Senolytics restore the contraction of the collagen gel based on senescent dermal fibroblasts culture
: A novel insight into the therapeutic approach to skin aging

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Abstract

Background: The removal of senescent cells (SNCs) has been proposed to be beneficial for improving various age-related diseases. However, little is known regarding senolytics, which selectively remove SNCs to reduce skin aging. In this study, we investigated the effects of senolytics on SNCs in terms of contractility of human dermal fibroblasts.

Methods: A collagen gel contraction assay was performed to investigate the contractility of senescent dermal fibroblasts (SDFs) induced by doxorubicin. The orientation of collagen fibers was observed using second harmonic generation (SHG) with multiphoton microscopy. ABT263, a drug that selectively eliminates SNCs, was used to evaluate the effects of senolytics on the recovery of collagen gel contraction.

Results: The contraction of SDF-containing 3D collagen gel (aged dermal model) was significantly less than that of non-senescent dermal fibroblast (nSDF)-containing collagen gel (young dermal model). Although thicker bundles and collagen fibril orientation were observed on SHG imaging in the young dermal model, they were not observed in the aged dermal model. Furthermore, the number of SDFs decreased in the aged dermal model treated with ABT263; consequently, gel contractility was restored.

Conclusion: Decreased contractility in the SDF collagen gel may cause morphological changes in the extracellular matrix with skin aging, and the elimination of SDFs remodels the collagen orientation and restores contractility in dermal fibroblasts. These results may lead to the development of novel therapeutic approaches against skin aging.

Keywords: senolytics, cellular senescence, skin aging, collagen contraction

Introduction

Cellular senescence is a programmed response to cell damage that leads to irreversible growth arrest. It can be triggered by various intrinsic and extrinsic stresses in the cells, such as telomere attrition, DNA damage, oncogene activation, oxidative stress, and inflammation. Senescent cells (SNCs) prevent genetically unstable propagation and promote wound healing, embryonic development, and tissue repair [1]. In contrast, recent studies have revealed that SNCs may damage surrounding tissues via the secretion various inflammatory mediators, growth factors, and extracellular proteases called senescence-associated secretory phenotype (SASP) factors [2]. As evidence suggests that SNCs accumulate with age and contribute to age-related pathologies in various organs [1], the removal of SNCs has been proposed as beneficial for improving age-related diseases. Indeed, many studies have reported that senolytics, which selectively eliminate SNCs, attenuate age-related deterioration of several organs [3].

The skin is the outermost part of the body and has three layers: the epidermis, dermis, and subcutaneous tissue. The dermis is primarily composed of fibroblasts, which synthesize the extracellular matrix (ECM) and generate mechanical forces within the ECM through their contractility. Collagen fibers are a major component of the ECM in the dermis, and the contraction of collagen fibers by dermal fibroblasts provide tensile strength and elasticity, which is critical for the maintenance of skin structure and remodeling. Thus, any disturbance to the collagen structure results in signs of skin aging such as wrinkling and sagging [4]. Although the collagen fiber density decreases with age, there is a paucity of information about the influence of aging on the contractility of dermal fibroblast collagen.

Increasing evidence shows that SNCs accumulate in the skin, specifically in the dermis, with age [5, 6], and senescent dermal fibroblasts (SDFs) are considered to be associated with skin aging [7]. However, the underlying mechanism of how these cells contribute to skin aging has not been fully elucidated. Moreover, whether the removal of SNCs will improve skin aging remains unknown. Therefore, in this study, we focused on the change in collagen contractility by SNCs and examined the effects of senolytics on collagen contraction as its characteristics are important for preventing skin aging. As a result, we found that the

contractility was weak and the collagen orientation was low in SDF-containing 3D collagen gel. We also found that kinesin superfamily genes expression was decreased in non-senescent fibroblast (nSDFs) co-cultured with SDFs. Moreover, we showed that the number of SDFs decreased drastically in the aged dermal model treated with ABT263 and consequently, gel contractility and collagen orientation were restored.

Materials and methods

Materials

Normal human dermal fibroblasts (NB1RGB) were purchased from Riken Cell Bank (Ibaraki, Japan). Anti-phospho histone H2A.X (Ser139) was obtained from Merck Millipore (Billerica, USA). Secondary antibodies were species-specific antibodies conjugated with Alexa Fluor 568. Chemical compounds used were doxorubicin (DRX, hydrocholoride) (Cayman Chemical, MI, USA), ABT263 (Cayman Chemical, MI, USA), and caspase inhibitor Z-VAD-FMK (Promega, WI, USA).

