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Age-Dependent Telomere-Shortening Is Repressed by Phosphorylated α-Tocopherol Together With Cellular Longevity and Intracellular Oxidative-Stress Reduction in Human Brain Microvascular Endotheliocytes

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Abstract Cellular life-span of neonatal human brain microvascular endotheliocytes (HBME) was estimated by population doubling levels (PDLs) for serial subcultivations until spontaneous proliferation stoppage, and was 2.4-fold longer for continuous administration with the 6-*O*-phosphorylated derivative (TocP) of α-tocopherol (Toc), being bioavailable owing to its water-solubility, or TocP plus 2-*O*-phosphorylated ascorbate (Asc2P), and 1.3-fold longer with Asc2P, at a dose of 150 μM, than for the non-administered control. Enlarged cell diameters indicative of cellular aging were repressed for TocP-administered cells as analyzed with a channelizer. Age-dependent shortening of telomeric DNA length (291 bp/PDL) was slowed markedly for TocP (165 bp/PDL) or TocP plus Asc2P, but slightly for Asc2P. Telomerase activity as assessed by the PCR-based TRAP method was detectable slightly at younger ages but no longer at middle ages for the non-administered cells, but, for TocP-administered cells, was intensely detected at younger ages and appreciably until middle ages. Intracellular TocP amounts were not changed age-dependently in contrast to a marked decrease in Toc which accrued from TocP esterolysis. This may be partly attributed to age-dependent changes in the lipid peroxidation product acrolein (ACR), which was abundant at older ages in non-administered cells, but scarcely in TocP-administered cells. Furthermore, intracellular reactive oxygen species (ROS) such as H_2O_2 and hydroperoxides as detected using the redox indicator CDCFH-DA was less abundant in TocP-administered cells than in non-administered cells. Thus the telomeric-DNA retention, concurrently with retained telomerase activity, was shown to be correlated with cellular longevity, and may be supported by diminished oxidative stress, in hydrophobic microenvironment, which can be achieved by TocP rather than AscP. J. Cell. Biochem. 102: 689–703, 2007.

Key words: α-tocopherol; reactive oxygen species (ROS); telomere length; lipid hydroperoxide; cellular aging; human microvascular endothelial cell

The biologically most active form out of diverse vitamin E analogues, α-tocopherol (Toc), is an important antioxidant in the cell membrane [Burton and Ingold, 1986]. It is an essential nutrient, and the major symptoms of vitamin E deficiency are neurological dysfunction, muscular weakness, arteriosclerosis, and reproductive failures [Sokol, 1988; Fukui et al., 2002; Upston et al., 2002; Young et al., 2005]. Toc, because of its hydrophobic molecular property, requires special transport mechanisms in the aqueous environment of the plasma, body fluids, and cells. Unlike other fat-soluble vitamins, Toc is transported by plasma lipoproteins [Kayden and Traber, 1993; Traber and Arai, 1999]. Recently, Toc-transfer-protein knockout (α-$TPP^{-/-}$) mice were used to evaluate its antioxidant effect in the absence of Toc [Hosomi et al., 1995; Arita et al., 1995]. Ascorbic acid (Asc) dissolved in the aqueous medium promotes a fast regenerative recycling of Toc radicals through the boundary layer between the aqueous medium

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and micelles [Diplock and Lucy, 1973; Fukuzawa et al., 1980].

Oxidative stress has been shown to be implicated in numerous neurological disorders including Parkinson's and Alzheimer's diseases (AD) and cerebral ischemia [Smith et al., 1995; Mark et al., 1996]. Therefore, antioxidant therapy may be so important as to be elicited toward potential regimens for neurodegenerative disorders. In fact, several studies demonstrated that antioxidants such as Toc and superoxide dismutase [Przedborski and Jackson-Lewis, 1998] exert cytoprotective effects against brain injury and delay the progression of AD [Carney et al., 1991; Smith et al., 1991; Takeda et al., 1998; Cherubini et al., 2005]. But the exact and detailed mechanisms underlying oxidative damages remain to be fully analyzed and understood.

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) are serious threats to health along with the induction of cell death, and provide damages to the cell membrane concurrently with accelerating the telomere-shortening through the instability of chromosome ends. There is an appreciable evidence that telomere-shortening acts as a biological clock in human or mammalian cells, limiting and commanding the maximum number of population doubling levels (PDLs) corresponding to cellular life-spans. Therefore, repressive regulation of intracellular oxidative stress can lead to enhancement of the cell growth potential together with an increase in telomeric repeats of chromosome ends [Mitsui and Schneider, 1976; Moyzis et al., 1988]. The plasma lipoproteins and cell membrane-associated phospholipids can be exempted from peroxidation upon exposure to aqueous peroxy radicals ($LOO^{\cdot}$) and hydroperoxides (LOOH) until depletion of endogenous Toc and Asc as ROS scavengers, resulting in suppression to lowering of the cell growth potential. Particularly, Toc can be retained for a period longer than those of other plasma ROS-scavengers such as SH compounds, Asc, bilirubin, and urate, and can undergo passive diffusion into the cellular membrane [Fujiwara et al., 1997; Huang et al., 2004; Raymond and Johnson, 2004; Saitoh and Miwa, 2004; Tam et al., 2005; Xiao et al., 2005].

In the present study, the effects of the novel water-soluble pro-vitamin E, α-tocopherol-6-*O*-phosphate (TocP) were examined by its preventing ability against aging-induced telomere shortening or membrane damages in cultured microvascular endothelial HBME cells in comparison with effects of Toc and Asc-2-*O*-phosphate (Asc2P). TocP is hypothesized to improve its drug bio-availability through the endowing with a slight hydrophilicity as compared with Toc, and is aimed in the present study at its localization in the vicinity of telomere and its surrounding nuclear matrix resulting in the cell longevity. Intracellular TocP was shown to be efficiently converted to Toc, and retained abundantly in the intracellular space through Toc transport, and TocP may act for a longer period than Toc. To clarify a possibility that TocP-derived Toc may protect the biomembrane or the lipophilic microenvironment in the vicinity of telomeric DNA from either endogenous oxidative stress during the aging process or LOOH-mediated cell-membrane degenerative propagation, we examined alterations of the telomere length and telomerase activity [Lucy, 1972; Fukuzawa et al., 1980; Upston et al., 1999; Na et al., 2004] during the aging process.

MATERIALS AND METHODS

Cell Cultures

Normal human brain microvascular endotheliocytes (HBME) were obtained from Applied Cell Biology Research Institute (ACBRI), and were cultured in a CS-C complete medium kit (Cell Systems Corporation, Kirland, WA) in a humidified atmosphere of 95% air and 5% CO_2. Cells were seeded on rat type I collagen-coated (Attachment Factor™) 100-mm dishes at densities of $5.0–6.5 \times 10^3$ cells/cm^2, and were continuously administered with TocP (150 μM) for each time of culture passage. Upon approximately each passage, the spent culture medium was replaced by the fresh medium. The subconfluent cells were thus detached from the substratum, and were enumerated upon each passage culture with a Coulter electric particle counter ZM. The PDL is regarded as zero for culture starting immediately after the established culture of the HBME cells, and is calculated for a PDL increase according to the following equation:

$$\log_2\left\{\frac{(\text{the number of collected cells})}{(\text{the number of seeded cells})}\right\}.$$

HBME cells fed with or without TocP were rinsed, trypsinized, and then enumerated.

Photography and Cell Size Distribution

Microscopic aspects of HBME cells were observed with a Nikon microscope DiaPhot and photographed with a Nikon digital camera E450. Cell size distribution was assessed for HBME cells, which were fed with or without Asc2P (Showa Denko Co., Tokyo) or TocP (Showa Denko Co.), were rinsed, trypsinized, and then analyzed with a Coulter counter ZM equipped with a channelyzer model 256^{TM} with calibration using PDVB latex particles (Becton-Dickinson, Mountainview, CA) of 5.1 and 13.7 μm in diameters.

