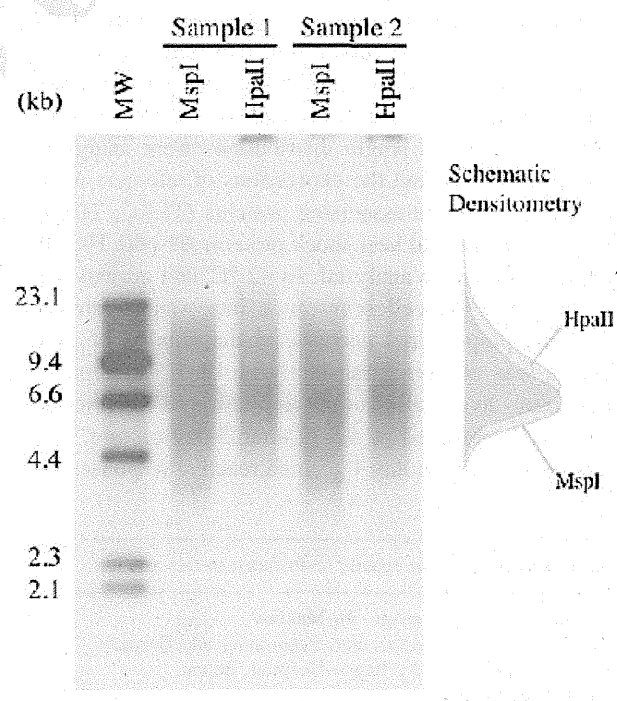

Fig. 1 Densitometric analysis of the isoschizomeric TRFs of *Msp*I- and *Hpa*II-digest. Representative Southern blot results of two samples digested with *Msp*I or *Hpa*II are shown. *Gray areas* on the *right* side depict *Hpa*II-densitometry and *Msp*I-densitometry, respectively

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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

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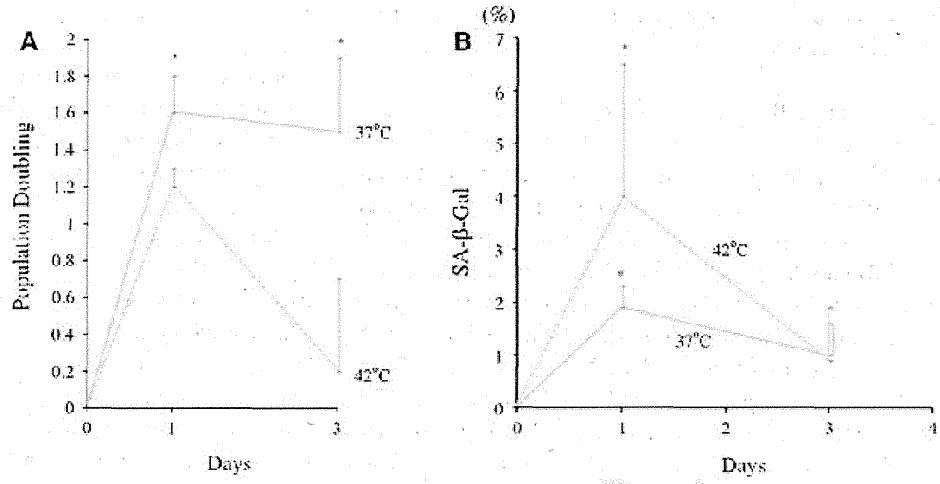

Fig. 2 The population doubling (PD) and the ratio of senescence-associated β-galactosidase (SA-β-Gal) staining of HUVECs cultured in the presence of H_2O_2. **a** The PD on day 1 and day 3 at 37 and 42 °C. The *horizontal bars* are standard deviations. $*p < 0.05$, at 37 vs. 42 °C. **b** The percentages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

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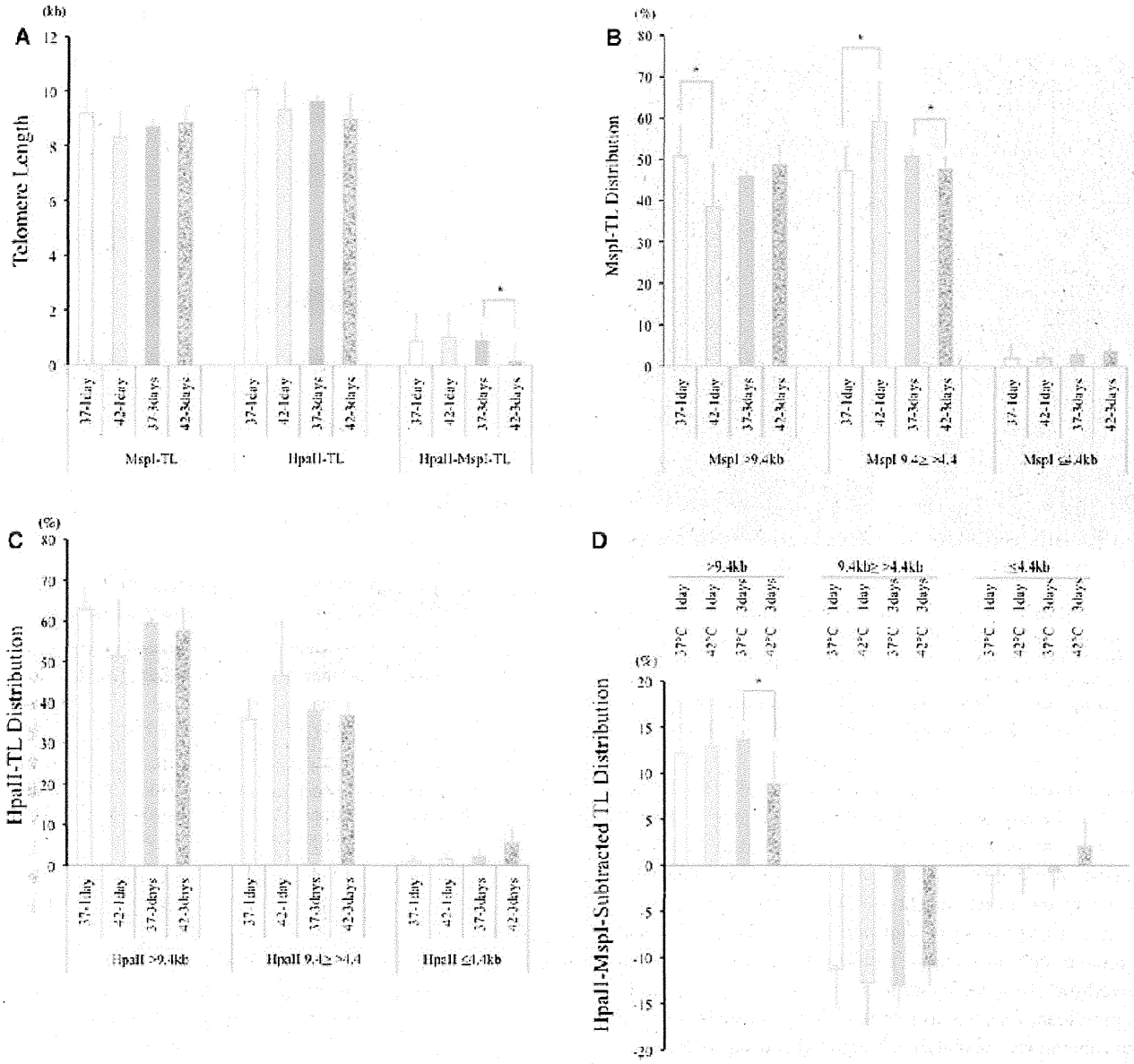