Cell culture

NB1RGB were maintained at 37°C and 5% CO_2 in minimum essential medium α (Thermofisher Scientific, MA, USA) containing 10% fetal bovine serum (FBS). For senescence induction, cells were treated with 200 ng/mL DRX for 3 days. Senescence-associated β-galactosidase (SA-β gal) staining was performed according to the β-galactosidase staining kit protocol (Clontech Laboratories, Inc., CA, USA). Briefly, cells treated with DRX were washed twice in phosphate-buffered saline (PBS), fixed for 5 min in fixing buffer, washed in PBS, and incubated in X-gal staining solution overnight. After staining, β-galactosidase-positive cells were determined by observation of blue cells under microscopy.

Immunofluorescence microscopy

Formalin-fixed fibroblast-containing 3D collagen gel was embedded in paraffin and sectioned to 10 μm thickness. After dewaxing and dehydration, sections were boiled in citrate buffer for 10 min and incubated with 3% BSA (Sigma-Aldrich, MO, USA) for 1 h at room temperature. Then, sections were incubated with the primary antibody overnight at 4°C, washed thrice, and incubated for 1 h with appropriate secondary antibodies conjugated with

fluorophore. Nuclear staining was performed with 4`,6-diamidino-2-phenylindole. Images were acquired on an all-in-one type fluorescence microscope (BZ-8000, Osaka, Japan).

Collagen contraction assay

The effects of senescent dermal fibroblasts on collagen gel contraction were determined using Cellmatrix type I-A (Nitta Gelatin, Osaka, Japan) according to the manufacturer's instruction. Briefly, fibroblasts cultured with or without DRX were trypsinized and suspended in a culture medium. Cells were counted and prepared in a suspension at a density of 1×10^6 cells/mL in collagen gel solution prepared on ice by mixing Cellmatrix type I-A: reconstitution buffer (Nitta Gelatin): 5 × DMEM in a ratio of 7:1:2. The mixture was added to each well of a 24-well culture plate. After 1 h of incubation at 37°C and 5% CO_2, a culture medium containing 10% FBS was added, and the cell-populated collagen gel was cultured for 3 days. After 3 days of culture, to initiate contraction, collagen gels were gently released from the sides of the culture dishes with a sterile spatula. Then, the culture medium was replaced with a fresh medium that did not contain a pharmacological reagent, 1 μM ABT263 or 1 μM ABT263 plus caspase inhibitor. The change in collagen gel size was observed at various times after release with microscopy (Keyence).

DNA microarray analysis

Non-senescent dermal fibroblasts were cultured with or without senescent fibroblasts using a culture insert system for 3 days. After culture, total RNA was extracted from non-senescent dermal fibroblast using ReliaPrep™ RNA Miniprep Systems (Promega, WI, USA). Human gene ST array was performed at Kurabo (Osaka, Japan).

Second harmonic generation (SHG) imaging

Collagen gel was fixed in 4% paraformaldehyde for 1 h at 4°C and then washed with PBS to remove excess fixative. The fixed collagen gel was observed with a multiphoton microscope. Multiphoton imaging was performed with an inverted microscope (Ti2-E, Nikon, Tokyo, Japan) equipped with an Apochromat LWD Lambda S 20XC WI objective lens (NA 0.95, Nikon) and a pulse laser with excitation at 880 nm. We used an emission short pass filter at 492 nm for SHG signal. Image acquisition was performed by NIS-Elements (Nikon).

Statistical analysis

Statistical significance was determined using Student's t-test and one-way analysis of variance. *P*-values <0.05 were considered significant.

Results

3D collagen gel contraction decreased in the aged dermal model

To explore how cellular senescence affects age-related dermal change, we first assessed the contractility of SDFs to collagen. Cellular senescence was induced by treatment with 200 ng/mL DRX. Then, we confirmed the SA-β gal staining and formation of phosphorylation of the Ser-139 residue of the histone variant H2AX (γH2AX), which are all senescence markers (Fig. 1). SDFs and nSDFs were embedded in 3D collagen gel, which were referred to as the aged and young dermal models, respectively, and the contraction of these collagen gels was observed. We found that the contraction of the aged dermal model was drastically lower than that of the young dermal model (Fig. 2). Next, we examined whether SDFs affect the contractility of nSDFs. Contraction was observed in 5×10^5 nSDFs or 1×10^6 nSDFs- collagen gel (Figs. 3A-II, III). However, in the co-culture gel (5×10^5 nSDFs and 1×10^6 SDFs) or in collagen gel containing only 1×10^6 SDFs, contraction was not observed (Figs. 3B-III, IV).

