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Modulation of cellular senescence in fibroblasts and dermal papillae cells *in vitro*

JAMES V. GRUBER, PHILIP LUDWIG, and ROBERT HOLTZ,
Arch Personal Care, South Plainfield, NJ 07080 (J.V.G.,P.L.),
BioInnovation Laboratories, Lakewood, CO 80235 (R.H.).

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Synopsis

A hexapeptide (Hexapeptide-11) of structure Phe-Val-Ala-Pro-Phe-Pro (FVAPFP) originally isolated from yeast extracts and later synthesized by solid state synthesis to high purity has demonstrated an ability to influence the onset of senescence in intrinsically aged fibroblasts, extrinsically aged fibroblasts, and extrinsically aged dermal papillae cells *in vitro*. The mechanism of senescence control is believed to be related to the peptide's ability to reversibly downregulate ataxia telangiectasia mutated (ATM) and p53 protein expression. The importance of p53 as the gatekeeping protein for monitoring cellular DNA damage is strategic for maintaining cellular health. ATM activates p53 by direct phosphorylation, causing cells to move into senescence which effectively moves them out of reproductive processes. Technologies that can influence ATM and p53 expression may offer unique benefits for controlling cellular senescence and effectively delaying cellular aging processes. The influence on ATM and p53 expression is noted to occur in both cell lines at peptide concentrations between 0.1% and 1.0%. The implications of these effects for aging benefits for skin and hair is important as, to date, no known small peptide has been suggested to demonstrate this effect in such a reversible and dose-dependent fashion.

INTRODUCTION

Cellular replicative senescence accompanies aging and is linked to multiple physical changes in humans ranging from wrinkling and thinning of the skin to hair loss (1). Replicative senescence is a fundamental feature in normal human cells and results partially from diminished telomere function at the Hayflick limit. The Hayflick limit (or Hayflick phenomena) is the number of times a normal cell population will divide before it stops, presumably because the telomeres reach a critical length (2,3). When cells reach replicative senescence they stop replicating DNA but continue metabolism (i.e., they continue to make ATP). Radiation and oxidative stress prematurely induces the same phenotypes as replicative senescence prior to the Hayflick limit. This process is known as stress-induced premature senescence (SIPS) (4).

Address all correspondence to James Gruber at vince.gruber@lonza.com.

Fibroblasts are cells that grow in the dermal layer of the skin and are responsible for expression of new collagen and elastin. Dermal papillae cells also grow in the dermis of the skin and are the cells responsible for expression of hair fibers. Dermal papillae cells from balding and non-balding individuals have recently been grown *ex vivo* (5). Dermal papillae cells from balding individuals exhibit signature protein markers indicating they are experiencing premature senescence. They have been found to express high levels of senescence-associated β-galactosidase (SA-β-Gal) and ataxia telangiectasia mutated (ATM) proteins, both being accepted markers for cellular senescence (5). Ultraviolet radiation (UVR)–stressed fibroblasts from aged skin also express high levels of these two proteins suggesting senescence plays a strategic role in extrinsic skin aging as well (6). For fibroblasts from aged skin, it has been determined that they express high levels of both ATM protein and SA-β-Gal indicating these cells are approaching or have reached cellular senescence (7).

Phenotypically, aging includes effects from the subcellular to macroscopic level. Such signs of aging may be induced or caused by intrinsic factors, e.g., chronological aging, or extrinsic factors, e.g., environmental damage, sunlight, UV, smoke, ozone, pollutants, stress, etc. Visible signs of skin aging include an increase of fine lines, wrinkles, large pores, and surface roughness. Recently, it was reported that in mice genetically modified to show accelerated senescence that selective removal of senescent cells demonstrated a significant improvement in the aging phenotype of the mice compared against controls in which the senescent cells were not removed (8). The study demonstrates that even though senescent cells make up only a small percentage of the total cells within the mice, the influence of these senescent cells on aging is profound.