Determination of Telomere Length by Southern Blots

Genomic DNA was extracted with a nucleic acid extraction kit IsoQuick (ORCA Research, Inc., Bothell, WA) from 1×10^6 cells of each passage collected when reaching 70% confluence, and quantified by fluorometry using Hoechst 33258 (Sigma) and NIH Image analysis for agarose minigel electrophorograms. Extracted DNA was completely digested with the restriction enzyme *Hinf I* (TaKaRa, Kyoto, Japan) to produce TRFs as previously described [Hiyama et al., 1995]. A portion (2 mg/lane) was loaded onto a 0.8% agarose gel, and electrophoresed at 35 V/cm for 20 h together with 1 kb-DNA Ladder (Gibco BRL, Grand Island, NY) and lambda DNA/Hind III digest (Nippon Gene, Tokyo) as size markers. DNA was depurinated by soaking gels in 0.2 N NaOH/0.6 M NaCl for 25 min, and transferred to a nitrocellulose membrane Optitran BA-S 85 (Schleicher & Schuel, Dassel, Germany). DNA was prehybridized with AlkPhos direct hybridization buffer (Amersham Biosciences, Buckinghamshire, UK) in 0.5 M NaCl, added by a blocking reagent to a final concentration of 4% (w/v) at 55°C, and hybridized in denatured alkaline phosphatase enzyme-labeled $(TTAGGG)_4$ (Qiagen Co. Tokyo). Membranes were washed in 2 M urea, 0.1% SDS, 50 mM sodium phosphate buffer pH 7.0, 10 mM $MgCl_2$, and 0.2% blocking reagent at 55°C. The membranes were placed in a clean container and added with 50 mM Tris-HCl buffer, 100 mM NaCl, and 2 mM $MgCl_2$ at pH 10. A detection reagent (Amersham Biosciences) was pipetted on the membranes, which were left for 2–5 min and underwent a chemiluminescent signal with a Kodak X-ray film Scientific Imaging Film, followed by densitometry with a Pharmacia laser densitometer UltroScan XL. Additionally, TRFs of each manner-treated cells of several randomly selected passages (including PDL zero) were simultaneously analyzed by Southern blots on the same single agarose gel, resulting in TRF lengths similar to those estimated from separate gels.

PCR-Based Assay for Telomerase Activity

Cells of each passage collected and frozen were assessed for telomerase activity by a telomerase assay kit TeloChaser (Toyobo, Osaka, Japan) [Tatematsu et al., 1996]. Briefly, 1×10^6 cells were lysed in a lysis solution followed by preparation of 1×10^5 cell-equivalent extracts. Telomerase reaction was conducted at 37°C for 30 min after mixing the cell lysate with PCR mix and Taq mix which were contained in a TeloChaser kit. PCR amplification was repeated by 28 cycles using an Astec thermal cycler PC-800 with a synchronized temperature shift at 95°C for 30 s, 68°C for 30 s, and 72°C for 45 s, respectively, as one cycle. PCR products of 20 μl/lane were loaded onto 10% polyacrylamide gel, and electrophoresed at 300 V/cm in 0.7× TBE, followed by staining with a SYBR Green I dye and the subsequent densitometry.

Cell Viability as Endogenous Mitochondrial (MT) Activity Using a WST-1 Dye

WST-1 assay was achieved to determine viable cell numbers. At different time points, the cells were rinsed with phenol red-free DMEM, then incubated for 3 h in phenol red-free DMEM medium containing 10% WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2-disulfophenyl)-2H-tetrazolium, monosodium salt; Dojn Laboratories Co., Kumamoto, Japan) at 37°C. Cell viability was determined based on MT ability to reductively convert a WST-1 dye to yellowish formazan, being indicative of the number of viable cells [Ishiyama et al., 1996]. The absorbance at 450 nm was read with an absorbance multiplate reader Microplate Photometer (Bio-Rad, San Jose, CA).

Intercellular Toc Concentrations

The synthetic compound TocP was continuously administered at a dose of 150 μM to HBME

cells upon each passage and medium exchange. Intracellular Toc amounts were measured by high-performance liquid chromatography (HPLC) analysis protocols [Yamauchi et al., 2002] as well as other vitamin E analogues analysis. After calculated for cell numbers, cells were suspended in ethanol solution. The cells' lysate was mixed with a standard solution containing 10 nmol/ml δ-Toc (200 μl), 5% pyrogallol and HCl (200 μL) in ethanol (0.5 ml). The mixture was diluted with 4 ml of hexane/ethylacetate (9:1) solution, and was subjected to freeze-thawing which was repeated three times. The mixture in cells lysates underwent shaking for 5 min, was centrifuged at 3000 rpm for 5 min at 4°C, and then was stored on ice. The hexane layer was saved, and the hexane extracts were evaporated under dryness. The residue was dissolved again with 30 μl of methanol/H_2O/trihydrogen phosphate (93:6:1) eluent solution. This solution was filtrated with an YMC membrane filter Duo-filter™. The chromatographic profiles of extracts from TocP-treated and untreated HBME cells are shown corresponding to a calibrator in Figure 4C–E. The TocP peak was well separately resolved and identified by comparing a peak retention time of Toc peak with that of δ-Toc added as an internal standard. Elution peaks of Toc and TocP show retention times at 5.3 min and 10 min, respectively; because there were no later eluting peak, further injections were made at intervals of 20 min (Fig. 4C–E).

Intracellular Oxidative Stress

HBME cells seeded at 1,000–1,500 cells/cm^2 were grown on a 24-well microplate in the presence or absence of 150 μM TocP in culture medium for 24 h, were rinsed three times with phosphate-buffered saline (PBS(−)) and replaced by the medium containing CDCFH (Molecular Probes, Eugene, OR) at a final concentration of 50 μM [Szejda et al., 1984]. After 30-, 60- and 90-min incubation for the control or TocP group, the fluorescence intensity was measured with a fluorescence plate reader CytoFluor 2350 (Millipore, Bedford, MA). The excitation and emission wavelengths used were 480 and 530 nm, respectively. Fluorescence of the oxidative form of CDCFH increased in a manner dependent on cell numbers and incubation times for viable cells, but not for methanol-killed cells similarly as compared with the blank.

Determination of Lipid Peroxidation

Lipid peroxidative products, Acrolein (ACR) were measured with use of anti-ACR rabbit antibody at a dilution rate of 1:3,000 (Nihonyushi Co. Ltd., Japan). ACR is known to preferentially react with lysyl residues resulting in the formation of the novel ACR-lysine adduct, N^{ε}-(3-formyl-3, 4-dehydropiperidino) lysine (FDP-lysine). FDP-lysine was found to potently deserve as an epitope of the antibody. Indeed, low-density lipoprotein (LDL) was demonstrated to be oxidatively modified and generate ACR-LDL adducts. Furthermore, after in vitro incubation of some proteins with glucose, ascorbate, and arachidonate, arachidonate was the only source of antigenic material, suggesting that polyunsaturated fatty acids may be a source of ACR that causes the production of protein-bound ACR [Uchida, 1998; Calingasan et al., 1999; Kondo et al., 2001; Luo et al., 2005]. Cells were cultured on cover glasses and incubated for 18–24 h in the culture medium containing 150 μM TocP. Then cells were rinsed three times with PBS(−), fixed with 4% paraformaldehyde by immersion for 10 min, and subsequently rinsed three times with PBS(−). Endogenous peroxides were inhibited with a final concentration of 0.3% H_2O_2 in methanol for 20 min. After washing, cells were incubated with the primary antibody in 0.1% Triton X-100 in PBS(−) at 2 h, then followed by addition with the FITC-conjugated secondary antibodies against rabbit IgG (1:1000) for 2 h in the same solution.

RESULTS

Effects of the Vitamin E Derivative TocP on Cellular Proliferation and Life-span of HBME

HBME were serially cultured in the presence or absence of the vitamin C derivative Asc2P, the vitamin E derivative TocP, or Asc2P plus TocP, which were always added at a dose of 150 μM upon each subculture and medium exchange. Cellular life-span, as estimated by a population doubling level (PDL), for TocP- or Asc2P plus TocP-administered HBME cells in culture was 2.4-fold longer than that of the non-administered control cells. In contrast, the solely Asc2P-administered cells were less markedly (1.3-fold) prolonged for their cellular life-span comparative with the control cells (Fig. 1B). The TocP-administered cells even at older ages

