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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

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Abstract The cellular life-span of cultivated human skin epidermis keratinocytes NHEK-F was shown to be extended up to 150% of population doubling levels (PDLs) by repetitive addition with two autooxidation-resistant derivatives of ascorbic acid (Asc), Asc-2-*O*-phosphate (Asc2P), and Asc-2-*O*-alpha-glucoside (Asc2G), respectively, but to be not extended with Asc itself. In contrast, hydrogen peroxide (H_2O_2) as dilute as 20 μM which was non-cytotoxic to the keratinocytes, or at 60 μM being marginally cytotoxic achieved the cellular longevity, unexpectedly, up to 160 and 120% of PDLs, respectively, being regarded as a hormesis-like stimulatory effect. The lifespan-extended cells that were administered with Asc2P, Asc2G, or 20 μM H_2O_2 were prevented from senescence-induced symptoms such as PDL-dependent enlargement of a cell size of 14.7 μm finally up to 17.4 μm upon Hayflick's limit-called loss of proliferation ability as estimated with a channelizer, and retained young cell morphological aspects such as thick and compact shape and intense attachment to the culture substratum even upon advanced PDLs, whereas other non-extended cells looked like thin or fibrous shape and large size upon lower PDLs. The PDL-dependent shortening of telomeric DNA of 11.5 kb finally down to 9.12–8.10 kb upon Hayflick's limit was observed in common for each additive-given cells, but was decelerated in the following order: 20 μM H_2O_2 > Asc2P = Asc2G > 60 μM H_2O_2 > Asc = no additive, being in accord with the order of cell longevity. Intracellular reactive oxygen species (ROS) was diminished by Asc2P, Asc2G or 20 μM H_2O_2, but not significantly by Asc or 60 μM H_2O_2 as estimated by fluorometry using the redox indicator dye CDCFH. There was no appreciable difference among NHEK keratinocytes that were administered with or without diverse additives in terms of telomerase activity per cell, which was 1.40×10^4–4.48×10^4 times lower for the keratinocytes than for HeLa cells which were examined as the typical tumor cells. Thus longevity of the keratinocytes was suggested to be achieved by slowdown of age-dependent shortening of telomeric DNA rather than by telomerase; telomeres may suffer from less DNA lesions due to the continuous and thorough repression of intracellular ROS, which was realized either by pro-vitamin C such as Asc2P or Asc2G that exerted an antioxidant ability more persistent than Asc itself or by 20 μM H_2O_2 which diminished intracellular ROS assumedly through a hormesis-like effect. J. Cell. Biochem. 93: 588–597, 2004.

Key words: telomeres; oxidative stress; L-Ascorbic acid-2-*O*-Phosphate; hormesis; cell ageing

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Repeated DNA sequences at ends of eukaryotic chromosomes, called telomeres, are destined to be shortened upon DNA lagging strands [Watson, 1972; Harley et al., 1990]. In addition to the end replication problem, however, there may be other causes responsible for telomere-shortening, among which direct injuries to telomeric DNA such as base lesions and strand cleavages that are induced by ultraviolet [Kruk et al., 1995; Oikawa et al., 2001] or hyperoxia [von Zglinicki et al., 1995; Honda et al., 2001] is considered to be the most influential, although not demonstrated yet to trigger age-dependent telomere-shortening. They are rather artificial agents which exogenously or transiently bring about a pathogenic heightened level of reactive oxygen species (ROS), whereas effects of a physiologic stationary level of ROS that is endogenously and continuously generated during normal aerobic metabolism [Cadenas and Davies, 2000; Leeuwenburgh and Heinecke, 2001] on telomere-shortening are unknown; still less explicit is the inhibitory effect of ROS-scavenging on telomere shortening.