Biochemical markers for replicative senescence have been identified and include the genes p53 (TP53), Sirtuin1 (SIRT1), ataxia telangiectasia mutated (ATM) and the related ataxia telangiectasia related protein (ATR) (9). In addition, Rad23 (RAD23), p21 (TP21), and p16 (TP16) have also been identified as important markers for cellular senescence. ATM protein plays a strategic role in DNA checkpoint response functions linked in part to ATM-directed phosphorylation/activation of p53 and a host of other cellular DNA-damage response proteins (10–12). ATM protein also plays a central role in signaling the presence of DNA double-strand breaks. Loss of ATM protein function is characterized by accelerated telomere loss, genomic instability, progressive neurological degeneration, and premature aging. ATM protein deficiency and telomere dysfunction likely act together to impair cellular and whole-organism viability. High ATM protein expression is associated with cells in senescence. Changes in expression of ATM protein can also be used as a biomarker to identify senescent cells.

Cellular senescence can be observed via various methods. Cellular senescence leads to an increase in SA-β-Gal activity, which can be used as a biomarker to identify senescent cells (13). SA-β-Gal is expressed by cells in either intrinsic or stress-induced cellular senescence. Senescent cells can also be noted by changes in the morphology of the cells.

It has been reported that extracts from yeast fermentation, in particular, *Saccharomyces cerevisiae*, have demonstrated wound healing properties (14–19). These physiological effects, which have been variously attributed to increased cellular oxygen consumption

(14,17,19), improved collagen synthesis (15,18,19), and increased blood vessel development (17), appear to be principally related to unique proteins and low molecular weight peptides that are enzymatically manufactured in the growing yeast (17,18). Undoubtedly, many of these proteins and peptides are small molecular weight fragments of larger signaling molecules. It is now widely recognized that low molecular weight proteins and nuclear fragments can play a role in upregulating important cellular growth factors that can lead to skin healing (20–23).

EXPERIMENTAL

PEPTIDE ISOLATION

The peptide used in the following studies was isolated and identified from a low molecular weight fraction of a ferment of *S. cerevisiae* (24). The peptide amino acid sequence was determined by Erdman Degradation at the University of California, Davis Molecular Structure Facility and indicated that the peptide was a hexapeptide comprised of Phenylalanine (Phe), Valine (Val), Alanine (Ala) and Proline (Pro), comprising the unique sequence Phe-Val-Ala-Pro-Phe-Pro (FVAPFP) (Figure 1). The peptide has been assigned the International Nomenclature Cosmetic Ingredient name, Hexapeptide-11. BLAST2® (Washington University, St Louis, MO) available with the National Center Biotechnology Information (NCBI) Sequence Viewer software (http://www.yeastgenomeorg/cgi-bin/blast-sgd.pl) was employed to match this peptide sequence against the entire protein dataset for *S. cerevisiae*. The sequence of amino acids that comprise Hexapeptide-11 appears in a number of yeast proteins; in particular, the amino acid sequence for Hexapeptide-11 can be found in stress-related proteins (hsp70), and transmembrane proteins as well as a number of proteins whose functions are presently unknown.

For the purposes of these studies, the hexapeptide was synthesized using solid state peptide synthesis techniques to a purity of 95% as determined by high-performance liquid chromatography (not shown). The peptide used in the following studies was the highly purified synthetic peptide and all concentrations shown are on a dry basis.

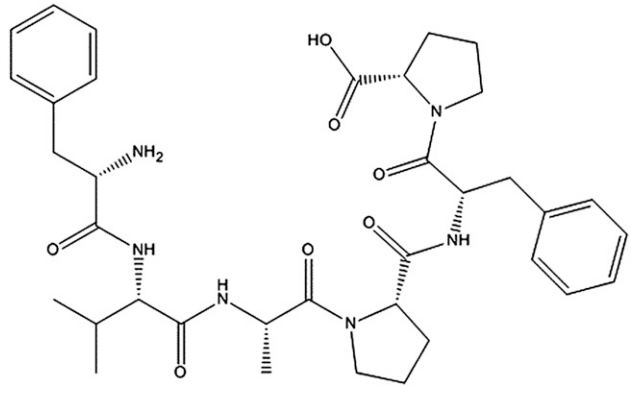

Figure 1. Structure of Hexapeptide-11.