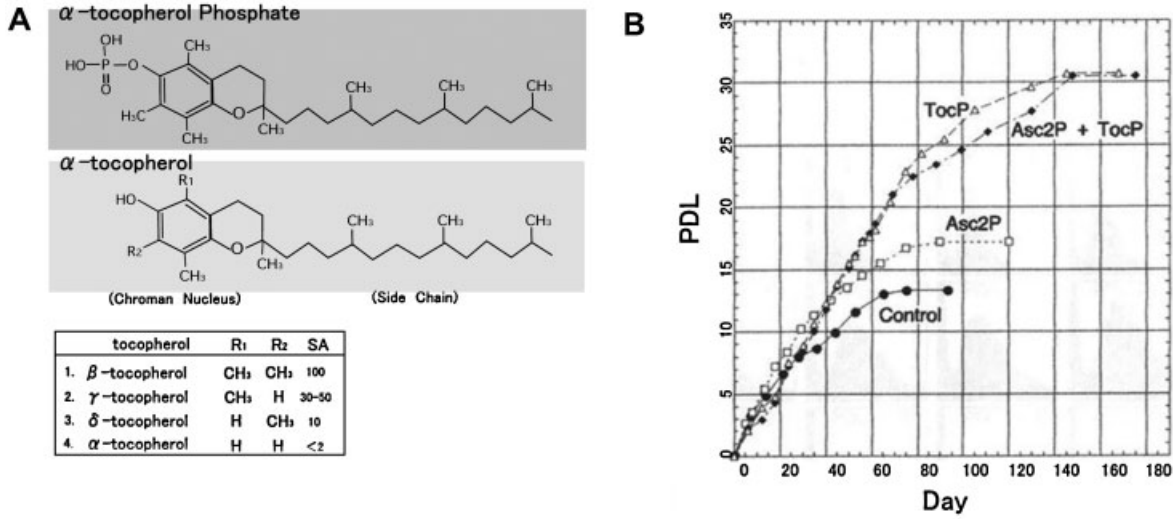


Fig. 1. Life-span prolongation by a-tocopheryl phosphate (TocP). Chemical structures of TocP, the growth rate, and life-span of human neonatal brain microvascular endotheliocytes (HBME) were assessed for the serial treatments in the presence or absence of TocP during the culture period. **A**: Chemical structures of TocP and α-tocopherol (Toc) are shown by schematic representation [Traber, 2005]. The properties of tocopherol analogues are known to exhibit relative differences in the specific activity (SA) for anti-sterility. **B**: The life-spans of HBME cells that were serially cultured in the presence or absence of the vitamin C derivative Asc2P, TocP, or Asc2P + TocP. The data shown are typical of three independent experiments.

were retained for their proliferation ability at the normal level nearly equal to the level of the non-administered control cells at an age as young as PDL 3.3, and were continued for their full ability via an aging period until the so-called Hayflick limit around the 50-day culture.

The Ability of TocP to Retain a Compact Cell Size and Cell Morphological Aspect

To examine the effects of TocP on age-dependent changes of cell size and morphology, a channelyzer was utilized for the particle-distribution analysis to a portion of harvested HBME cells that were serially subcultivated in the absence or presence of TocP of 150 μM until spontaneous stoppage of cell division. Administration with TocP exerted an inhibitory effect on a PDL-dependent increment in the cell size (Fig. 2B) and the senescent cell features in microscopic photographs, as shown for three typical periods such as relatively young, middle, and old ages (Fig. 2A). Enlarged cell diameter is known as one of the symptoms of cell aging to be inversely correlated with cellular senescence [Mitsui and Schneider, 1976]. The cell diameter of TocP-administered cells was suppressed up to 6.8–8.2%, and was as long as 16.8 μm even at an old age of PDL 15.3 being more compact than 18.3 μm at a PDL as low as 9.4 of the control (Fig. 2B). In contrast, solely Asc2P-administed cells were not markedly suppressed for the enlargement of cell size.

Slow-down of Age-dependent Telomere Shortening and Telomerase in TocP-Administered Cells

DNA extracts from HBME cells of each culture passage were restrictively digested to produce terminal restriction fragments (TRFs) consisting of both entire telomeric and partial subtelomeric regions, and analyzed by Southern blots [Hiyama et al., 1995]. The TRF patterns in Figure 3A were converted to scores of their average telomeric DNA lengths in Figure 3B. The TRF lengths indicative of telomeric DNA lengths were markedly shortened at a rate of 291 bp/PDL for the control cells, while TRF lengths in TocP-treated cells were considerably maintained at 165 bp/PDL in a degree similar to that of Asc2P + TocP-treated cells (158 bp/PDL). The solely Asc2P-treated cells were only slightly reduced for the telomere-shortening rate down to 227 bp/PDL relative to the control. The slowed rate of age-dependent shortening of TRF lengths was shown to be in proportion to longevity of the cellular life-span for TocP-treated cells that were most markedly maintained out of all examined cells. Telomerase, an intracellular reverse transcriptase that elongates or retains

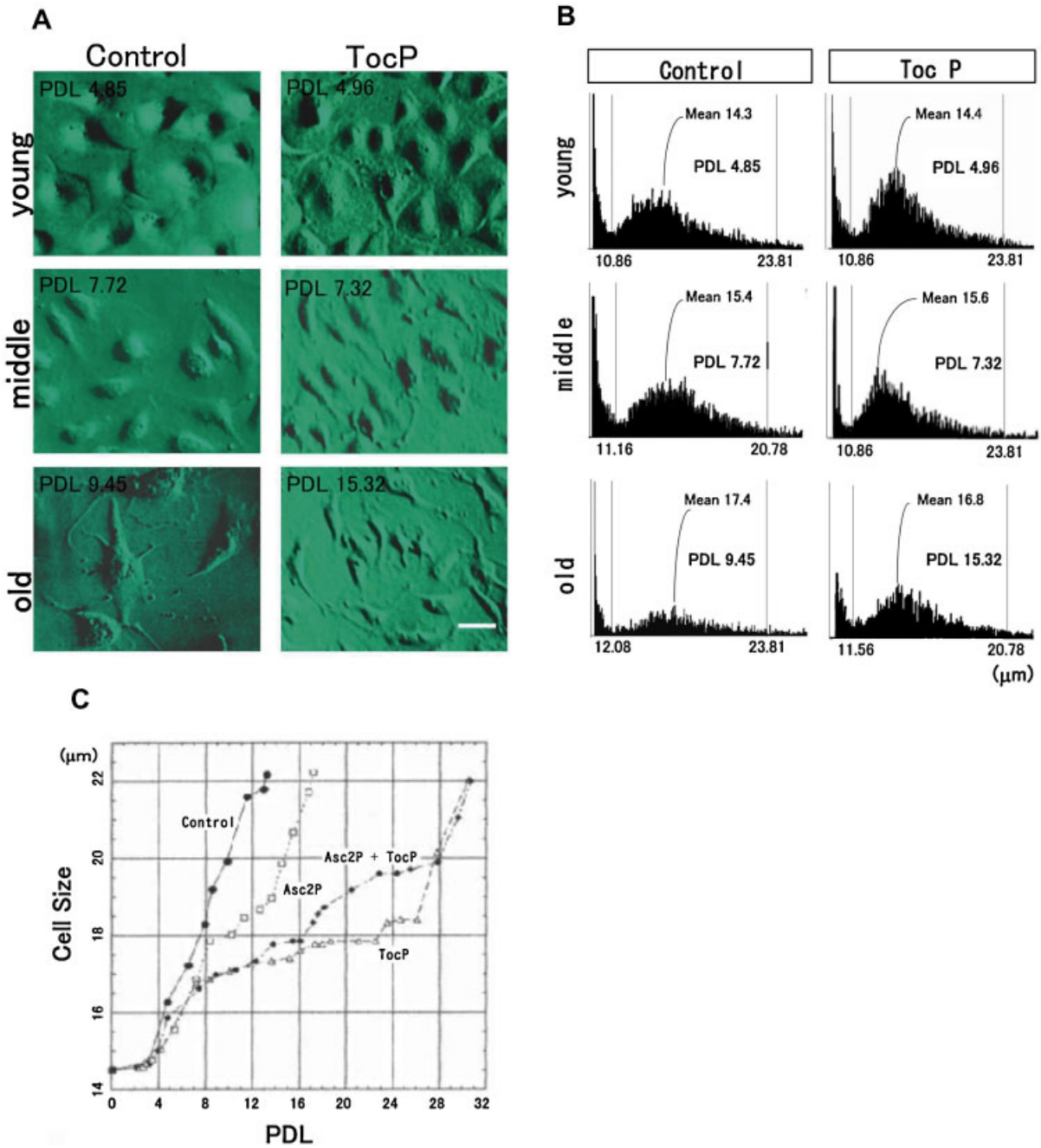
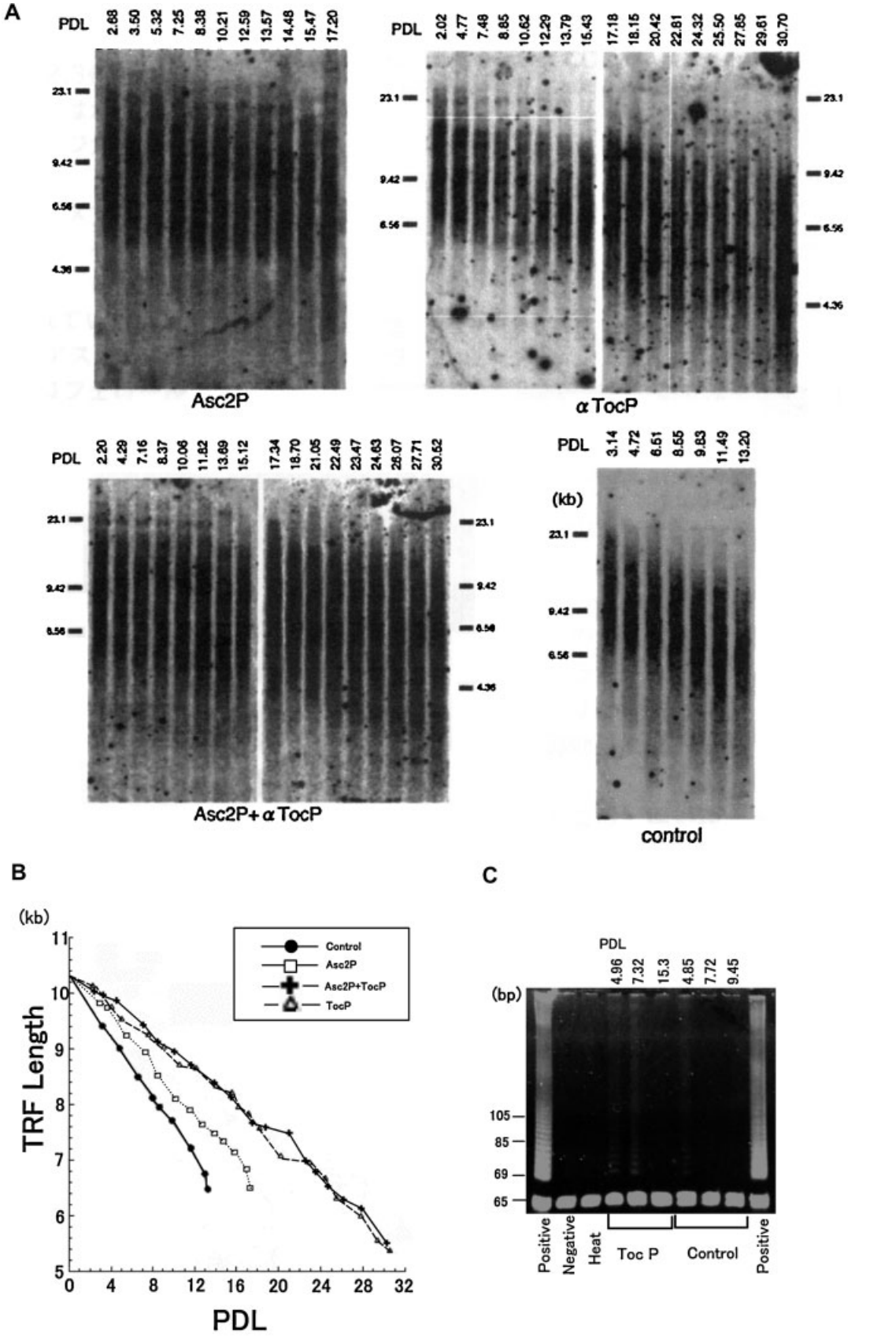