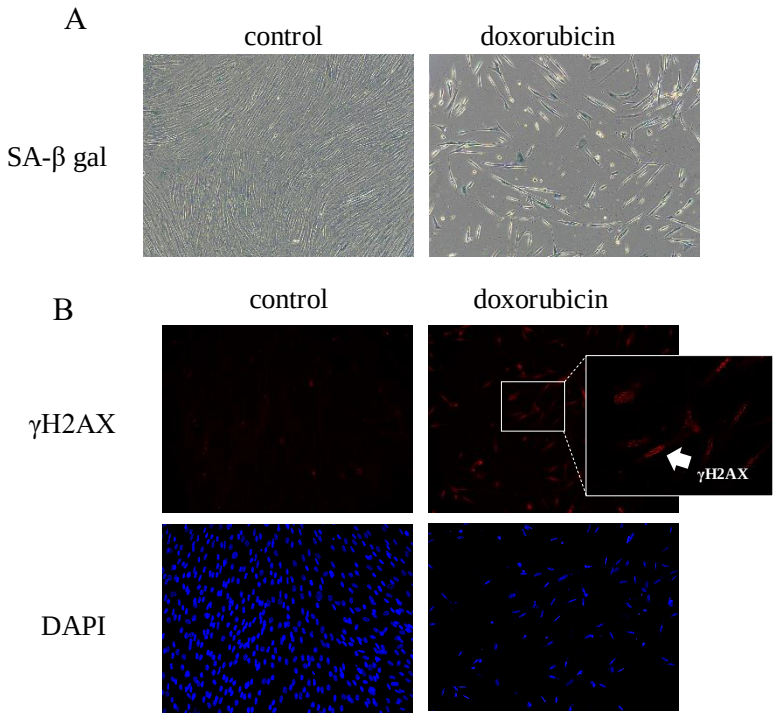


Fig. 1. Cellular senescence is induced by doxorubicin. Human dermal fibroblasts were treated with doxorubicin for 3 days. After treatment, these cells were subjected to SA-β gal staining (A), and immunofluorescence staining for senescence marker (γH2AX) and DAPI (B).

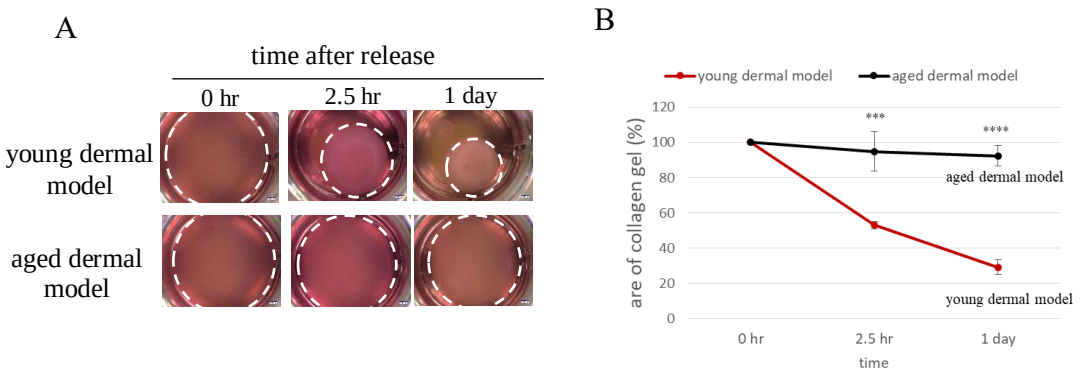


Fig. 2. The contraction of collagen gel is decreased in aged model. The contraction of non-senescent dermal fibroblast (nSDF) and senescent dermal fibroblast (SDF) containing collagen gel , referred to as young dermal model and dermal model respectively, were observed with microscopy at indicated times after release from dish (A). The acquired images were analyzed with ImageJ and the area of collagen was calculated (B). ***$P < 0.001$, ****$P < 0.0001$ aged model vs young model, t-test