FIBROBLASTS STUDIES

DNA microarray. Human dermal fibroblasts were obtained from Cascade Biologics (Grand Island, NY), seeded into T-25 flasks, and grown at 37 ± 2°C and 5 ± 1% CO_2. Upon reaching confluency, the cells were treated with Hexapeptide-11 for 24 h after which total RNA was isolated using an RNAqueous Kit (Ambion Inc., Austin, TX) per the manufacturer's instructions. After purification, the total RNA was prepared for array use by first amplifying the RNA using a MessageAmp aRNA Kit (Ambion), and then fluorescently labeling the aRNA with Cy3 or Cy5 using an ASAP Labeling Kit (Perkin Elmer, Boston, MA), both per the manufacturer's instructions. To purify the fluorescently labeled aRNA, a microcon YM-30 filter column was inserted into a collection tube and filled with 400 µl of TE buffer. The Cy3 and Cy5 probes were combined and then added to the microcon filter and thoroughly mixed with the TE buffer. The filter was centrifuged at 12,000 rpm for 8 min and the flow-through was discarded. The column was then washed twice with 400 µl of TE buffer, discarding the flow-through each time. After the final wash, the filter column was inverted, placed into a new collection tube, and centrifuged at 2000 rpm for 2 min to collect the probe.

The fluorescently labeled aRNA was applied to the DNA microarray chips (Agilent Technologies, Santa Clara, CA) and the chip was hybridized overnight and washed per the manufacturer's recommended protocol. After washing, the microarrays were scanned with an Axon GenePix 4100A Scanner (Molecular Devices, Sunnyvale, CA) with the scanning resolution set to 5 µm and analyzed with GenePix Pro software. During the initial scan, the PMT gains for the scanner were adjusted such that the Cy5/Cy3 image count ratios were between 0.88 and 1.12.

Fluorescence intensities for the microarrays were subjected to global normalization. The total fluorescent signal for both dyes was normalized with a correction factor that would make the ratio of total intensities for both dyes equal to one. For this study, a Cy3/Cy5 (untreated/treated) fluorescence intensity ratio greater than 1.3 or less than 0.7 (this relates to a change in gene expression of at least ±30%) was used as the cutoff for up- and downregulated genes, respectively (25). In addition, the fluorescence intensity of the gene marker had to be greater than the background intensity.

Fibroblast SIPS. For the fibroblast SIPS experiment, fibroblasts were prepared in the same fashion noted above for the array work. The cells were treated for 24 h with the hexapeptide at various concentrations between 0.01% and 2.0% except the untreated control cells which were treated only with normal cell culture media. At the end of the 24-h incubation, the cells were exposed to a sublethal dose of hydrogen peroxide (H_2O_2) (150 µM, diluted in cell culture media) for 2 h (26). Following the H_2O_2 exposure period, the media was removed and replaced with fresh, normal cell culture media and the cells were allowed to grow for an additional 24 h after which changes in ATM expression were assessed using an immunoblotting-based technique.

Fibroblast intrinsic replicative senescence. Human neonatal fibroblasts were obtained after primary culture (passage 1), seeded into a set of T-75 flasks in 3 ml/flask of fibroblast growth media, and grown at 37 + 2°C and 5 + 1% CO_2. The cells were expanded through six passages (one passage was defined as growing the cells until the flask was confluent and then splitting the cells 1:2, thus one passage was roughly equal to one population doubling). After the 6th passage, the fibroblasts were split into different treatment groups and treated with the various test materials through passage 18. At passage 18, a portion of the fibroblasts were used to assay

changes in ATM and SA-β-Gal, while the remaining fibroblasts were cultured for an additional week (approximately 2 additional passages) in the absence of test materials.

