Knockdown of paraoxonase 1 expression influences the aging of human dermal microvascular endothelial cells

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The Master's Thesis submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science

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ABSTRACT

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Vascular endothelial cells participate in the regulation of both blood homeostasis and the interaction of blood with surrounding tissues. Skin is one of the most commonly studied tissues for microcirculation research due to its close correlation of cutaneous vascular function, aging and age-related cardiovascular events. To elucidate proteins that determine this correlation between endothelial cell function and aging in the vascular environment of the skin, two-dimensional electrophoresis coupled with matrix-assisted laser desorption/ionization-time of flight-mass spectrometry were carried out on the plasma samples from six donors in their 20s (young) and six donors in their 60s (old). Two-dimensional gel spots from old donors were compared with two-dimensional gel spots from young donors, and 496 paired spots were observed. Among the 496 paired spots analyzed, 45 proteins, including paraoxonase 1 (PON1) and transthyretin, were downregulated by more than two-fold, and 34 proteins, including Zinc finger protein 774, were upregulated by more than two-fold in the plasma of old donors compared to their amounts in the plasma of young donors. PON1 was selected in this study for further

characterization due to its primary location of synthesis in the liver and its association with high-density lipoprotein (HDL) metabolism after secretion into plasma. HDL-associated PON1 has been reported to reduce oxidative stress in lipoproteins and atherosclerotic lesions. To elucidate the role of PON1 on skin aging and determine how it controls cellular senescence, the characteristics of PON1 in human dermal microvascular endothelial cells (HDMECs) were determined. When the expression of endogenous PON1 was knocked-down by small interfering RNA targeting PON1, HDMECs showed characteristic features of cellular senescence such as increases in senescence-associated β-galactosidase stained cells and enlarged and flattened cell morphology. These features were found in aged HDMECs at passage 20 as well. At 48 h post-transfection, the protein expression of p16 in PON1 siRNA-treated HDMECs was higher than that in scrambled siRNA-treated HDMECs. In addition, the expressions of moesin and rho GTP dissociation inhibitor, additional age-related candidate biomarkers, were decreased by PON1 knock-down in HDMECs.

In conclusion, these results suggest that PON1 functions as an aging-related protein and plays an important role in the cellular senescence of HDMECs.

Key words: aging, paraoxonase 1, human dermal microvascular endothelial cell, plasma

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I. INTRODUCTION

Skin aging is in compliance with the aging of cells that compose the skin. To understand skin aging, studies need to examine the main cells in the skin of the aging, including keratinocytes, fibroblasts and vascular endothelial cells. Vascular endothelial cells participate in the regulation of both blood homeostasis and the interaction of blood with surrounding tissues. The normal function of these cells, which have the capacity to generate new blood vessels and maintain their integrity, is important for inflammatory reactions, wound healing, and tissue growth. However, the proliferation of endothelial cells is limited, and, in a similar fashion as other cells, they undergo irreversible growth arrest or senescence, reducing the function of the cells.[1-4] The skin is one of the most commonly studied tissues for microcirculation research due to the close correlation among cutaneous vascular function, aging and age-related cardiovascular events. Cellular senescence can be triggered or accelerated by various stresses such as telomere shortening, DNA damage, oxidative stresses, glycation or overexpression of certain oncogenes, by which cells lose the

ability to proliferate.[5-10]

Major alterations known to occur during the aging process include changes in the structure and integrity of proteins. Proteomic analysis of aged tissue and body fluids using two-dimensional electrophoresis and matrix-assisted laser/desorption ionization – time of flight mass spectrometry (MALDI-TOF MS) can provide a proteome profile that reflects the cellular aging status. Proteomics allows for the identification of protein structure, function and characteristics and can be applied to the study of aging processes.[11, 12] Previous proteomic studies have reported that several proteins related to the cytoskeleton, stress response, metabolism, protein biosynthesis, nucleoplasmic transport and DNA repair and maintenance were differentially expressed in aging populations.[2, 13, 14] However, the utility of identifying aging-related proteins as markers of aging and the determinations of their roles in the process remain unclear.