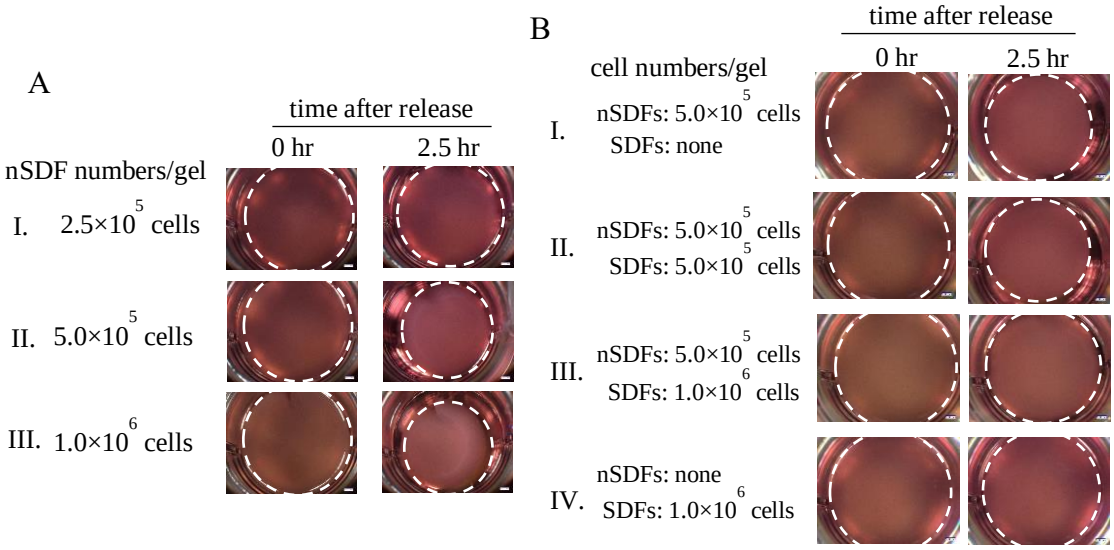


Fig. 3. The collagen gel contraction is decreased by SDFs even in the presence of an adequate number of nSDFs sufficient to contract the gel. The different numbers of nSDFs were embedded in collagen gels and the numbers of nSDFs capable of contracting the collagen gel was determined (A). nSDFs and SDFs of each cell number were embedded in the same collagen gel, and the contraction of collagen gel was observed 2.5 hr after release from dish (B).

Expression of the kinesin family gene decreased in nSNCs co-cultured with SNCs

To further investigate the effects of SDFs on the ability of nSDFs to contract collagen gel, we performed a comprehensive analysis of the change in the gene expression in nSDFs co-cultured with SDFs using a culture insert system to verify the interaction between SDFs and

A

Name of gene	Gene Symbol	Fold change co-culture vs. alone	UniGene ID
integrin, alpha 2	ITGA2	0.894	Hs.482077
integrin, alpha V	ITGAV	0.896	Hs.436873
integrin, beta 1	ITGB1	0.953	Hs.643813
integrin, beta 2	ITGB2	1.044	Hs.375957
fibronectin 1	FN1	0.992	Hs.203717
matrix metallopeptidase 1	MMP1	1.321	Hs.83169
matrix metallopeptidase 2	MMP2	1.104	Hs.513617
matrix metallopeptidase 9	MMP9	1.045	Hs.297413
tubulin, alpha 1a	TUBA1A	0.698	Hs.654422
tubulin, beta class I	TUBB	0.870	Hs.636480
tensin 1	TNS1	0.945	Hs.471381
tensin 2	TNS2	0.915	Hs.6147
tensin 3	TNS3	0.983	Hs.520814
tensin 4	TNS4	1.121	Hs.438292