For the analysis of ATM expression, at the end of the treatment period, the culture media was removed and the cells were washed once with phosphate buffered saline (PBS). After removing the wash, 1 ml of RIPA buffer (50 m*M* TRIS, pH 7.4, 150 m*M* NaCl, 1 m*M* PMSF, 1 m*M* EDTA, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) was added to the flasks and they were incubated on ice for 15 min on a rocking platform to lyse the cells. The cell lysates were then transferred to 1.5 ml tubes and centrifuged for 5 min at 14,000 rpm (4°C). The supernatant was retained and stored at −75°C. The protein concentration of the supernatant was determined using a BCA Protein Assay (Pierce, Rockford, IL). ATM content was then determined using an ELISA-based assay.

SA-β-Gal staining. Prior to staining, the fibroblasts were washed once with PBS and then fixed for approximately 6 min in a fixing solution (2% formaldehyde and 0.2% glutaraldehyde in PBS). After fixing, the cells were washed three times with PBS and stained using a Senescent Cells Staining Kit (Sigma-Adrich, St. Louis, MO) per the kit's instructions. The cells were then incubated at 37°C overnight in a non-CO_2 incubator. On the following day, the staining solution was removed and replaced with PBS. The cells were then photographed microscopically, and the number of stained cells (SA-β-Gal positive) in each field was counted.

Dermal papillae studies. Human dermal papillae cells were seeded into 12 plates in dermal papillae growth well medium (DPGM) and grown at 37 + 2°C and 5 + 1% CO_2 until confluent with a media change every 48 to 72 h as needed. Once the cells were confluent, the cell culture media was replaced with PBS and the cells were irradiated with 20 mJ/cm^2 UVB. After the UVB irradiation, the PBS was removed and replaced with cell culture media supplemented with the various test materials. Nonsupplemented DPGM was used as the untreated control. In addition, one set of cells was not exposed to UVB and served as the non-UVB treated control. After the addition of the media, sets of cells were cultured for 48 h. At the end of the incubation period, the cells were assayed for changes in SA-β-Gal activity as described above.

Statistical analysis. Treatments were compared via an ANOVA with a subsequent post hoc analysis (Newman–Keuls Multiple Comparison) using Graphpad Prism Software. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

GENOMIC RESPONSE OF HEXAPEPTIDE-11 ON NORMAL HUMAN FIBROBLASTS

The purified hexapeptide was initially examined for cytotoxic effects on normal human dermal fibroblasts. No cytotoxic effects were noted up to 1.0% of hexapeptide treatment (data not shown). Hexapeptide-11 was then evaluated at 0.001% and 1.0% concentrations on normal human dermal fibroblasts for 24 h by gene microarray analysis to determine the biological effect that Hexapeptide-11 has ATM gene expression (Figure 2). Both concentrations demonstrated statistically significant reductions in ATM gene expression as shown by their corresponding ratio of medians being less than 0.7 (25).

1.0% Hexapeptide/Fibroblasts		
Ratio of Medians (635/532)	GeneName	Description
0.654	ATM	Ataxia telangiectasia mutated, a serine/threonine kinase involved in apoptosis, DNA stability, cell cycle, and radiation response
0.001% Hexapeptide/Fibroblasts		
Ratio of Medians (635/532)	GeneName	Description
0.577	ATM	Homo sapiens ataxia telangiectasia mutated (includes complementation groups A, C and D) (ATM), transcript variant 1, mRNA [NM_000051]

Figure 2. Summary of ratio of medians for two concentrations of Hexapeptide-11, 0.001% and 1.0% for ATM expression after 24 h treatment on normal human dermal fibroblasts.

INFLUENCE OF HEXAPEPTIDE-11 ON STRESSED-INDUCED PREMATURELY SENESCENT FIBROBLAST CELLS

Premature cellular senescence can be induced by treating normal human dermal fibroblasts with H_2O_2 (26). The hexapeptide delays senescence in H_2O_2 stress-induced prematurely senescent dermal fibroblasts as measured by ATM protein expression in a dose-dependent fashion at peptide concentrations of 1.0% or more, but not at levels 0.1% and below (Figure 3). Interestingly, the influence of the peptide on ATM expression at concentrations greater than 1% does not continue to show increasing suppression of ATM protein expression, but instead shows a leveling effect up to 2% of hexapeptide concentration. The reasons for this leveling effect are unknown at this time, but additional studies reported here were typically run at concentrations no greater than 1.0% because of these initial dose findings.