Fig. 2. Retention of compact cell size as a hallmark for cell juvenility. **A**: Cell morphological photographs were shown for control and TocP-treated cells at each passage of relatively young, middle, or old age. **B**: Dependence of mean cell size on PDLs. These data are obtained based on three independent experiments that were conducted with dishes in duplicate for cells that were subjected to each treatment, SD of which is indicated by the bar. **C**: The life-spans of HBME cells that were serially cultured in the presence or absence of the vitamin C derivative Asc2P, TocP, or Asc2P + TocP. PDL-dependent change of cell size of HBME cells was diversely treated as in Figure 1B. Scale bar, 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 3. Telomeric DNA length and telomerase. **A**: Age-dependent shortening of telomere length for graded PDLs in HBME cells by Southern blot analysis. **B**: Terminal restriction fragments (TRFs) were analyzed by length of densitometic telomere fragments as a hallmark for telomere length of HBME cells that were serially subcultivated in the presence of 150 μM Asc2P, 150 μM TocP, Asc2P + TocP of equimolar 150 μM and control (no additive). **C**: Telomerase activity in 1.0×10^5 cells was measured by the PCR-based TRAP method, and shown by electrophorograms of Southern blots, where the sum of brightness of faint ladder-shaped several bands correlates with degrees of telomerase activity as compared with wide bands as the internal standard in the bottom of all lanes. Extracts from HeLa cells (1.0×10^2 cells) were similarly analyzed as the positive control. Note some faint bright bands in two left TocP lanes and one left control lane. The data shown are typical of three independent experiments.


A
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αTocP
Asc2P+αTocP
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TocP
C
PDL
(bp)
Positive
Negative
Heat
Toc P
Control
Positive

Fig. 3.

telomeres, was measured for each passage of telomeric repeats [Piatyszck et al., 1995] (Fig. 3C). Non-administered control cells and 150 μM TocP-administered young, middle, or old age cells displayed a prominent telomerase-mediated 6-bp-interval ladder-like pattern using PCR-based modified TRAP assay (Fig. 3C). TocP-administered cells exhibited some detectable telomerase activity at the young age (PDL 4.9) and middle age (PDL 7.3), but not detectable at the old age (PDL 15.3), whereas the control cells have a significantly detectable telomerase activity only at the young age (PDL 4.8) but no activity at the middle (PDL 7.7) and old ages (PDL 9.4). These results indicate that TocP activated telomerase activity through administration to cells, but exerted no direct effect on telomerase reaction and the inherent level of telomerase of HBME cells was slightly detected only at the young age, but thereafter rapidly disappeared age-dependently in the absence of TocP, whereas the telomerase activity was retained until the middle age in the presence of TocP.

Cellular Metabolic Level Associated With MT Activity

To examine an effect of TocP on the endogenous MT activity, HBME cells were exposed to TocP of the indicated concentrations below 300 μM for 12 h in the same way as done for the cell viability assay by the WST-1 method. All data represent that wide-ranged concentrations at 1–500 μM were preliminarily examined for activation to MT dehydrogenases resulting in the determination of the optimal concentration at 150 μM of TocP (data not shown). Viable cells that exhibit MT dehydrogenases such as succinate dehydrogenase are capable of reducing the WST-1 dye to generate the yellowish formazan. The MT activity was of the highest degree in cells that were administered with TocP of 125 μM, nearly equal to 150 μM where cellular aging was examined, out of graded doses of TocP. Particularly, the TocP-derived effects influenced the MT activity in a dose-dependent manner below 150 μM of TocP, although the MT activity was gradually reduced at the range of 200–300 μM TocP (Fig. 4A). All TocP-treated cells examined were augmented for MT activity as compared with untreated cells. MT activities that may be putatively affected by TocP were examined for MT dehydrogenase as a typical redox-related indicator, but not yet for other functions.

Intercellular TocP and Toc Contents and TocP-to-Toc Conversion Efficacy

It should be clarified whether TocP of 150 μM that was administered to HBME cells was taken up into cells and converted by enzymatic dephosphorylation to Toc. The amounts of TocP in the extracts from 5.0×10^5 cells were shown not to be obviously different among the non-added middle-age cells, TocP-added middle-age, and old-age cells (PDL 7.7, 7.3, and 15.3, respectively) by an HPLC technique with fluorometric detection (Fig. 4B, middle). The ratio of TocP-to-Toc conversion that may be executed assumedly by diverse types of intracellular phosphatases was drastically decreased during aging from 147.3 nmol at the middle age to 11.7 nmol at the old age per 5.0×10^5 cells (Fig. 4B, top; 4D and E). These changes of Toc contents are presumably attributed either to age-dependent reduction of the number of Toc transporters concurrently with promotion of oxidative collapse of Toc [Sato et al., 1991; Arita et al., 1995; Hosomi et al., 1995] or to the diminished activity of intracellular phosphatases. The efficacy of Toc-converting ratio to intracellular TocP between ToP-treated middle- and old-age cells significantly reduces; this change in rate suggests that TocP-active sites are localizing for the cell membrane or cytosol.

In Situ Detection of Cellular Oxidative Stress

Non-administered old-age cells (PDL 9.4) became notably fluorescent after loading with CDCFH-DA, whereas TocP-added old-age cells (PDL 15.3) did not appear appreciably fluorescent (Fig. 5A), suggesting that generation of intracellular ROS might be markedly scavenged by Toc that was yielded by dephosphorylation of administered TocP. The level of intracellular ROS in HBME cells was significantly lower after 90-min incubation for the TocP-added old-age (PDL 15.3) and middle-age (PDL 7.3) cells than for the non-added control old-age (PDL 9.4) and middle-age (PDL 7.7) cells (Fig. 5B). The level of intracellular ROS in TocP-added old-age cells was suppressed 3.4 times lower than that in the control old-age group. Intercellular ROS in each age cells was indicated in Figure 5C. In the TocP-added cells, the ROS-attributed fluorescence was repressed as indicated by no transcendence over 500 fluor.