B

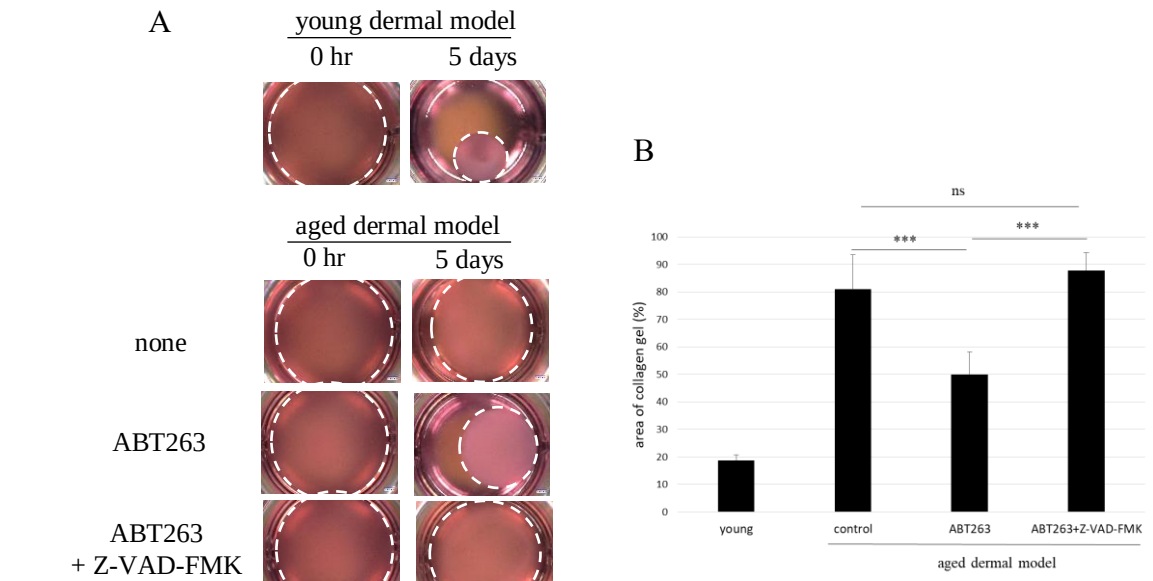
Name of gene	Gene Symbol	Fold change co-culture vs. alone	UniGene ID
kinesin family member 20A	KIF20A	0.111	Hs.718626
kinesin family member 11	KIF11	0.188	Hs.8878
kinesin family member 20B	KIF20B	0.300	Hs.240
kinesin family member 14	KIF14	0.308	Hs.3104
kinesin family member 23	KIF23	0.312	Hs.270845
kinesin family member 2C	KIF2C	0.324	Hs.720061
kinesin family member 15	KIF15	0.331	Hs.658939
kinesin family member 4A	KIF4A	0.395	Hs.648326
kinesin family member 18A	KIF18A	0.466	Hs.301052
kinesin family member 22	KIF22	0.567	Hs.612151
kinesin family member 18B	KIF18B	0.596	Hs.135094
kinesin family member C1	KIFC1	0.609	Hs.436912
kinesin family member 4B	KIF4B	0.765	Hs.567824
kinesin family member 3A	KIF3A	0.799	Hs.43670
kinesin family member 24	KIF24	0.823	Hs.710246
kinesin family member 27	KIF27	0.839	Hs.697514
kinesin family member 5B	KIF5B	0.842	Hs.327736
kinesin family member 5C	KIF5C	0.848	Hs.435557 // Hs.660699
kinesin family member 19	KIF19	0.921	Hs.372773
kinesin family member 21A	KIF21A	0.956	Hs.374201
kinesin family member 28P	KIF28P	0.967	---
kinesin family member 2A	KIF2A	0.984	Hs.558351
kinesin family member 13A	KIF13A	0.998	Hs.94499
kinesin family member 21B	KIF21B	1.000	Hs.169182
kinesin family member 5A	KIF5A	1.000	Hs.151219
kinesin family member 7	KIF7	1.000	Hs.513134
kinesin family member 2B	KIF2B	1.000	Hs.226805 // Hs.744513
kinesin family member 3C	KIF3C	1.000	Hs.21611
kinesin family member 1A	KIF1A	1.000	Hs.516802
kinesin family member 16B	KIF16B	1.000	Hs.101774
kinesin family member 9	KIF9	1.000	Hs.373947
kinesin family member 25	KIF25	1.000	Hs.150013
kinesin family member 6	KIF6	1.000	Hs.588202
kinesin family member 13B	KIF13B	1.000	Hs.444767
kinesin family member 12	KIF12	1.000	Hs.28149
kinesin family member 26A	KIF26A	1.016	Hs.134970
kinesin family member 1B	KIF1B	1.036	Hs.97858
kinesin family member 17	KIF17	1.055	Hs.130411
kinesin family member C3	KIFC3	1.100	Hs.23131
kinesin family member 1C	KIF1C	1.132	Hs.435120
kinesin family member 26B	KIF26B	1.160	Hs.368096
kinesin family member C2	KIFC2	1.213	Hs.528713
kinesin family member 3B	KIF3B	1.224	Hs.369670

Table 1. SDFs affect the expression of gene in nSDFs. nSDFs were cultured with SDFs using a culture insert system for 3 days. After culture, comprehensive gene expression in the cultured cells was analyzed by DNA microarray. The changes in expression of integrin, fibronectin, matrix metallopeptidases, tubulins and tensins, and kinesin superfamily gene were shown in the Table 1A and 1B, respectively.