It is not elucidated in telomerase gene-transfected cells [Shay et al., 1998; Yudoh et al., 2001] whether retention of telomeric length may elongate cellular life-span or not. The cumulative frequency of cell divisions, designated as a population doubling level (PDL), is defined to be maximum when normal somatic cells exhaust the finite replicative capacity [Hayflick and Moohead, 1961]. The loss of doubling potential of mortal cells may be at least partly due to progressive shortening of telomeres down to a length of the permissive limit [Harley et al., 1990; Figueroa et al., 2000] that may induce chromosomal instability [Counter et al., 1992; de Lange, 1992]. To examine effects of scavenging of intracellular ROS on both rates of shortening of telomeric DNA and retention of cellular replicative capacity, we intended to analyze neonatal human epidermal karatinocytes NHEK-F as the representative of nontransformed skin cells which play a crucial role in skin cares such as protection against ultraviolet injuries, prevention from wrinkles and anti-aging of skin [Kaji and Matsuo, 1979; Giacomoni et al., 2000; Scharffetter-Kochanek et al., 2000; Nusgens et al., 2001]. As an intracellular ROS-scavenger exogenously added to NHEK-F keratinocytes, we have focused ascorbic acid (Asc), which is known to diminish humoral ROS most efficiently out of diverse antioxidant biomolecules such as SH groups, alpha-tocopherol, bilirubin and urate naturally contained in human plasma [Frei, 1991]. We firstly tried to serially subcultivate NHEK-F keratinocytes in the presence of Asc, and failed in both the artificial slowdown of age-dependent shortening of telomeres and extension of cellular life-span assumedly owing to lability of Asc in aqueous solution [Bode et al., 1990]. So we examined whether telomere shortening can be prevented by the auto-oxidation-resistant derivative of vitamin C, Asc2P (ascorbic acid-2-*O*-phosphate) [Yamamoto et al., 1990; Kanatate et al., 1995] or Asc2G (ascorbic acid-2-*O*-alpha-D-glucoside) [Miyai et al., 1997; Tatemoto et al., 2001], that is phosphorylated or glucosylated at the 2,3-enediol moiety of an Asc molecule, respectively. In addition, to expect promotive effects of pro-oxidants on telomere shortening [von Zglinicki et al., 2000; Bar-Or et al., 2001; Sozou and Kirkwood, 2001], hydrogen peroxide was added to the keratinocytes at doses of a trace amount so as not to influence cell proliferation. In the present study, we showed that Asc2P or Asc2G, but not Asc itself, succeeds in the artificial slowdown of age-dependent telomere shortening, but, unexpectedly, that hydrogen peroxide of a trace amount is more advantageous over both the Asc derivatives in terms of extensive effects of cellular life-span and slowdown effects on telomere shortening.

MATERIALS AND METHODS

Cell Culture

Neonatal human foreskin epiderm-derived keratinocytes NHEK-F (Kurabo Industries, Osaka, Japan) being mycoplasma-free were grown in Humedia-KB2 medium (Kurabo) supplemented with bovine pituitary extract (0.4% v/v), human epidermal growth factor (0.1 ng/ml), insulin (10 μg/ml), hydrocortisone (0.5 μg/ml), gentamycin (50 μg/ml), and amphotericin B (50 ng/ml) (Complete Humedia-KG2) in a humidified atmosphere of 5% CO_2/95% air at 37°C, and collected at 70% confluence. Cells were fed with or without Asc, Asc2P, or Asc2G of 130 μM successively upon every culture passage. Simultaneously, other cells were successively treated with H_2O_2 of 20 or 60 μM being uncytotoxic. Cells were enumerated upon every passage with a Coulter electric particle counter for substratum-attaching cells. Spontaneously

detaching cells were as few as below the detectable limit except for H_2O_2-treated cells of terminal passage. PDL is regarded as zero for culture starting immediately after the primary culture of neonatal human epidermal karatinocytes, and calculated to increase according to the equation: log2 {(the number of collected cells)/(the number of seeded cells)}.

Cell Size Distribution

NHEK-F cells fed with or without Asc (Sigma Chemicals, Tokyo, Japan), Asc2P (Showa Denko Co., Tokyo, Japan) or Asc2G (Wako, Osaka, Japan) were rinsed, trypsinized, and then analyzed with a Coulter counter ZM equipped with a channelyzer model 256 with calibration using PDVB latex particles (Becton-Dickinson, Mountainview, CA) of 11.2 and 23.5 μm in diameter.

Determination of Telomere Length by Southern Blots

Genomic DNA was extracted with 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 electrophorogram. Extracted DNA was completely digested with the restriction enzyme Hinf I (TaKaRa, Kyoto, Japan) to produce terminal restriction fragments (TRFs) as previously described [Hiyama et al., 1995]. A portion (2 μg/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, Japan) 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, and add blocking reagent to afinal concentration of 4% (w/v) at 55°C, and hybridized in denatured alkaline phosphatase enzyme-labelled (TTAGGG)4 (QIAGEN KK, Tokyo, Japan). Membranes were washed in 2 M Urea, 0.1% SDS, 50 mM Na phosphate pH 7.0, 10 mM $MgCl_2$, 0.2% Blocking reagent at 55°C. Place the membranes in a clean container and add 50 mM Tris, 100 mM NaCl, 2 mM $MgCl_2$ pH 10.0. Pipette detection reagent (Amersham Biosciences) on the membranes and leave for 2–5 min and underwent chemilunescent 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 telomerase assay kit TeloChaser (TOYOBO, Osaka, Japan) [Tatematsu et al., 1996]. Briefly, 1×10^6 cells were lysed in lysis solution followed by preparation of 1×10^5 cell-equivalent extracts. Telomerase reaction was conducted at 37°C for 30 min and there was mixed with PCR mix, Taq mix (in TeloChaser). PCR amplification was repeated by 28 cycles using an Astec thermal cycler PC-800 with 95°C for 30 s, 68°C for 30 s, and 72°C for 45 s 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 SYBR Green I and densitometry.