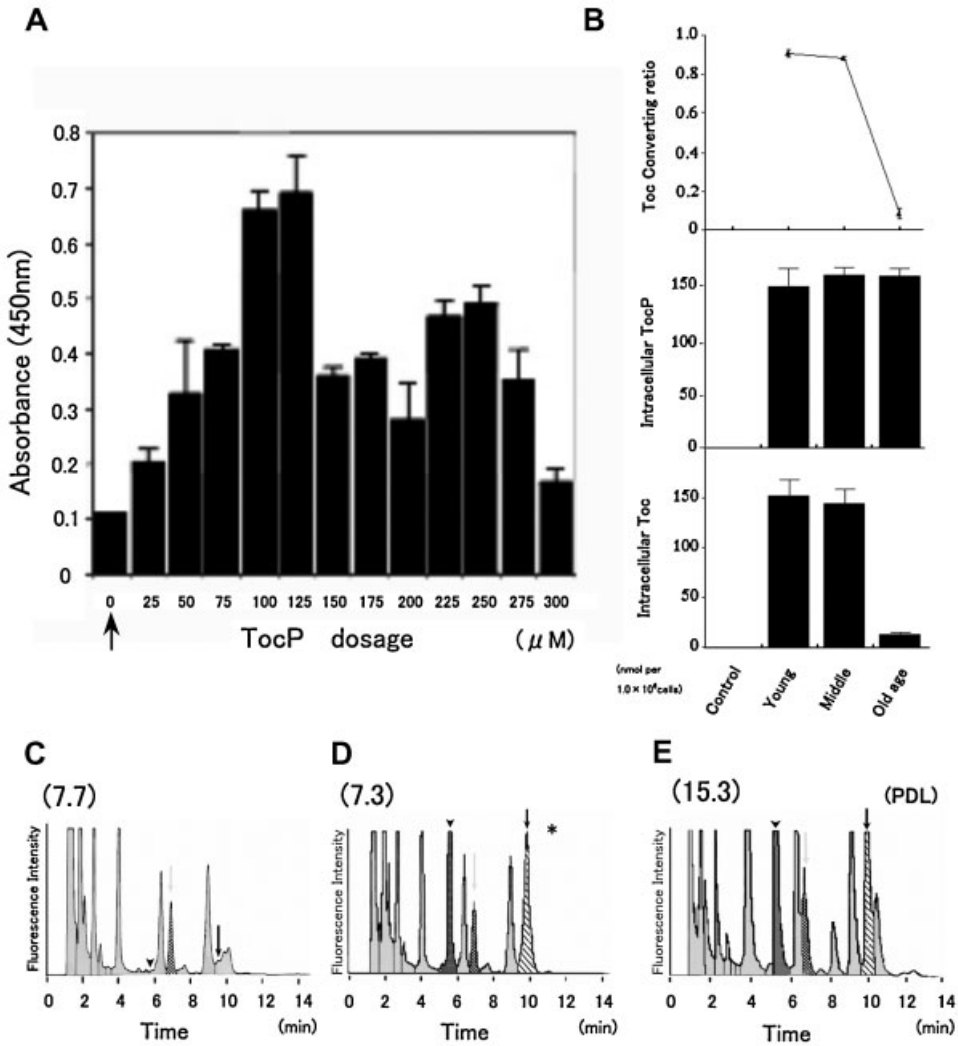
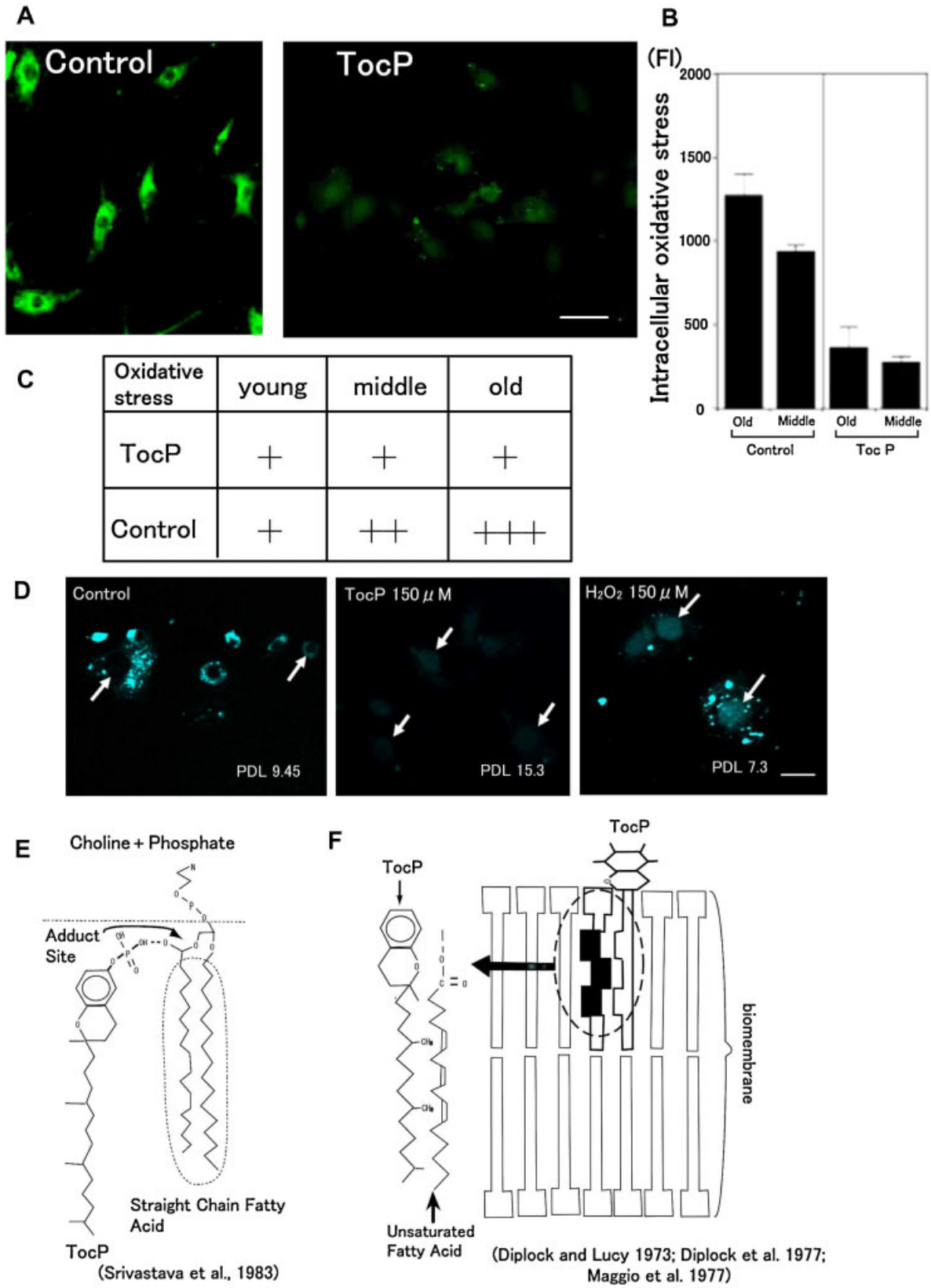


Fig. 4. Promotion of metabolic level and intracellular uptake of TocP. The TocP-induced promotion of basic metabolic levels of HBME cells and intracellular uptake of TocP. **A**: As an indicator for intercellular metabolism level, MT dehydrogenase activity was estimated by formazan-based WST-1 assay for cells that were administered with TocP at graded doses. The control cells that were administered with TocP-lack medium are shown by the arrow. HBME cells were plated at the density of $1.0–1.5 \times 10^4$ cells/cm^2. After preincubation for 24 h, the medium was replaced by fresh FBS-free medium containing diverse concentrations of TocP of 0–300 μM. The data shown are typical of three independent experiments. **B**: The age-dependent change of intercellular TocP and Toc contents in TocP-administered HBME cells as analyzed by HPLC and fluorometry. Toc conversion ratio represents a ratio of intracellular Toc content versus intracellular TocP content. **C–E**: HPLC chromatograms with detection by fluorometry were obtained at 15-fold dilution of cell lysates by mobile phase solution (asterisk) for extracts from: (C) the control middle-aged (PDL 7.7) cells that were cultured in TocP-lack culture medium, (D) the middle-aged (PDL 7.3) and (E) old-aged (PDL 15.3) cells that were administered with TocP of 150 μM. Retention times of Toc (long black arrow) and TocP (arrowhead) are indicated together with a peak of δ-Toc added as the internal standard at 10 nmol/ml (long gray arrow).

unit/0.1 ml during the culture period regardless of cellular ages.

