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Senescent phenotypes of skin fibroblasts from patients with Tangier disease

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Abstract

Tangier disease (TD) is characterized by a deficiency of high density lipoprotein (HDL) in plasma and patients with TD have an increased risk for coronary artery disease (CAD). Recently, we reported that fibroblasts from TD exhibited large and flattened morphology, which is often observed in senescent cells. On the other hand, data have accumulated to show the relationship between cellular senescence and development of atherosclerotic CAD. The aim of the present study was to investigate whether TD fibroblasts exhibited cellular senescence. The proliferation of TD fibroblasts was gradually decreased at population doubling level (PDL) ~10 compared with control cells. TD cells practically ceased proliferation at PDL ~30. DNA synthesis was markedly decreased in TD fibroblasts. TD cells exhibited a higher positive rate for senescence-associated β-galactosidase (SA-β-gal), which is one of the biomarkers of cellular senescence *in vitro*. These data showed that TD cells reached cellular senescence at an earlier PDL compared with controls. Although, there was no difference in the telomere length of fibroblasts between TD and controls at the earlier passage (PDL 6), the telomere length of TD cells was shorter than that of controls at the late passage (PDL 25). Taken together, the current study demonstrates that the late-passaged TD fibroblasts showed senescent phenotype *in vitro*, which might be related to the increased cardiovascular manifestations in TD patients.

Keywords: ABCA1; Cdc42; SA-β-gal; Senescence; Tangier disease; Telomere

Tangier disease (TD) is characterized by a marked deficiency of high density lipoprotein (HDL) in plasma and the accumulation of cholesteryl esters in many tissues such as tonsils, liver, spleen, intestinal mucosa, peripheral nerves, and cornea. The patients with TD have an increased risk for coronary artery disease (CAD) [1]. This disorder is caused by the mutations in the ATP-binding cassette transporter-A1 (ABCA1) gene [2–4]. Obviously, the higher susceptibility for CAD is to some extent attributed to the deficiency of HDL, which is the major player in the reverse cholesterol transport. However, the pathophysiology of this disorder has not been completely understood yet.

Cells from patients with TD were known to have some cell-biological abnormalities such as defective cholesterol efflux and development of Golgi apparatus [1]. We recently presented that the expression of small G protein [5],

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Cdc42Hs, which has various cell-biological functions such re-arrangement of actin–cytoskeletons and vesicular transport [5], was markedly decreased in skin fibroblasts and monocyte-derived macrophages from TD patients [6]. We found that Cdc42 was decreased in aged fibroblasts *in vitro* and *in vivo* [7]. Furthermore, we raised a hypothesis that Cdc42 plays a role in intracellular transport and export of lipids from the cells [5,7]. We and others have reported TD cells were characterized by the presence of enlarged morphology with altered actin–cytoskeletons [5,8], which is often observed in aged cells *in vitro* and *in vivo* [9].

It is known that human cultured skin fibroblasts ceased proliferation and exhibited senescence after serial passaging *in vitro*, which is termed replicative senescence. Cells from various types of aging syndromes such as Werner syndrome and Hutchinson-Gilford progeria had a reduced proliferative capacity *in vitro* and exhibited cellular senescence at the earlier passage [11]. On the other hand, although the underlying mechanisms still need to be examined, data have accumulated to show that cellular senescence *in vitro* may be related to many human disorders including cardiovascular diseases other than aging syndromes [12].

From the above observations, we have tested a hypothesis that cells from patients with TD may exhibit senescent phenotype *in vitro*. In the present study, we demonstrate that TD fibroblasts showed senescent phenotypes *in vitro*, such as accelerated replicative senescence at earlier PDL in association with shortening of telomere.

Materials and methods

Cells and culture. Skin fibroblasts were obtained from two unrelated TD patients (TD1 and TD2 showed in Table 1) who were diagnosed by both classical TD phenotypes and mutations in the ABCA1 gene [11,13]. Both patients had an apparent CAD. The following three fibroblasts cell lines served as controls. Human normal fibroblasts from a 55-year-old male (N55) were obtained from KURABO. Co. Ltd. (Japan), when the PDL was 6. The other normal human fibroblasts from 24-, and 48-year-old female (N24 and N48) were obtained with an informed consent. These subjects had no clinical complications. For the study of aging, all fibroblast cell lines were carefully established at Health Science Research Resources Bank (HSRRB, Japan) and Osaka University [7]. The cells were cultured according to the standard conditions in modified Eagle's medium (MEM) supplemented with L-glutamine, nonessential amino acids, and 10% of fetal calf serum in a humidified 5% CO_2 controlled incubator at 37 °C. The cells were passaged by 1:4 split to increase cumulative cell population doubling level (PDL) by 2 on passage.