Intracellular Oxidative Stress

NHEK cells seeded at 2,500 cells/cm^2 were grow on a 24-well microplate in the presence or absence of Asc2P, Asc2G, and H_2O_2 for 8 days, were rinsed three times day with HuMedia-KG2 and replaced by the medium containing 10 μM CDCFH (Molecular Probes, Eugene, OR) [Szejda et al., 1984]. After 15 min incubation, the fluorescence intensity was measured with a fluorescence plate reader CytoFluor 2350 (Millipore, Betford, MA). The excitation and emission wave lengths 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 the blank.

RESULTS

Effects of Anti-Oxidants or Pro-Oxidants on Proliferation Ability of Human Keratinocytes

We intended to examine effects of anti-oxidants or pro-oxidants on cellular life-spans and lengths

of telomeric DNA in neonatal human foreskin epidermal keratinocytes NHEK-F, which can be regarded as the representative of human skin that may be the target tissue for cosmetics and be most directly affected by ultraviolet ray or environmental temperature and moisture. Firstly effects of morphological aspects of NHEK-F keratinocytes were examined for cells that were serially subcultivated in the presence or absence of diverse additives such as Asc, Asc-2-*O*-phosphate (Asc2P), or Asc-2-*O*-alpha-glucoside (Asc2G) at 130 μM as the anti-oxidant or hydrogen peroxide (H_2O_2) at 20 or 60 μM as the pro-oxidant (Fig. 1). None- or Asc-added NHEK-F cells at the maximal PDL corresponding nearly to Hayflick limit showed some symptoms characteristic for senescent cells such as thin and enlarged cellular outlook and sparseness in cell population even after a sufficient culture period under nutrient-rich conditions, whereas Asc2P- or Asc2G-added cells looked young at the similar PDL. Unexpectedly hydrogen peroxide-added NHEK-F keratinocytes retained morphological aspects typical of young cells. The maximum PDL was unchanged for Asc-added NHEK-F cells as compared with the none-added control cells, whereas 1.5-fold enhancement was observed for Asc2P- or Asc2G-added cells (Fig. 2a). More marked enhancement (1.6-fold) of the maximum PDL was achieved by cells that were repetitively administered with hydrogen peroxide as dilute as 20 μM. Upon addition with hydrogen peroxide at 60 μM, in contrast, less marked enhancement (1.2-fold) was observed. The ratio of PDL of additive-receiving cells versus PDL of non-added control cells at each culture period was shown to be lowered at the initial period after addition with hydrogen peroxide at 60 μM, and be thereafter restored (Fig. 2b), suggesting the threshold concentration of hydrogen peroxide for an subacute cytotoxicity and cellular tolerance. Dependence of cell viability of NHEK-F keratinocytes on concentrations of H_2O_2 that was added for 8 days as estimated by WST-1 assay principally measuring mitochondrial dehydrogenase activity (Fig. 2c). The cell viability was shown to be unaltered upon addition with hydrogen peroxide at 60 μM, but significantly promoted at 20 μM to be 108% relative to the non-added control.

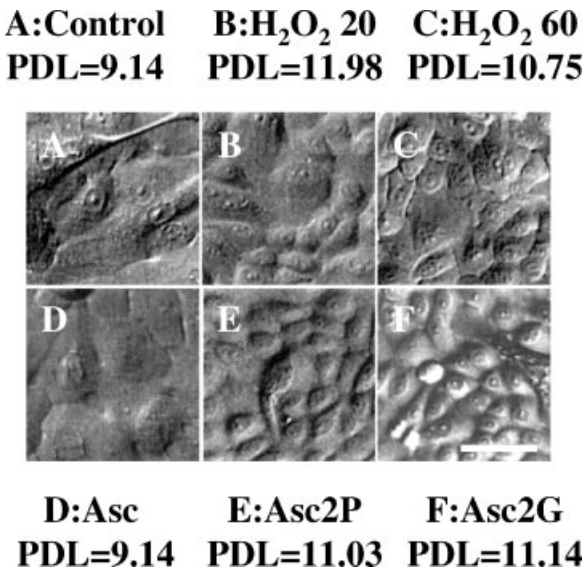

Fig. 1. Morphological aspects of neonatal human skin epidermal keratinocytes NHEK-F that were serially subcultivated in the presence or absence of diverse additives such as ascorbic acid (Asc), Asc-2-*O*-phosphate (Asc2P), or Asc-2-*O*-alpha-glucoside (Asc2G) at 130 μM or hydrogen peroxide (H_2O_2) at 20 or 60 μM (bar: 50 μm). None- or Asc-added NHEK-F cells were photographed at the maximal population doubling level (PDL), and cells that were administered with other additives were at PDLmax of 10.75–11.98. The photographs shown are typical of three independent experiments, each of which was conducted in duplicate.