Immunocytochemical Detection of the Oxidative Stress-Associated Substance ACR

To determine whether the cell membrane that is composed of polyunsaturated fatty acids may be injured, the production of protein-bound ACR was examined by immunocytochemical stain (Fig. 5D). The protein-bound ACR was detected abundantly in the non-added control cells of older age at PDL 9.4, but scarcely detected in TocP-added cells of older age (Fig. 5D, left and middle), and markedly detected in H_2O_2-treated cells of middle age even at PDL as low as 7.3 in the absence of TocP owing to acute drastic oxidative stress on the cell membrane (Fig. 5C, right).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Straight Chain Fatty Acid
TocP
(Srivastava et al., 1983)
F
TocP
TocP
biomembrane
Unsaturated Fatty Acid
(Diplock and Lucy 1973; Diplock et al. 1977; Maggio et al. 1977)

Fig. 5.

DISCUSSION

The present study demonstrates that the Toc derivative TocP has a potent prolonging effect on the cellular life-spans of HBME, because the maximal replication level was expanded for TocP-treatment culture (Fig. 1B). HBME cells grow at a doubling period of 1–2 days, which is a normal rate as a normal human cell strain in contrast to most types of well-spread cell lines. The TocP-treated culture effectively repressed either the age-dependent cell-size enlargement (Fig. 2C) as a symptom of cellular aging, or the age-dependent decreasing of telomeric repeats in the chromosome ends (Fig. 3B). Intracellular oxidative stress including hydroperoxide and $LOO^{\cdot}$ during the aging process was significantly reduced in TocP-treated endotheliocytes as shown by the redox indicator CDCFH analysis (Fig. 5B) and ACR immunostain (Fig. 5D), suggesting a close correlation between diminishment of the oxidative stress and prolongation of cellular life-spans, both of which were executed by TocP. TocP significantly prevented age-dependent decreases in telomerase activity in addition to those of telomeric repeats, each of which might be associated with the level of intracellular oxidative stress; and increased oxidative stress in old passage cells is a well-known phenomenon as one of major causes for cellular senescence, and may be attributed to increased ROS amounts due to age-dependent accumulated error catastrophe of metabolic machineries.

The change of telomeric repeats has been reported to be correlated with cellular age [Moyzis et al., 1988]. However, it is poorly understood whether antioxidants such as vitamin E may give an influence on telomere length and telomerase and on the level of intracellular oxidative stress from a viewpoint of the aging. We reported that the TRF, a hallmark of telomere lengths, is shortened along with cellular aging in human umbilical endothelial cells (HUVEC) and human epidermal keratinocytes (NHEK), where the age-dependent shortening of TRF can be slowed down together with both elongation of cellular life-span and retention of telomerase activity by maintaining the intracellular Asc concentration at the higher level [Fujiwara et al., 1997; Furumoto et al., 1998; Yokoo et al., 2004], and the decline in TRF is known to take place concurrently with DNA single strand cleavages by hyperoxia treatment or high-concentration hydrogen peroxide [Von Zglinicki et al., 2000]. Age-dependent shortening of telomeres has been elucidated in the present study to be closely related to cellular senescence, whereas it remains to be elucidated in the forthcoming study whether telomere shortening activates or merely results from senescence by detecting telomere dysfunctional foci. Both the persistent retentions of telomerase expression and full telomeric length cannot be conducted by TocP, but might be achieved by gene technological methods resulting in life-span extension rather than immortalization.

Intracellular processing of TocP is considered to occur around the cell membrane boundary, where Toc may be converted from TocP by diverse types of phophatases. We observed no significant difference between the solely TocP treatment and the TocP plus Asc2P treatment in terms of age-dependent telomere shortening (Fig. 3B). In terms of telomere shortening that is expressed as per PDL and may be affected by more long-term ROS exposure for slowly doubling cells, HBME cells exerted a value

Fig. 5. Intracellular oxidative stress and TocP-disposition into the intracellular space. **A**: HBME were serially subcultivated in the presence or absence of TocP, and stained with the redox indicator CDCFH-DA, a membrane-permeable dye precursor. After 90-min dye-staining, fluorographs are shown for the non-administered control middle-aged cells and the TocP-treated older-age cells. The scale bar, 20 μm. **B**: Intracellular oxidative stress is evaluated by fluorometry using CDCFH-DA and expressed in fluorescence unit for the middle- and older-age HBME cells that were serially cultured in the presence or absence of TocP of 150 μM. **C**: Intracellular oxidative stress levels are semi-quantitatively expressed for the control and TocP-treated cells in diverse age states as assessed by CDCFH-DA fluorometry, and are categorized according to degrees of CDCF-attributed florescence intensity (fluor. unit) as follows: +, 100–500 fluor. unit; ++, 500–1000 fluor. unit; +++ 1000 fluor. unit at a sent number of 7 for a fluorometer. **D**: HBME cells under diverse conditions underwent immunostain for ACR as an indicator for intracellular lipid peroxidation. ACR stain was conducted for the control, 150 μM TocP- and 150 μM H_2O_2 (for 30 min)-treated cells. Note arrows in that the faint stain in TocP-cells, the cytoplasm-preferable stain in the control cells, and the stain directional to the peri-nuclear loci and cell destructive fragments in H_2O_2-treated cells. The scale bar, 10 μm. **E**: A phosphate-OH moiety of TocP forms an adduct with the fatty acid ester portion of a phosphatidyl choline component in the membrane bilayers through hydrogen bond. **F**: TocP achieves a better fitting to the arachidonic acid portion of the membrane phospholipid like a key-to-keyhole mode. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

higher than those of other types of cells assumedly because of higher oxygen consumption of the brain and a mechanical injuries due to speedy blood stream as their in vivo milieu. The combined effect of vitamins E and C was indistinguishable from that of either single vitamin in terms of TRF length by Southern blots in a way similar to that for cell replication levels (Figs. 1B and 3B) or cellular oxidative stress (data not shown). The results imply that 150 mM TocP may be necessary and sufficient for appreciable protection of the cell membrane from aging-associated ROS injuries. Telomerase is known to be composed of both a catalytic core of a reverse transcriptase (hTERT) as a whole molecule and an RNA template component (hTR) [Greider and Blackburn, 1989; Feng et al., 1995; Lingner et al., 1997; Meyerson et al., 1997; Nakamura et al., 1997], and has a slight activity in normal human somatic cells. Telomerase activity in HBME as detected by PCR-based TRAP assay (Fig. 3C) was at the only faint level for the non-treated control cells, and disappeared during the aging process from young to middle ages, whereas the age-dependent diminishment of telomerase activity was appreciably slowed down for TocP-treated cells, suggesting the repressive effect of TocP/Toc on age-dependent disappearance of telomerase activity [Hiyama et al., 1996]. The amount of endogenous vitamin E that has been originally contained in the intracellular space may be at least partially responsible for telomere retention, but too short to appreciably retain the telomere length, because an age-dependent telomere-shortening was rapid for non-administered cells in spite of a significant content of intracellular vitamin E as quantified by HPLC. In addition, the direct targeting sites by TocP to telomere and telomerase may be potently different from each other, but may be at least in part in common with repressive effects against ROS-induced lesions either in the RNA template and reverse transcriptase protein portions of telomerase or in the vicinity of telomere and the nuclear matrix. TocP activated telomerase activity through administration to cells, but exerted no direct effect on telomerase reaction. A large amount of data and evidences have been necessitated in the present study only for confirmation for a closely positive correlation between oxidative-stress repression and telomere-shortening inhibition, and it should be therefore analyzed in the next study from a viewpoint of the causal relationship between oxidative stress and telomere dysfunction or life-span.