To examine the influence of Hexapeptide-11 on intrinsic aging, normal human dermal fibroblasts were grown through 24 population doublings without additional added oxidative stress. Topical application of Hexapeptide-11 for the last 18 cycles showed a dose-dependent, statistically significant reduction of both ATM protein expression and SA-β-Gal expression, Figures 4 and 5, respectively. The effect on ATM protein and SA-β-Gal expression was reversible after 1 week of peptide removal indicating that influence of the hexapeptide on these cellular markers is not permanent.

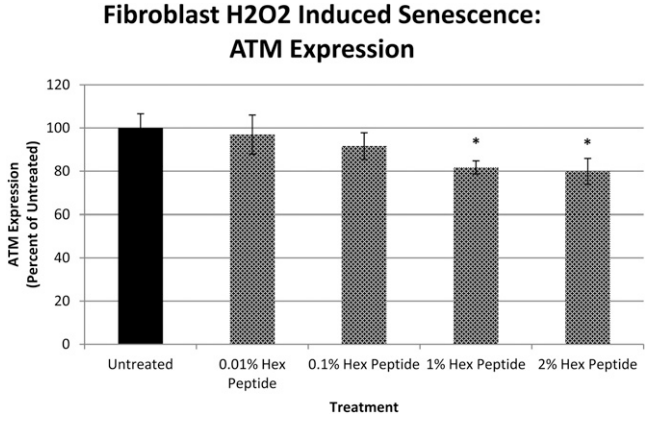

Figure 3. Changes in ATM protein expression in H_2O_2 prematurely stressed normal human dermal fibroblasts treated with various concentrations of Hexapeptide-11.

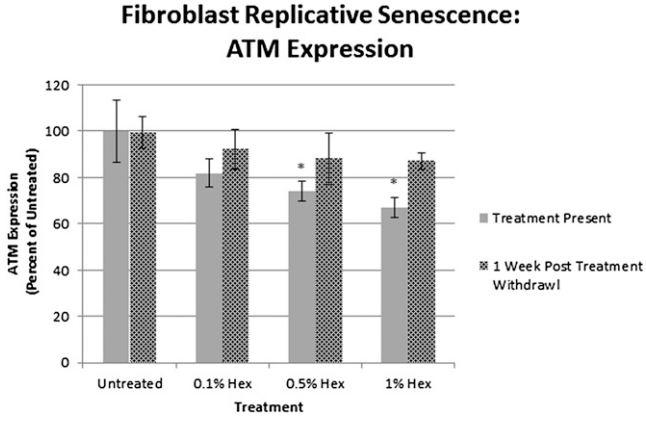

Figure 4. Expression of ATM protein in normal dermal fibroblasts aged for 22 population doubling cycles and treated for the last 18 cycles with Hexapeptide-11. Data for the same fibroblasts then tested one week after removal of the peptide from the media.

INFLUENCE OF HEXAPEPTIDE-11 ON PREMATURELY SENESCENT DERMAL PAPILLAE CELLS

Dermal papillae cells grow at the base of the hair shaft and are responsible for the growth of new hair fibers in anagen phase hair growth cycles (27). Dermal papillae cells from balding and non-balding individuals have been grown *ex vivo* (5). It has been demonstrated that dermal papillae cells taken from balding areas of the scalp show a higher level of senescence cellular subpopulations compared to dermal papillae from non-balding areas. For the purposes of this study, an *in vitro* senescence testing model was developed using UV to push dermal papillae cells into premature senescence. Dermal papillae cells were exposed to 20 mJ/cm^2 UVB for a period of time previously determined to not be cytotoxic to the cells, but known to elicit expression of measureable quantities of SA-β-Gal. After the irradiation, the cells were incubated for 48 h with various concentrations of the Hexapeptide-11, after which SA-β-Gal activity was determined. Exposure of dermal