SDFs. As a result of the DNA microarray, we found that gene expression either increased or decreased in nSDFs co-cultured with SDFs compared with nSDFs alone. However, we did not find a change in the expression of genes associated with cell adhesion to collagen fibril, such as integrin and fibronectin, in nSDFs co-cultured with SDFs (Table 1A). By contrast, the expression of several kinesin superfamily genes, which play roles in intracellular transport, were drastically decreased in nSDFs co-cultured with SDFs compared with that in nSDFs alone (Table 1B).

Senolytics recovered the contraction of the aged dermal model

Finally, to investigate the effects of removing of SNCs from the aged dermal model on their contractility, SDF-containing collagen gel was treated with ABT263, which is a known senolytic drug [10]. As shown in Fig. 4A and B, contraction of the aged dermal model was recovered by treatment with ABT263. By contrast, simultaneous treatment of the aged dermal model with ABT263 and Z-VAD-FMK, an apoptosis inhibitor, suppressed the recovery of collagen contraction caused by ABT263 (Fig. 4A, B). We then addressed the number of SNCs in the aged dermal model treated with ABT263 by immunofluorescence against γH2AX. The number of γH2AX-positive cells in the aged dermal model treated with ABT263 was reduced compared with that in controls (Fig. 4C). To further verify the effects of senolytics on the aged dermal model, we observed the orientation of collagen fibers in the dermal model using SHG with multiphoton microscopy. Although thicker bundles and collagen fibril orientation were observed on SHG imaging in the young dermal model, they were not found in the aged dermal model (Fig. 5). By contrast, following treatment with ABT263, thicker bundles were observed in the aged dermal model. These data suggest that senolytics recover the collagen contractility in aged dermis accompanied by the remodeling of collagen fibril bundles.



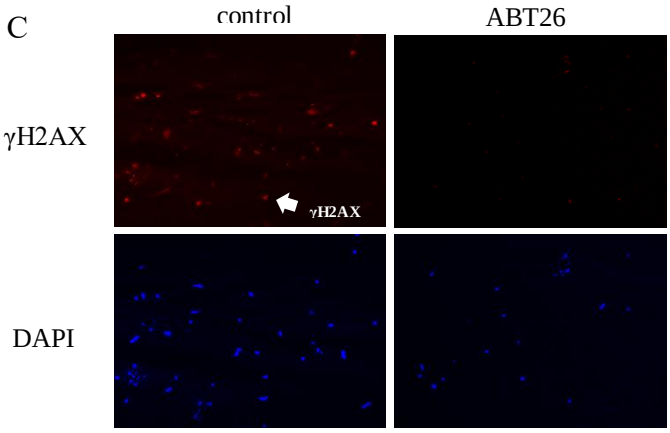


Fig. 4. ABT263 restores the contraction of aged dermal model and reduce senescent dermal fibroblasts in aged dermal model. The contraction of aged dermal model in the presence or absence of ABT263 with or without Z-VAD-FMK was observed (A). The acquired images were analyzed with ImageJ and the area of collagen was calculated (B). Senescence marker (γH2AX) in aged dermal model treated with ABT263 was detected by immunofluorescence (C). ***$P < 0.001$, one-way ANOVA