Aging-related differences in blood components could be associated with the aging of endothelial cells. To elucidate proteins that reveal this correlation between endothelial cell function and aging in the vascular environment of the skin, two-dimensional electrophoresis coupled with matrix-assisted laser desorption/ionization-time of flight-mass spectrometry were carried out on the plasma from six donors in their 20s (young) and six donors in their 60s (old). Two-dimensional gel spots from old donors were compared with two-dimensional gel spots from young donors and 496 paired spots were observed. Among the 496 paired spots, 45 proteins, including paraoxonase 1 (PON1) and transthyretin, were downregulated by more than two-fold, and 34 proteins, including Zinc finger protein 774, were upregulated by more than two-fold in the plasma of old donors compared to young donors. PON1 was selected in this study for further characterization due to its primary location of synthesis in the liver and its association with high density lipoprotein (HDL) metabolism after secretion into plasma. HDL-associated PON1 has been reported to reduce

oxidative stress in lipoproteins and atherosclerotic lesions.[15-20] The PON1 gene is activated by PPAR-γ, which increases the synthesis and release of PON1 enzyme from the liver, thereby reducing atherosclerosis. PON1 is present at the cellular level in a variety of tissues, where it can confer local protection against oxidative damage. A number of studies have shown that PON1 activity deceases with age. Reduced serum PON1 activity in humans is correlated with increased serum levels of oxidative lipids. PON1 is one of the candidate genes potentially involved in human longevity.[21-29]

Because endothelial cells are an important factor in atherosclerosis that is known to be reduced by PON1, we performed a transfection experiment using human dermal microvascular endothelial cells (HDMECs). To elucidate the role of PON1 on skin aging and how it controls cellular senescence, the characteristics of PON1 in HDMECs were determined. When the expression of endogenous PON1 was knocked-down by small interfering RNA targeting PON1, HDMECs showed characteristic features of cellular senescence, such as increases in senescence-associated β-galactosidase stained cells and enlarged and flattened cell morphology. These features were found in aged HDMECs at passage 20 as well. At 48h post-transfection, the protein expression of p16 in PON1 siRNA-treated HDMECs was higher than that in scrambled-siRNA treated HDMECs. In addition, the expressions of moesin and rho GTP dissociation inhibitor (Rho-GDI), additional age-related candidate biomarkers, were decreased by PON1 knock-down in HDMECs.

Thus, the aim of this study was to determine a novel protein that functions as an aging-related protein and to characterize the functional role of the selected PON1 protein in the cellular senescence of HDEMCs.

II. MATERIALS AND METHODS

1. HDMEC culture

Human dermal microvascular endothelial cells were purchased from Lonza (Walkersville, MD). These cells were prepared and treated in a tissue incubator using EGM-2MV medium (Lonza) containing human epidermal growth factor, hydrocortisone, gentamicin, amphotericin-B, 5% fetal bovine serum, vascular endothelial growth factor, human fibroblast growth factor-B, R_3 - insulin growth factor-1 and ascorbic acid. Cultures were maintained from passage 3 to passage 20 at 37℃ in a humidified incubator with 5% CO_2. The growth medium was changed the day after seeding and every other day thereafter.

2. Proteome analysis

A. Isolation of human plasma

Peripheral blood samples were obtained from six healthy donors in their 20s (referred to hereafter as 'young' donors) and six healthy donors in their 60s (referred to hereafter as 'old' donors). This study was approved by the Institutional Review Board of Severance Hospital, and informed consent was obtained from all participants. Plasma was isolated from the blood samples by the Ficoll gradient using Ficoll-Plaque Plus (GE HealthCare, Uppsala, Sweden) according to the instructions of the manufacturer.

B. Depletion of high abundance proteins by affinity chromatography

Plasma samples were processed to deplete high abundance proteins using the multiple affinity removal system column which contains antibodies to albumin, immunoglobulin G (IgG), antitrypsin, immunoglobulin A (IgA), transferrin, and haptoglobin linked to the gel matrix and the reagent kit from Agilent

Technologies (Palo Alto, CA). Cibacron blue affinity chromatography of plasma was performed following the method of Travis et al.[30] Briefly, 1ml of plasma was applied onto a column of Blue Sepharose CL6B (GE HealthCare, Upssala, Sweden) that was pre-equilibrated with 0.05M Tris-HCL/0.05M NaCl at pH 8.0. The column was washed with the same buffer to collect unbound proteins. The bound proteins were desorbed from the column with 0.05M Tris HCl/0.5M KCl at pH 8.0. For the immunoaffinity chromatography, 90 mL of 5-times diluted plasma was loaded onto the multiple affinity removal column (4.6 x 50 mm, Agilent Technologies, Palo Alto, CA), and the flow-through fractions and bound fractions were collected according to the manufacturer's protocol. The collected protein fractions were analyzed by separation on 12% SDS-PAGE and staining with Coomassie blue G-250.