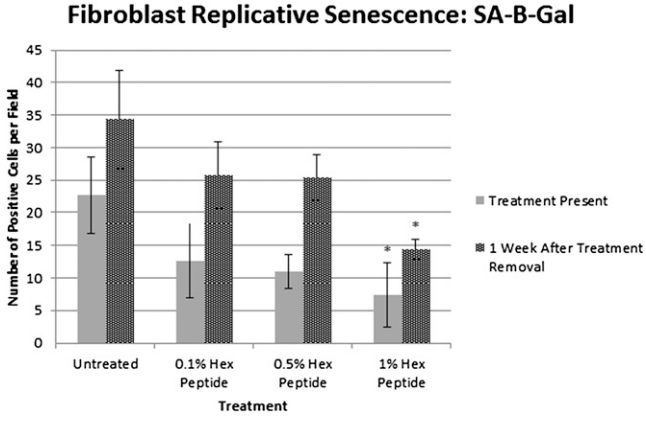

Figure 5. Expression of SA-β-Gal protein in normal dermal fibroblasts aged for 22 population doubling cycles and treated for the last 18 cycles with Hexapeptide-11. Data for the same fibroblasts then tested one week after removal of the peptide from the media.

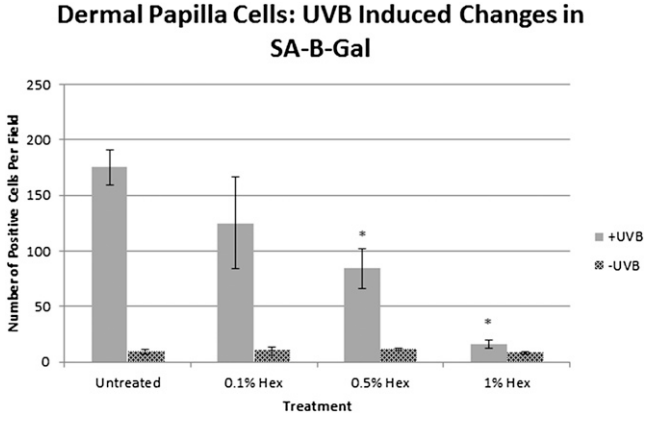

Figure 6. Analysis of SA-β-Gal expression in UV-induced prematurely senescent human dermal papillae cells compared against nonirradiated cells.

papillae cells to UV radiation causes an increase in the expression of SA-β-Gal indicating the cells are expressing biochemical signatures of premature senescence (Figure 6). Treatment with Hexapeptide-11 shows a dose-dependent, statistically significant decline in SA-β-Gal indicating reduced senescence at the 0.5% and 1.0% treatment levels.

CONCLUSION

Senescence, whether replicative or stress-induced, can be detected in both fibroblasts and dermal papillae cells through analysis of ATM protein expression and SA-β-Gal activity. A hexapeptide originally isolated from *S. cerevisiae* fermentation lysates and later synthesized at high purity when applied to both intrinsically and extrinsically aged fibroblasts *in vitro* was found to reduce gene expression of ATM and SA-β-Gal proteins in a dose-dependent fashion. Application of Hexapeptide-11 reduced ATM protein expression in fibroblasts that have undergone SIPS using H_2O_2 and those grown through intrinsic aging cycles. SA-β-Gal activity was reduced in intrinsically aged fibroblasts exposed to Hexapeptide-11 as well.

A method of using UV light to induce SIPS in dermal papillae was also developed. It was shown that Hexapeptide-11 also decreased SA-β-Gal activity in UV-irradiated dermal papillae cells. This suggests that the hexapeptide can delay senescence in a second dermal cell line. The influence of Hexapeptide-11 on senescence-associated aging appears to be broadly applicable and reversible. To date, this may represent the first known peptide to possess these attributes. The impact of these findings is being further substantiated on *ex vivo* models. The results of these findings will be reported at a later date.

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