Fig. 3 The telomere length, the telomere length distribution of HUVECs exposed at different temperatures. The mean telomere lengths (**a**) and the telomere length distributions (**b–d**) are shown. The *Hpa*II–*Msp*I-subtracted percentages of telomere length range indicated (>9.4, 9.4–4.4, or <4.4 kb) 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

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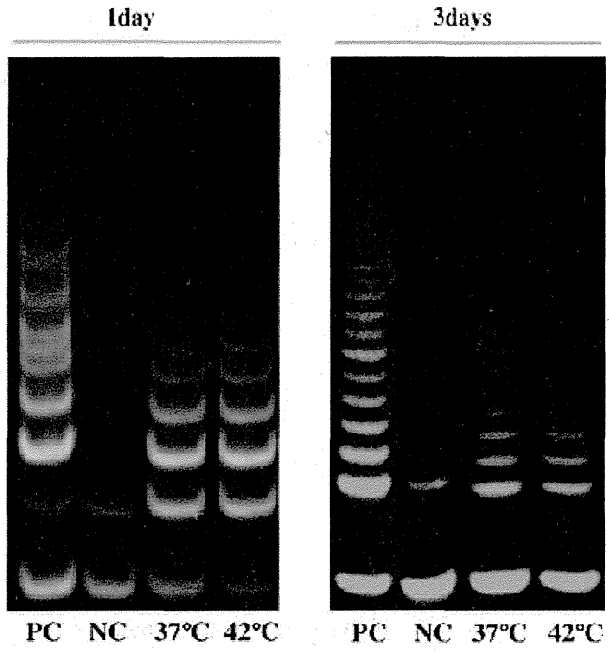

Fig. 4 The telomerase activity of endothelial cells at different temperatures. The relative telomerase activity was compared as a proportional ratio of the density the ladder of a sample to that of the mean value at 37 °C. The *panels* show photographs of representative TRAP assay results for HUVECs.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

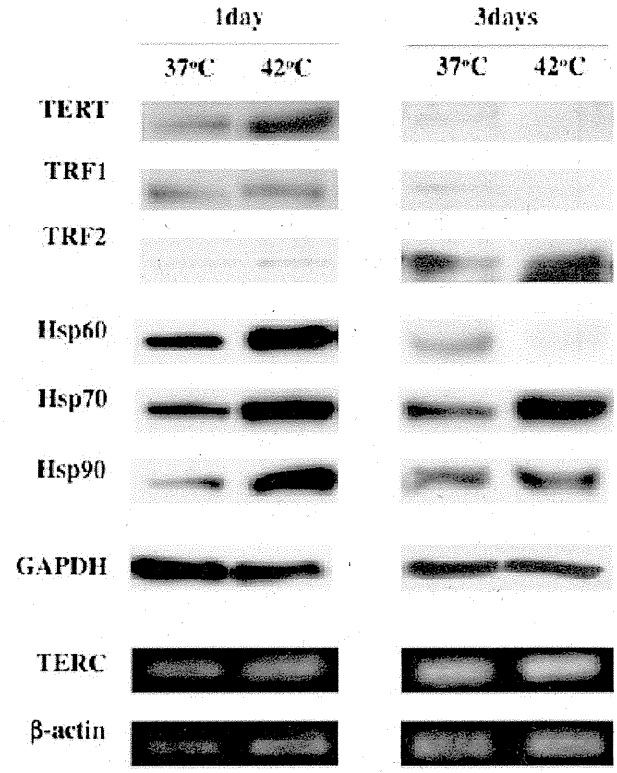

Fig. 5 The expression levels of TERC, telomere-associated proteins, and heat shock proteins of HUVECs cultured on day 1 and day 3 at different temperatures. The *panel* shows representative Western blot analysis results of telomere-associated proteins (TERT, TRF1, and TRF2), heat shock proteins (Hsp60, 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

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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.

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