C. Two-dimensional gel electrophoresis and image analysis

The plasma was mixed with rehydration buffer (8M urea, 2% CHAPS, 1% IPG buffer, and 50 nM DTT) for 2h at room temperature and then electrofocused for a total of 56,000 Vh in 18 cm DryStryp (pH 3-10) with the Amercham IPGphore (both from Amersham Biosciences, Piscataway, NJ). After electrofocusing, the gel was equilibrated for 15 min with a buffer containing 10 mg/ml dithiothreitol (DTT), 8M urea, 2% (w/v) SDS and 30% (v/v) glycerol and then for another 15 min with a second buffer containing the same ingredients, with the exception that DTT was replaced with 25 mg/ml iodoacetamide. Two-dimensional SDS-PAGE was performed on a 12% polyacrylamide gel using a PROTEIN*w*II multi-cell (Bio-Rad, Hercules, CA). The protein spots were visualized by Coomassie Blue G-250 staining. The images of the protein spots on the stained 2D-gels were analyzed by PDQuest (version 7.1, Bio-Rad).

D. Mass spectrometry

Protein spots were analyzed by matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF MS) using M@LDITM-LR (Micromass, Berverly, MA) after tryptic digestion. The raw mass spectra were analyzed to obtain a list of monoisotopic peaks using the ProteinLynx Global SERVER 2.0 program from Waters (http://www.waters.com). Protein candidates were identified using the Swiss-Prot database with a peptide mass error tolerance of 50 ppm. When the search results were ambiguous, the remaining tryptic mixture, after MALDI-TOF mass spectrometry, was subjected to ESL-tandem mass spectrometry using Q-TOP API US (Micromass).

3. Transfection of PON1 siRNA

To suppress PON1 expression, HDMECs were transfected with 30 nM stealth RNAi™ siRNA (HSS108253; #1, HSS108254; #2, HSS108255; #3) (Invitrogen, Carlsbad, CA) using RNAiMAX lipofectamine transfection reagent (Invitrogen). One day before transfection, HDMECs were seeded at a density of 1×10^5 cells on 60 mm plates in growth medium without antibiotics. At 30-50% confluence, cells were transfected with PON1 siRNA or scrambled siRNA. The medium was changed after 5 hours. The cells were incubated for 24-48 hours at 37℃ in a humidified incubator with 5% CO_2.

4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from HDMECs using an RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's recommendations. cDNA was synthesized from 1 μg of extracted total RNA using AccuPower RT PreMix (Bioneer, Seoul, Korea) and a PCR system 2700 (Applied Biosystems, Foster

City, CA). Reverse transcriptase-polymerase chain reaction amplification was carried out using 1 μg of cDNA as the template in 20 μl of 20 pmole primer with AccuPower PCR PreMix (Bioneer, Seoul, Korea). The PON1 primers were 5'-TATTGTTGCTGTGGGACCTGAG-3' (forward) and 5'-CACGCTAA ACCCAAATACATCTC-3' (reverse). The GAPDH primers were 5'-CATTGC CCTCAATGACCACT-3' (forward) and 5'-TCCTTGGAGGCCATGTAGAC-3'. The products of reverse transcription reactions were denatured for 1 min at 94℃, followed by 30 cycles of amplification (1 min of denaturation at 94℃, 1 min of annealing at 63℃, and 1 min of elongation at 72℃), and extension at 72℃ for 10 min. The PCR fragments were visualized on 2% agarose gels containing ethidium bromide.

5. Western blot analysis

Total protein was isolated from HDMECs, and 20 μg of protein was resolved by 12% SDS-PAGE. These proteins were then transferred to an NC membrane according to standard procedures. The membrane was blocked with 5% v/v skim milk in a TBS-T buffer (TBS with 0.05% w/v Tween-20) and reacted with the PON1 (Abcam, Cambridge, MA), p16, moesin, and Rho-GDI (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies (diluted 1:1000 in 2% v/v skim milk in the TBS-T buffer) for 12h on a rocking platform at 4℃. The membrane was then washed three times for 15 min with the TBS-T buffer and incubated for 1h with 2% skim milk in the TBS-T buffer containing horseradish peroxidase-conjugated anti-rabbit, anti-mouse, and anti-goat antibody (diluted to 1:2000). The hybridized membrane was washed in TBS-T buffer and signals were visualized using a chemiluminescent ECL detection kit (Santa Cruz Biotechnology, Santa Cruz, CA). GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a control for protein loading.