Correlation of Cellular Ages and Cell Sizes

NHEK-F keratinocytes were serially subcultivated with or without diverse additives, and were analyzed for the cell size with a Coulter channelizer. The resultant histograms (Fig. 3a) and curves for dependence of cell size on PDL (Fig. 3b) were obtained. The cell size became larger from 14.7 to 17.4 μm as cellular age advanced from PDL 4.1 to 9.1 for non-added cells. In contrast, enlargement of cell size per PDL was moderated for lifespan-elongated cells that were administered with Asc2P of 130 μM or hydrogen peroxide of 20 μM.

Correlation of Telomeric DNA Lengths and Cellular Ages

NHEK-F keratinocytes were serially subcultivated with or without diverse additives, and analyzed for lengths of telomeric DNA by Southern blots of TRFs of DNA that was extracted from cells at each passage (Fig. 4a), and by densitography of the resultant smear bands (Fig. 4b). The rate of age-dependent shortening of telomeric DNA per PDL was in the order as follows: non-added or Asc-added cells >60 μM hydrogen peroxide-added cells > Asc2P- or Asc2G-added cells >20 μM hydrogen

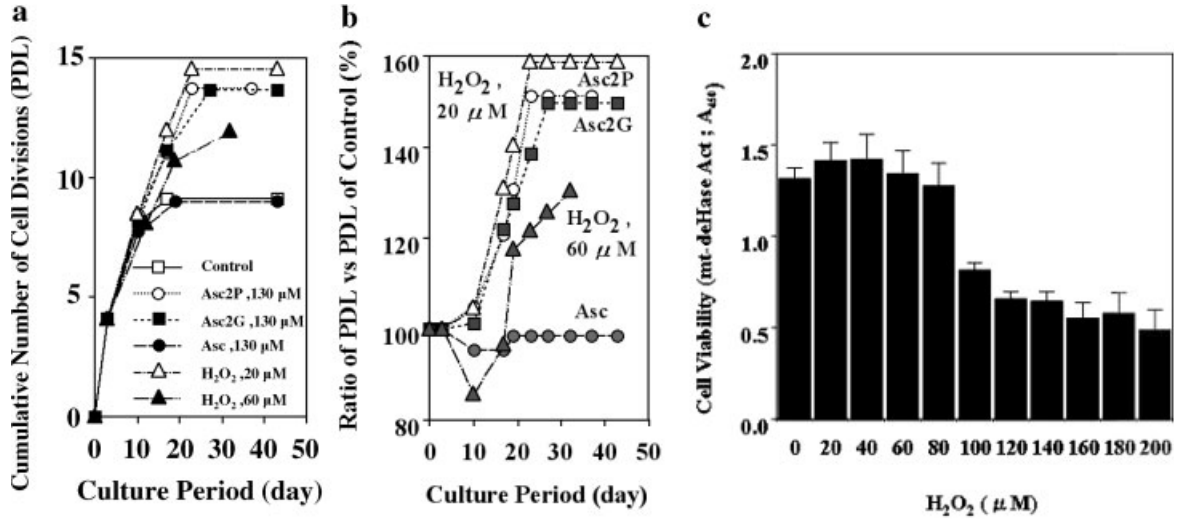

Fig. 2. Effects of Asc, its derivatives or hydrogen peroxide on proliferation ability of human keratinocytes NHEK-F. Cells that were isolated from the epidermis at the neonatal stage were serially cultivated via repetition passages in the presence or absence of diverse additives such as Asc, Asc2P, or Asc2G at 130 μM or H_2O_2 at 20 or 60 μM. **a**: The cumulative number of cell divisions that NHEK-F cells has achieved so far was estimated at each culture period upon subcultivation, and is expressed as PDL. **b**: The ratio of PDL of additive-receiving cells versus PDL of non-added control cells is calculated at each culture period. **c**: Dependence of cell viability of NHEK-F cells on concentrations of H_2O_2 that was added for 8 day (per one passage) as estimated by WST-1 assay principally measuring mitochondrial dehydrogenase activity using formazan-forming redox-indicator dye. The data shown are typical of three independent experiments, each of which was conducted in duplicate.

peroxide-added cells (Fig. 4c). This order was inversely correlated with that of the maximum PDL. The results showed that enhancement of maximum frequency of cell divisions may be attributed to retention of telomere lengths larger than the critical lower limit. The cells that were added with 20 μM hydrogen peroxide were shown to potently supply all descendent cells being 41.5-fold as many as non-added control cells did until loss of proliferating ability during cellular aging.