Table 1
Clinical profile of the patients with Tangier disease

	TD1	TD2	Normal range
Sex	Male	Male	
Age (yr)	51	57	
TC (mmol/l)	0.72	0.78	(<6.85)
TG (mmol/l	2.60	2.00	(<1.80)
HDL-C (mmol/l)	0.16	0.13	(0.80–1.80)

Age: age when skin fibroblasts were obtained.
TC: plasma total cholesterol level.
TG: plasma triglyceride level.
HDL-C: plasma high density lipoprotein–cholesterol level.

Cell proliferating rate. Cell proliferating rate was analyzed by the method as described by Mathon et al. [12]. Human fibroblasts (3×10^5 cells) were plated onto 9 cm-plastic dishes (SUMILON, Sumitomo Bakelite Co. Ltd., Japan) under conditions as detailed above. Every 4 days, cells were trypsinized, counted with a Coulter counter, and reseeded at a constant density. The proliferating rate of cells was determined as the ratio of total cell number trypsinized to the initial cell number seeded before 4 days.

DNA synthesis analysis. Human fibroblasts (10^4 cells/well) were seeded in 96-well plastic plates (FALCON culture plate, Becton–Dickinson Labware). DNA synthesis of cells was analyzed by bromodeoxyuridine (BrdU) incorporation assay method, using commercial kit (Cell proliferation ELISA system, Amersham).

Staining for senescence-associated β-galactosidase (SA-β-gal). Cells were washed twice with PBS and fixed with PBS containing 2% formaldehyde and 0.2% glutaraldehyde. Cells were then stained with SA-β-gal staining solution (1 mg/mL of X-gal, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM potassium ferrocyanidine, 5 mM potassium ferricyanide, and 40 mM citric acid/sodium phosphate buffer, at pH 6.0) for overnight [13].

Telomere length analysis. Purified genomic DNA (1 μg) from human fibroblasts (at PDL 6 and 25) was digested by an optimized mixture of frequently cutting restriction enzymes (*Hin*f1 and *Rsa*1). Following DNA digestion, the DNA fragments were electrophoresed through a 0.8% agarose gel and transferred onto a nylon membrane for Southern blotting. Telomere length was analyzed by using *Telo* TAGGG telomere length assay kit (Roche, Germany). The exposed X-ray film was scanned with a densitometer. The mean of telomere length was calculated as $\Sigma(OD_i)/\Sigma(OD_i/L_i)$, where OD_i is the the chemiluminescent signal and L_i is the length of the terminal restriction fragments at position *i*.

Statistical analysis. Results are expressed as means ± SD. Statistical significance was assessed by Student's *t* test for paired values and set at $P < 0.05$.

Results

Decreased proliferating rate and DNA synthesis of TD fibroblasts

To investigate the ability of proliferation of TD fibroblasts, we examined the proliferating rate at various PDLs (Fig. 1A). Although, the proliferating rates of three controls cell lines (N24: a young-aged normal cell line, N48 and N55: age-matched cell lines for TD patients) remained constant with continual passaging (PDL ~30), the proliferation of TD cells (TD1 and TD2) became slower at PDL ~10. Both TD1 and TD2 cells practically ceased proliferation at PDL ~30. Next, we examined DNA synthesis by BrdU incorporation assay method. Fig. 1B shows that DNA syntheses were significantly decreased at PDL 18 in TD cells, compared with those of the normal controls. It is known that the passaged fibroblasts had limited life span *in vitro* called "replicative senescence". These results suggest that TD cells might reach the replicative senescence at an earlier PDL.

Increased number of senescence-associated β-galactosidase (SA-β-gal) positive cells in Tangier disease

In order to further characterize TD cells from the viewpoints of cellular senescence, we analyzed the expression of