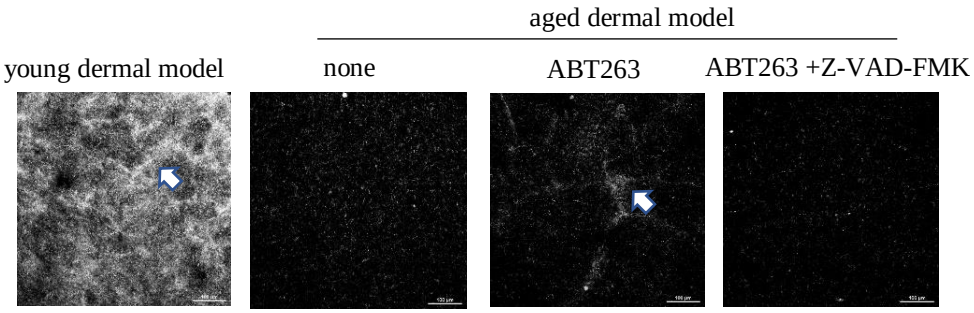


Fig. 5. ABT263 recovers the collagen bundle in aged dermal model. The orientation of collagen fiber in young dermal model and aged dermal model in the presence or absence of ABT263 with or without Z-VAD-FMK were observed using the second harmonic generation (SHG) with multiphoton microscopy. Arrowhead indicated the bundle of collagen fibril.

Discussion

Growing evidence shows that SNCs accumulate in the skin, specifically in the dermis, with aging, and that senescent dermal fibroblasts are associated with alterations in skin physiology and morphology. However, the details of the mechanisms are unknown. In this study, we investigated the effects of senescence of dermal fibroblasts on collagen contraction using a dermal model. We found that the SDFs induced by DRX had a lower ability to contract collagen gel than nSDFs (Fig. 2). This result was consistent with those of previous studies that showed collagen gels containing replication-aged fibroblasts had reduced contraction [8]. The decrease in collagen contractility of fibroblasts reduces the skin's mechanical properties and the appearance of wrinkles [4]. This suggest that senescence of fibroblasts is involved in the formation wrinkles. Interestingly, even in nSDFs that could sufficiently contract collagen gel, contraction was disturbed in the presence of a certain number of SDFs (Fig. 3). One of the major characteristics of SNCs is the release of SASP factors such as inflammatory cytokines, growth factors, and proteases. Although some SASP factors are important for stable cell-cycle arrest in SNCs, many SASP factors are thought to have adverse effects on the surrounding cells, such as causing a chronic inflammatory environment [2]. Thus, the decrease in collagen contractility observed in nSDFs coexisting with SDFs, as observed in this study, might have been triggered by SASP factors released from the SDFs. A comprehensive analysis of the gene expressions in nSDFs co-cultured with SDFs using a culture insert revealed that the expression of many kinesin superfamily genes in the nSDFs co-cultured with SDFs was reduced (Table 1). In addition, the comparison of gene expressions between nSDFs and SDFs showed that the expression of the kinesin superfamily genes was decreased in SDFs (data not shown). Kinesin is a motor protein that moves along microtubules using the energy generated by ATP hydrolase that plays a role in the transport of vesicles and organelles and regulates microtubule dynamics. Microtubules are involved in maintaining the cell structure and pseudopodical activity in dermal fibroblasts [9]. Therefore, it is likely that kinesin regulates dermal fibroblast contractility. Although the detailed mechanism of collagen contraction by fibroblasts has not been clarified, a decrease in kinesin expression in SDFs and nSDFs co-cultured with SDFs may be related to the decreased contractility.

Senolysis, which selectively removes SNCs, is attracting attention as an approach to SNC-related diseases. Senolytics improve symptoms in various age-related diseases [3]. In this study, we investigated the effect of ABT263, which eliminates SNCs by inducing apoptosis, on the decrease in collagen contraction associated with cellular senescence. Consequently, ABT263 recovered the contractility of SDF-containing collagen gel (aged dermal model) (Fig. 4A, B). Additionally, the number of SDFs in the aged dermal model treated with ABT263 decreased (Fig. 4C). Furthermore, simultaneous treatment of the aged dermal model with ABT263 and an apoptosis inhibitor canceled the recovery of collagen contraction by ABT263. These results indicate that collagen contraction is restored by the removal of SDFs. Additionally, collagen fiber orientation in the aged dermal model resulted from ABT263 treatment (Fig. 5). These results show that senolytics induce collagen matrix remodeling in aged skin, and that senotherapeutics are a promising approach against skin aging.

Conclusion

Our findings demonstrate that the decreased ability of SNCs to contract collagen gel might cause dermal ECM morphological changes with skin aging, and that the elimination of SNCs restores collagen gel contractility. These results may lead to novel therapeutic advances against skin aging.

Conflict of Interest Statement

The authors have no conflict of interest.

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