Contents of Intracellular Peroxides

To clarify why a rate for an age-dependent telomere shortening and a life-long cell-supplying ability were affected by the anti-oxidants

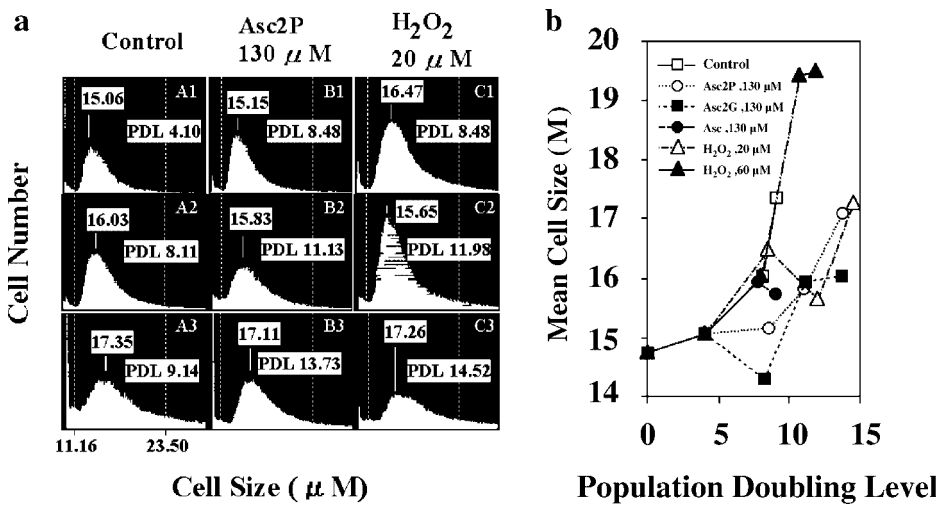

Fig. 3. Correlation of cellular ages and cell sizes of NHEK-F cells that were serially subcultivated with or without diverse additives. **a**: Histograms for distribution of cell sizes as determined by a Coulter channelizer. **b**: Dependence of cell sizes on PDL. The data shown are typical of three independent experiments, each of which was conducted in duplicate.

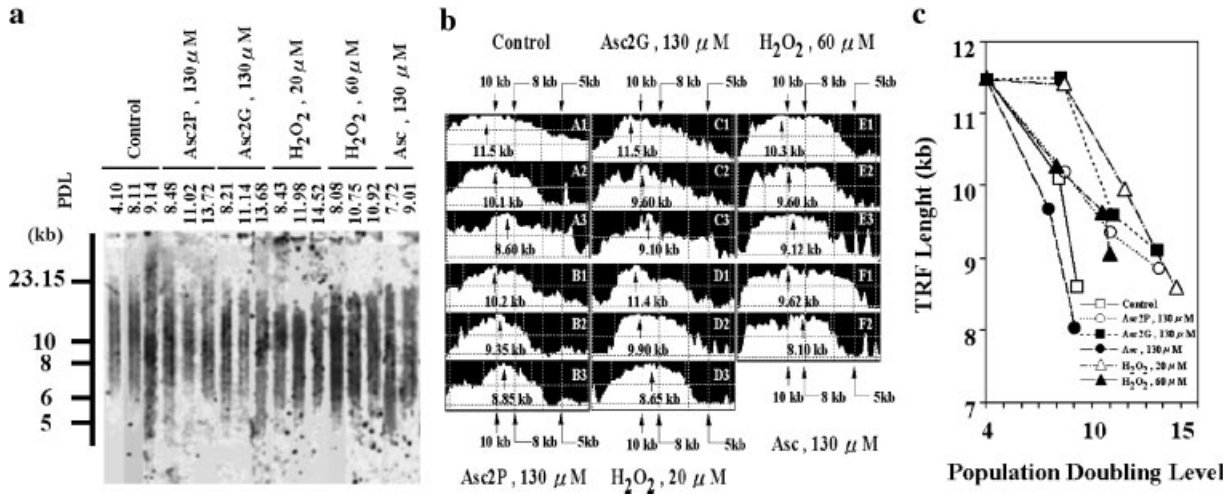

Fig. 4. Correlation of telomeric DNA lengths and cellular ages of NHEK-F cells that were serially subcultivated with or without diverse additives. **a**: Southern blots of terminal restriction fragments (TRFs) of DNA that was extracted from each passage of NHEK-F cells. **b**: Densitograms of the Southern blots that were obtained. The most dense position is indicated by an up-oriented arrow. The half-maximal density is indicated by a lateral dotted line. **c**: Curves for dependence of lengths of TRF nearly equal to those of telomeres on PDL.