Toc is the most active type out of diverse tocopherols in terms of antioxidant activities including a scavenging ability against $LOO^{\cdot}$ and δ-tocopherol is the least active ($\alpha > \beta = \gamma > \delta$). The antioxidant activity of vitamin E is based on the facility with which the hydrogen on 6-hydroxyl group of the chroman ring moiety can be donated to neutralize a free radical resulting in the formation of a more stable tocopheroxyl radical (Fig. 1A). Here, esterification by phosphate on the 6-hydroxyl group of the chroman nucleus of a Toc molecule (Fig. 1A) enables an excellent availability of TocP in the aqueous solution owing to its diminished hydrophobic molecular property relative to Toc. TocP may be localized preferentially in the cell membrane or less hydrophobic loci through its molecular property being more hydrophilic than Toc. The phosphate group of TocP can be adducted with the ester moieties of unsaturated acids such as arachidonic acid in the cell membrane, being like a relationship between key and keyhole (Fig. 5E and F). Thus, TocP compounds may be able to be easily incorporated into the biomembrane, as TocP may be localized preferentially in the cell membrane or less hydrophobic loci through its molecular property being more hydrophilic than Toc. In contrast to no significant difference in levels of intracellular TocP from young to old ages for TocP-treated cells, a lowered level of Toc in old age versus that in young or middle age (Fig. 4B) may be attributed to either a lowered efficiency of the dephosphorylated conversion of intracellular TocP to Toc or a more frequent consumption of the converted Toc in older ages.

Recently, it has been reported that severity of oxidative stress is reduced age-dependently together with an increased genomic stability as indicated by both decreased oxidative stress and mitochondria deletions in heart and muscle tissues [Cutler, 2005]. Marked dependence of MT dehydrogenase activity as an indicator for intracellular basic metabolism on TocP doses of 0–300 mM in the middle-age cells (Fig. 4A) may ensue from marked influences of antioxidants such as Toc converted from TocP on MT metabolism. We found that TocP promoted the activity of MT dehydrogenases such as succinate dehydrogenase most efficiently at 100–125 mM, whereas TocP, at 150 mM near the plateau

(Fig. 4A) for maximal retention of the MT function, was shown to execute cell longevity (Fig. 1B) and telomere retention (Fig. 3B). Thus intracellular ROS in the absence of any antioxidants can contribute to diminishment of the enzyme activity of mitochondria dehydrogenase and to some membrane injuries such as abolishment of membrane proteins including phospholipase A2 [Adibhatla et al., 2003].

To examine whether TocP could influence ROS levels in HBME cells, we quantified by fluorometry using the dichlorodihydrofluorescein derivative CDCFH-DA as a redox indicator. CDCFH-DA is taken up into cells, and undergoes esterolysis to be converted into the membrane-impermeable form, which is subjected to the subsequent oxidation to a highly fluorescent CDCF form primarily by H_2O_2, hydroxyl radicals, and diverse peroxides [Szejda et al., 1984; Haugland, 1996]. Repressive effects of TocP treatment on intracellular oxidative stress were observed in CDCFH fluorometry. Intracellular ROS distributed in both the cytoplasm by TocP administration, showing that TocP-derived Toc can prevail all over the intracellular space through the nuclear membrane or Toc receptors rather than localize in the membrane. Hydrogen peroxides and hydroxyl radicals under physiological conditions in terms of oxygen partial pressures and ROS pertinent concentrations may be selected in order to analyze the mechanism of normal aging. Intracellular distribution of the lipid peroxide-bound products, ACR-lysine additive compounds such as oxidative LDL was investigated in HBME cells. Repressive effects of TocP treatment on intracellular oxidative stress were observed in ACR immunostain, demonstrating a practical ability of TocP-derived Toc to scavenge ROS of diverse species such as hydroperoxides, hydrogen peroxides, and ACR that are frequently generated in the intracellular space rather than in the cell-free chemical reaction. Cell morphological enlargement may be associated with intracellular accumulation of the waste products such as ACR and malondialdehyde (MDA) [Richter et al., 2005] and other oxidative products [Selvakumar et al., 2005], and was shown (Fig. 2C) to be closely correlated with the cellular life-spans among the control, and TocP-, TocP + Asc2P-, and Asc2P-administered HBME, additionally suggesting that anti-aging effects of TocP may be attributed to an antioxidant ability of TocP-derived Toc. Toc molecules exist in the cell membrane through hydrophobic interactions [Diplock and Lucy, 1973; Diplock et al., 1977; Maggio et al., 1977; Fragata and Billemare, 1980; Srivastava et al., 1983]. Therefore, the microenvironment around nuclear matrix where telomere region casts an anchor may be more hydrophobic than vicinity of other chromosomal DNA portions, and is therefore considered to be easy to capture TocP so as to protect, the adjacent phospholipid portions from oxidative decomposition.

We suggest that TocP at the effective dose for HBME cell longevity may be more safe than traditional non-natural cerebral drugs because it is an ester of two natural ingredients such as vitamin E and the DNA component phosphate in addition to no inhibitory effect on cell proliferation within wide-ranged doses. In future, neurodegenerative diseases are increasingly serious problems, and should be cured by therapeutic agents including anti-dementia drugs and simultaneously prevented as an everyday care by anti-aging countermeasures with the use of safety vitamins such as TocP that can expand the cell longevity of HBME as well as that of neuronal and glial cells together with interruption of cellular damage progresses.

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REFERENCES

Adibhatla RM, Hatcher JF, Dempsey RJ. 2003. Phospholipase A2, hydroxyl radicals, and lipid peroxidation in transient cerebral ischemia. Antioxid Redox Signal 5: 647–654.

Arita M, Sato Y, Miyata A, Tanabe T, Takahashi E, Kayden HJ, Arai H, Inoue K. 1995. Human alpha-tocopherol transfer protein: cDNA cloning, expression and chromosomal localization. Biochem J 306:437–443.

Burton GW, Ingold KU. 1986. Vitamin E: Application of the principles of physical organic chemistry to the exploration of its structure and function. Acc Chem Res 19:194–201.

Calingasan NY, Uchida K, Gibson GE. 1999. Protein-bound acrolein: A novel marker of oxidative stress in Alzheimer's disease. J Neurochem 72:751–756.

Carney JM, Starke-Reed PE, Oliver CN, Landum RW, Cheng MS, Wu JF, Floyd RA. 1991. Reversal of

age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin-trapping compound N-tert-butyl-a-phenylnitrone. Proc Natl Acad Sci USA 88:3633–3636.

Cherubini A, Ruggiero C, Polidori MC, Mecocci P. 2005. Potential markers of oxidative stress in stroke. Free Radic Biol Med 39:841–852.

Cutler RG. 2005. Oxidative stress and aging: Catalase is a longevity determinant enzyme. Rejuvenation Res 8:138–140.

Diplock AT, Lucy JA. 1973. The biochemical mode of action of vitamin E and selenium: A hypothsis. FEBS Lett 29:205–210.

Diplock AT, Lucy JA, Verrinder M, Zieleniewski A. 1977. alpha-Tocopherol and the permeability to glucose and chromate of unsaturated liposomes. FEBS Lett 82:341–344.

Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC, Yu J, Le S, West MD, Harley CB, Andrews WH, Greider CW, Villeponteau B. 1995. The RNA component of human telomerase. Science 269:1236–1239.

Fragata M, Billemare F. 1980. Model of singlet oxygen scavenging by a-tocopherol in biomembranes. Chem Phys Lipids 27:93–99.

Fujiwara M, Nagao N, Monden K, Misumi M, Kageyama K, Yamamoto K, Miwa N. 1997. Enhanced protection against peroxidation-induced mortality of aortic endothelial cells by ascorbic acid-2-O-phosphate abundantly accumulated in the cell as the dephosphorylated form. Free Radic Res 27:97–104.

Fukui K, Omoi NO, Hayasaka T, Shinnkai T, Suzuki S, Abe K, Urano S. 2002. Cognitive impairment of rats caused by oxidative stress and aging, and its prevention by vitamin E. Ann N Y Acad Sci 959:275–284.

Fukuzawa K, Chida H, Suzuki A. 1980. Fluorescence depolarization studies of phase transition and fluidity in lectin liposomes containing a-tocopherol. J Nutr Sci Vitaminol 26:427–434.

Furumoto K, Inoue E, Nagao N, Hiyama E, Miwa N. 1998. Age-dependent telomere shortening is slowed down by enrichment of intracellular vitamin C via suppression of oxidative stress. Life Sci 63:935–948.

Greider CW, Blackburn EH. 1989. A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. Nature 337:331–337.

Haugland RP. 1996. Handbook of Fluorescent Probes and Research Chemicals 6th Ed. Eugene, OR: Molecular Probes Eugene, p 377–398.

Hiyama E, Yokoyama T, Hiyama K, Michio Y, Santo T, Kodama T, Ichikawa T, Matsuura Y. 1995. Alteration of telomeric repeat length in adult and childhood solid neoplasias. Int J Oncol 6:13–16.

Hiyama E, Hiyama K, Tatsumoto N, Kodama T, Shay JW, Yokoyama T. 1996. Telomerase activity in human intestine. Int J Oncol 9:453–458.

Hosomi A, Arita M, Sato Y, Kiyose C, Ueda T, Igarashi O, Arai H, Inoue K. 1995. Affinity for alpha-tocopherol transfer protein as a determinant of the biological activities of vitamin E analogs. FEBS Lett 409:105–108.