or pro-oxidant used, amounts of intracellular peroxides in NHEK-F keratinocytes that were serially subcultivated with or without diverse additives were assessed by fluorometric assay using the peroxide-susceptible and membrane-permeable fluorescent dye CDCFH-DA (Fig. 5). The dye is known to enter into cells without appreciable cytotoxicity, be deacetylated within the cell to the membrane-impermeable form CDCFH, and be converted by intracellular peroxides to the fluorescent oxidative form CDCF [Szejda et al., 1984]. Fluorescence in CDCFH-DA-loaded NHEK-F keratinocytes was diminished to 57% by hydrogen peroxide at 20 μM which was markedly effective for retention of telomeric lengths and cellar longevity, but was scarcely diminished at 60 μM which was less effective. Appreciable diminishment of peroxide-associated fluorescence was also observed for Asc2P- or Asc2G-added keratinocytes that retained telomeric lengths and showed cellular longevity, where as no significant diminishment was not observed for Asc-added keratinocytes.

Alteration of Telomerase Activity

Cell extracts were prepared at the indicated PDLs from NHEK-F keratinocytes that have been repetitively administered with Asc, its derivatives or H_2O_2, and were quantified for telomerase activity by PCR-based TRAP method (Fig. 6a) and the subsequent densitometry (Fig. 6b). The TRAP products looked like a ladder of DNA with $(81 + 6\times$ N) base pairs. Although a cellular age of PDL 4.10, activity of telomerase activity was slightly high, there was no significant difference in telomerase activities at all cultivation periods examined. Furthermore telomerase activity of NHEK keratinocytes is very feeble as compared with that of HeLa cells (Fig. 6c).

DISCUSSION

The present study demonstrates that cellular life-spans as indicated by the maximal PDL of normal human epidermal keratinocytes NHEK in culture can be markedly extend by repetition addition with the pro-vitamin C, Asc2P, and Asc2G, into the culture medium (Fig. 2). The extended cellular life-spans were also acquired, unexpectedly, by repetition addition with hydrogen peroxide at a dose of critical trace but not at doses above the definite upper threshold concentration (Fig. 2). One possibility responsible for these cellular longevity with the pro-vitamin C or hydrogen peroxide may be the difference of cellular loss during passage such as cell detachment from the culture substratum and cytodifferentiation to the corneum between the examined and control cell populations. NHEK has been reported to be inclined to be cornified in response to changes in concentrations of extracellular calcium ions

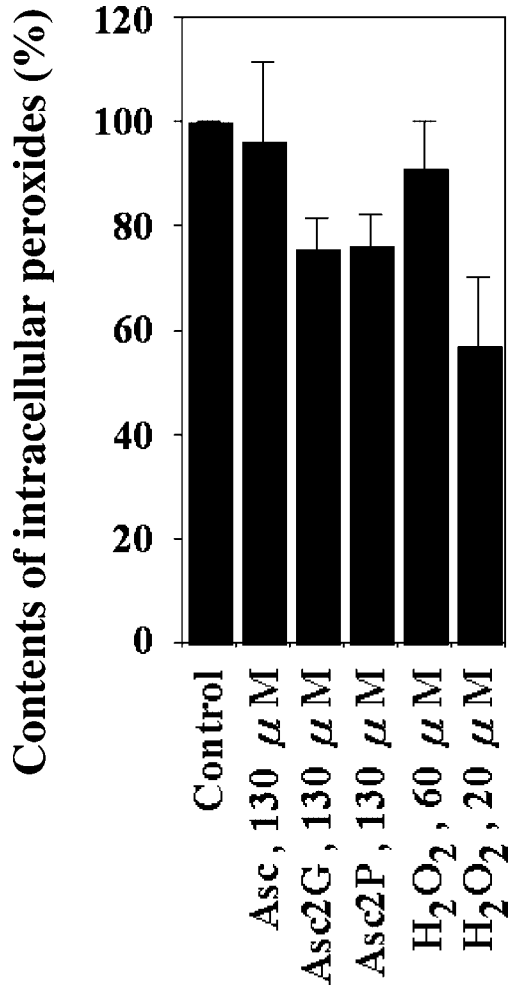

Fig. 5. Contents of intracellular peroxides at PDLs of 4.10 in NHEK-F cells that were serially subcultivated with or without diverse additives as assessed by fluorometric assay using the peroxide-susceptible dye CDCFH-DA. The data shown are typical of three independent experiments, each of which was conducted for culture in duplicate and for fluorometry in triplicate.

[Boyce and Ham, 1983]. The numbers of cells collected from the culture medium were therefore measured on the occasions of culture-medium exchange and passage, and were estimated to be negligible because cornified cells or floating cells were scarcely counted. Thus the observed elongation of the maximal PDL of NHEK cells was shown to correctly reflect a genuine preventive effects on cellular senescence. Furthermore the cell populations with extended maximal PDL showed both the repressed enlargement of the cell size indicative of cellular senescence [Simons and Van den Broek, 1970] and the slowdown of PDL-dependent shortening speed of telomeric DNA lengths as indicated by lengths of TRFs (Fig. 4). Other possibilities for the cellular longevity of NHEK cells may be explained in terms of telomerase and the ROS as well-known age-controlling factors. The difference between the examined and control NHEK cells was not significantly observed for activity of telomerase (Fig. 6), which was very feeble as detected even by PCR-based amplification method. On the other hand, the ROS level was decreased in the pro-vitamin C- or hydrogen peroxide-fed keratinocytes with extended maximal PDL (Fig. 5). The prolongation of life-spans attributable to the decreased ROS has been reported to be enabled also in vivo by diet calorie restriction [Dhahbi et al., 1998; Lipman et al., 1998] or medication with deprenyl [Kitani et al., 1999] and in vitro by addition with vitamin E for some studies [Yu et al., 1998], hydrocortisone [Bodey et al., 1997] or centrophenoxine (mecrofenoxate) [Sogawa and Kubo, 2000] and via induction of promoted resistance against oxidative stress also by targeted mutation of the mouse *p66shc* gene [Migliaccio et al., 1999] Telomeric DNA as indicated by TRF shortens in human lung fibroblastic cells from 12–15 kb to 5–8 kb along with cellular aging [Harley et al., 1990]. Cellular aging is considered to be substantially prescribed by some relevant genes, and their exhibition and regulation may be appreciably attributed to generation and distribution of ROS. Shortening of TRF is known to take place concurrently with DNA single strandcleavages by hyperoxia treatment or high-concentrations hydrogen peroxide [von Zglinicki et al., 2000]. Our previous study shows that TRF is shortened along with cellular aging in human umbilical endothelial cells HUVEC, where the age-dependent shortening of TRF can be slowed down together with both elongation of cellular life-spans and retention of telomerase activity by maintaining the intracellular Asc concentration at the higher level [Furumoto et al., 1998]. Physiological conditions in terms of oxygen partial pressures and hydrogen peroxide concentrations may be selected in order to analyze the mechanism of normal aging [Petersen et al., 1998; von Zglinicki et al., 2000]. In this present study, for the reason, the concentration the range of hydrogen peroxide that added to keratinocytes was selected at levels as low as 20–60 μM in which long-term cultivation is possible without either acute and chronic cytotoxicities or cell degeneration. The maximal PDL of keratinocytes was however extended by