Huang Z, Senoh Y, Katoh S, Miwa N. 2004. Preventive effects of a water-soluble derivative of chroman moiety of vitamin E on lipid hydroperoxide-induced cell injuries and DNA cleavages through repressions of oxidative stress in the cytoplasm of human keratinocytes. J Cell Biochem 92:425–435.

Ishiyama M, Tominaga H, Shiga M, Sasamoto K, Ohkura Y, Ueno K. 1996. A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. Biol Pharm Bull 19:1518–1520.

Kayden HJ, Traber MG. 1993. Bsorption, lipoprotein transport, and regulation of plasma concentrations of vitamin E in humans. J Lipid Res 34:343–358.

Kondo M, Oya-Ito T, Kumagai T, Osawa T, Uchida K. 2001. Cyclopentenone prostaglandins as potential inducers of intracellular oxidative stress. J Biol Chem 276:12076–12083.

Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V, Cech TR. 1997. Reverse transcriptase motifs in the catalytic subunit of telomerase. Science 276:561–567.

Lucy JA. 1972. Functional and structural aspects of biological membrane: A suggested structural role for Vitamin E in the control of membrane permeability and stability. Ann N Y Acad Sci 203:4–11.

Luo J, Uchida K, Shi R. 2005. Accumulation of acrolein-protein adducts after traumatic spinal cord injury. Neurochem Res 30:291–955.

Maggio B, Diplock AT, Lucy JA. 1977. Interactions of tocopherols and ubiquinones with monolayers of phospholipids. Biochem J 161:111–121.

Mark RJ, Blanc EM, Mattson MP. 1996. Amyloid beta-peptide and oxidative celluler injury in Alzheimer's disease. Mol Neurobiol 12:211–224.

Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, Ziaugra L, Beijersbergen RL, Davidoff MJ, Lui Q, Bacchetti S, Haber DA, Weinburg RA. 1997. hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. Cell 90:785–795.

Mitsui Y, Schneider EL. 1976. Relationship between cell replication and volume in senescent human diploid fibroblasts. Mech Ageing Dev 5:45–56.

Moyzis RK, Buckingham JM, Cram LS, Dani M, Deaven LL, Jones MDM, Ratliff RL, Wu JR. 1988. A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. Proc Natl Acad Sci 85:6622–6626.

Na M, Bae K, Sik Kang S, Sun Min B, Kuk Yoo J, Kamiryo Y, Senoo YI, Yokoo S, Miwa N. 2004. Cytoprotective effect on oxidative stress and inhibitory effect on cellular aging of Terminalia chebula fruit. Phytother Res 18:737–741.

Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, Harley CB, Cech TR. 1997. Telomerase catalytic subunit homologs from fission yeast and human. Science 277:955–959.

Piatyszck MA, Kim NW, Weinrich SL, Hiyama K, Hiyama E, Wright WE, Shay JW. 1995. Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). Methods Cell Sci 17:1–15.

Przedborski S, Jackson-Lewis V. 1998. Experimental developments in movement disorders: Update on proposed free radical mechanisms. Curr Opin Neurol 11: 335–339.

Raymond LJ, Johnson WT. 2004. Supplemental ascorbate or alpha-tocopherol induces cell death in Cu-deficient HL-60 cells. Exp Biol Med 229:885–894.

Richter T, Munch G, Luth HJ, Arendt T, Kientsch-Engel R, Stahl P, Fengler D, Kuhla B. 2005. Immunochemical crossreactivity of antibodies specific for "advanced glycation endproducts" with "advanced lipoxidation endproducts. Neurobiol Aging 26:465–474.

Saitoh Y, Miwa N. 2004. Cytoprotection of vascular endotheliocytes by phosphorylated ascorbate through suppression of oxidative stress that is generated immediately after post-anoxic reoxygenation or with alkylhydroperoxides. J Cell Biochem 93:653–663.

Sato Y, Hagiwara K, Arai H, Inoue K. 1991. Purification and characterization of the alpha-tocopherol transfer protein from rat liver. FEBS Lett 288:41–45.

Szejda P, Parce JW, Seeds MS, Bass DA. 1984. Flow cytometric quantitation of oxidative produce formation by polymorphonuclear leukocytes during phagocytosis. J Immunol 133:3303–3307.

Selvakumar E, Prahalathan C, Mythili Y, Varalakshmi P. 2005. Beneficial effects of DL-alpha-lipoic acid on cyclophosphamide-induced oxidative stress in mitochondrial fractions of rat testis. Chem Biol Interact 152: 59–66.

Smith CD, Carney JM, Starke-Reed PE, Oliver CN, Stadtman ER, Floyd RA, Markesbery WR. 1991. Excess brain Protein oxidation and enzyme dysfunction in normal ageing and in Alzheimer disease. Proc Natl Acad Sci USA 88:10540–10543.

Smith MA, Sayre LM, Monnier VM, Perry G. 1995. Radical ageing in Alzheimer's disease. Trends Neurosci 18:172–176.

Sokol RJ. 1988. Vitamin E deficiency and neurologic disease. Annu Rev Nutr 8:351–373.

Srivastava S, Phadke P, Govil G, Rao CNR. 1983. Fluidity, permeability and antioxidant behaviour of model membranes incorporated with a-tocopherol and vitamin E acetate. Biochim Biophys Acta 734:353–362.

Takeda A, Yasuda T, Miyata T, Goto Y, Wakai M, Watanabe M, Yasuda Y, Horie K, Inagaki T, Doyu M, Maeda K, Sobue G. 1998. Advanced glycation end products co-localized with astrocytes and microglial cells in Alzheimer's disease brain. Acta Neuropathol (Berl) 95:555–558.

Tam LS, Li EK, Leung VY, Leung VY, Griffith JF, Benzie IF, Lim PL, Whitney B, Lee VW, Lee KK, Thomas GN, Tomlinson B. 2005. Effects of vitamins C and E on oxidative stress markers and endothelial function in patients with systemic lupus erythematosus: A double blind, placebo controlled pilot study. J Rheumatol 32: 275–282.

Tatematsu K, Nakayama J, Danbara M, Shionoya S, Sato H, Omine M, Ishikawa F. 1996. A novel quantitative 'stretch PCR assay' that detects a dramatic increase in telomerase activity during the progression of myeloid leukemias. Oncogene 13:2265–2274.

Traber MG, Arai H. 1999. Molecular mechanisms of vitamin E transport. Annu Rev Nutr 19:343–355.

Traber MG., 2005. Vitamin E regulation. Curr Opin Gastroenterol 21:223–227.

Uchida K, Kanematsu M, Sakai K, Matsuda M, Hattori N, Mizuno Y, Suzuki D, Miyata T, Noguchi N, Niki E, Osawa T. 1998. Protein-bound acrolein: Potential markers for oxidative stress. Proc Natl Acad Sci USA 95: 4882–4887.

Upston JM, Terentis AC, Stocker R. 1999. Tocopherol-mediated peroxidation (TMP) of lipoproteins: Implications for vitamin E as a potential antiatherogenic supplement. FASEB J 13:977–994.

Upston JM, Terentis AC, Morris K, Keaney JF Jr, Stocker R. 2002. Oxidized lipid accumulates in the presence of a-tocopherol in atherosclerosis. Biochem J 363:753–760.

Von Zglinicki T, Pilger R, Sitte N. 2000. Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts. Free Radic Biol Med 28:64–74.

Xiao L, Takada H, Maeda K, Haramoto M, Miwa N. 2005. Antioxidant effects of water-soluble fullerene derivatives against ultraviolet ray or peroxylipid through their action of scavenging the reactive oxygen species in human skin keratinocytes. Biomed Pharmacother 59: 351–358.

Yamauchi R, Noro H, Shimoyamada M, Kato K. 2002. Analysis of vitamin E and its oxidation products by HPLC with electrochemical detection. Lipids 37:515–522.

Yokoo S, Furumoto K, Hiyama E, Miwa N. 2004. Slow-down of age-dependent telomere shortening is executed in human skin keratinocytes by hormesis-like-effects of trace hydrogen peroxide or by anti-oxidative effects of pro-vitamin C in common concurrently with reduction of intracellular oxidative stress. J Cell Biochem 93:588–597.

Young JF, Rosenvold K, Stagsted J, Nielsen JH, Andersen HJ. 2005. Significance of vitamin E supplementation, dietary content of polyunsaturated fatty acids, and preslaughter stress on oxidative status in pig as reflected in cell integrity and antioxidative enzyme activities in porcine muscle. J Agric Food Chem 53:745–749.