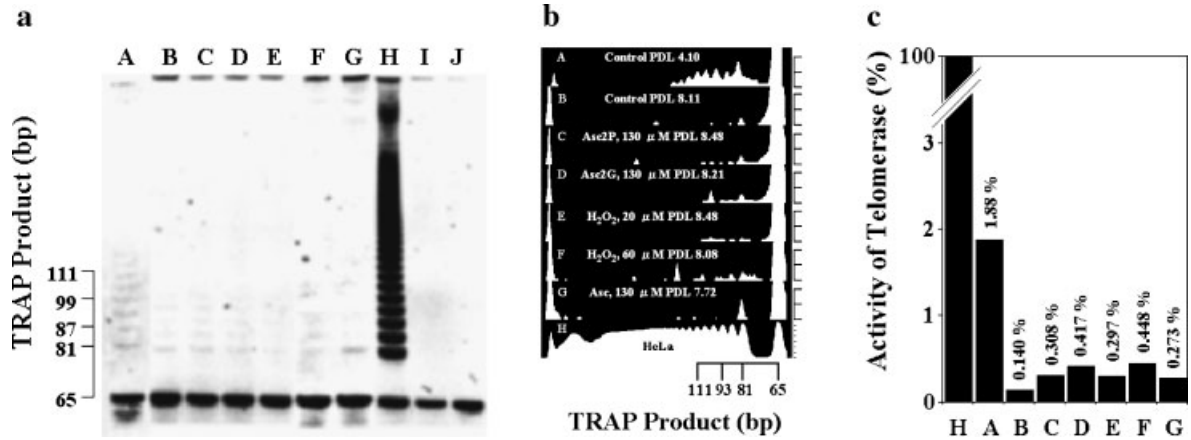

Fig. 6. Telomerase activity of NHEK-F cells that were serially subcultivated in the presence or absence of diverse additives. Cell extracts were prepared from NHEK-F keratinocytes of 1×10^5 cells at the indicated PDL that have been repetitively administered with Asc, its derivatives or H_2O_2, and underwent TRAP-based telomerase reaction. The TRAP products that were amplified by PCR were stained with SYBR Green I (**a**) and expressed for the resultant values of telomerase activity (**c**), estimated by densitometry (**b**). A & B, NHEK-F cells administered without additives; C–G, NHEK-F cells repetitively administered with diverse additives (PDL; A: 4.10, B: 8.11, C: 8.48, D: 8.21, E: 8.48, F: 8.08, G: 7.72); H, HeLa cells of 2.5×10^4 cells as the positive control; I, heat-inactivated extracts from HeLa cells treated similarly as for H; J, lysis solution alone as the negative control. The data shown are typical of three independent experiments, each of which was conducted for cultivation in duplicate and for PCR in duplicate.

hydrogen peroxide at 20 μM, which can be designated as a type of hormesis that is defined to stimulate some biological events through a cytotoxicity as feeble as below the threshold lower limit, although it may be in appearance contrary that there are two longevity effectors belonging to the prooxidant and antioxidant such as pro-vitamin C in the same cell strain. The longevity effect was also exerted by a diminishment of intracellular ROS that can be executed in spite of administration with hydrogen peroxide of a trace. Thus telomeric age-dependent shortening can be partly controlled by artificial diminishment of intracellular ROS although most of its shortening may be destined to be inevitable. Although vitamin C (ascorbic acid: Asc) is usually difficult for maintenance of its higher concentrations within a cell due to both the instability in aqueous solution and the exhaustive consumption against cellular oxidative stress, pro-vitamin C such as Asc2P can maintain the intracellular Asc concentrations at higher levels through gradual release of Asc from pro-vitamin C owing to enzymatic esterolysis [Kanatate et al., 1995; Fujiwara et al., 1997; Furumoto et al., 1998; Liu et al., 2000]. The intracellular accumulation of abundant Asc can realize an intracellular ROS-scavenging, which achieves both the alleviation of the DNA replication-independent unscheduled shortening of telomeric DNA as indicated by the TRF shortening speed and the resultant extension of the cellular life-spans as indicated by the maximal PDL. Since the repressive effect on cellular aging due to dilute hydrogen peroxide attenuated the intracellular ROS level (Fig. 5), ROS below the cytotoxic level can accomplish the induced promotions of both gene exhibitions of a diverse species of antioxidant enzymes such as superoxide dismutases and restructurings of uptake and distribution of antioxidant low-molecular substances such as Asc. In the nematode *Caenorhabditis elegans* and *Drosophila melanogaster*, there are some reports concerning hormesis that enables the life-span extension also in vivo due to feeble ROS stimulus. [Le Bourg et al., 2001; Cypser and Johnson, 2002]. The present study may be the first one, within our knowledge, that hormesis can be closely correlated with repressive effects on telomere shortening. Control of cellular aging by administration with Asc or its derivatives and a trace of hydrogen peroxide was achieved by appreciable elimination of intracellular ROS. In the number of supplied cells derived from an ancestor cell, a life-long cell-supplying ability was markedly enhanced for administration with Asc derivatives or hydrogen peroxide on basis of increased PDLmax as compared with the unadministered control. In the adult cells, it is always stimulated by detrimental factors, such as UV and oxygen [Ahmed et al., 1999;

Toussaint et al., 2000], which bring about diverse damages at the organ or cellular level, and the resultant repair sometimes makes accumulation of error in DNA. The cell which received intense damage becomes extinct and needs to be transposed to a new cell. On the other hand, the ROS-induced DNA injuries within a cell can also be prevented by Asc or its derivatives [Kanatate et al., 1995]. The exhibition of telomerase activity is very low in adult somatic cells, but is closely correlated with both the quantity of intracellular ROS and the maximal life-span as indicated by PDLmax. This suggests that the shortening speed of telomeric DNA is adjusted at least in part by intracellular ROS quantity and telomerase activity as well as by a cumulative frequency of DNA replications. And if a correlation between telomerase and tumorigenesis [Ueda et al., 1997] is not clarified, the method of ROS repression by the anti-oxidant agents is regarded as the most suitable mean utilized for cell aging control.

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