6. Senescence-associated β-galactosidase (SA-β-gal) staining

SA-β-gal activity in cells was measured using a cell senescence assay kit (ScienCell Research Labs, San Diego, CA). The cells were fixed by incubating with 2 ml of working fixing solution for 5 min at room temperature. The fixed cells were then washed three times with phosphate buffered saline (PBS) and stained by incubating with working staining solution for 12h, protected from light, at 37℃. After incubation, the cells were washed three times with PBS and then kept in PBS at 4℃. The percentage of blue cells observed under a light microscope was calculated.

7. Phase-contrast microscopic examination

Phase-contrast microscopy (Olympus, Tokyo, Japan) was used to confirm the senescence of HDMECs and to examine the morphologic changes of transfected HDMECs.

8. MTT [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide] assay

HDMECs were seeded at 2 x 10^4 cells in 96-well plates and transfected with PON1siRNA. Cell proliferation was estimated with colorimetiric assay using 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) staining. Cell viability was assessed by measuring absorbance at 570 nm with a molecular devices spectra Max 340 microplate reader. Experiments were repeated three times, and data are presented as mean ± standard deviation (SD).

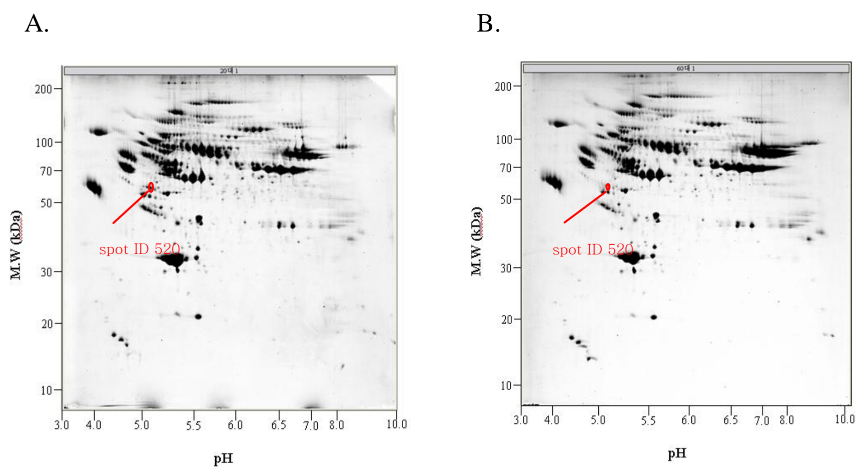
9. Immunohistochemical staining

Stored tissues were cryosectioned to 8 μm, attached to silane-coated slides (Muto Pure Chemicals, Tokyo, Japan), fixed in acetone for 15 min and washed with PBS. Immunohistochemical staining was performed using the Histostain®-Plus Bulk Kit (Invitrogen, Camarillo, CA). To suppress nonspecific binding, the samples were placed in a serum blocking solution for 1h. Mouse anti-human PON1 monoclonal antibodies (Abcam, Cambridge, MA) diluted 1:100 with PBS were used and incubated at 4℃. The biotinylated secondary antibody was incubated for 1h at room temperature. The enzyme conjugate was incubated for 1h and 3-amino-9-ethylcarbazole (AEC) solution was used as the substrate. Sections were counterstained with hematoxylin before being examined under a light microscope.

Ⅲ. RESULTS

1. Identification of proteins differentially expressed in the plasma of young and old donors

To discover aging-related proteins in plasma, we used 2-DE coupled with LC-MS/MS to analyze plasma from six donors in their 20s and six donors in their 60s. Approximately 496 paired spots and five non-paired spots were detected on each 2-DE gel. Among the 496 paired spots, 45 proteins, including paraoxonase1 (PON1) and transthyretin, were downregulated more than two-fold, and 34 proteins, including Zinc finger protein 774, were upregulated in plasma of old donors by more than two-fold compared with the same proteins in plasma of young donors (Fig. 1). Information on protein function was retrieved from Swiss-Prot and GenBank databases and relevant references therein. Proteins that changed by more than two-fold were identified by MS or MS/MS (Table. 1).



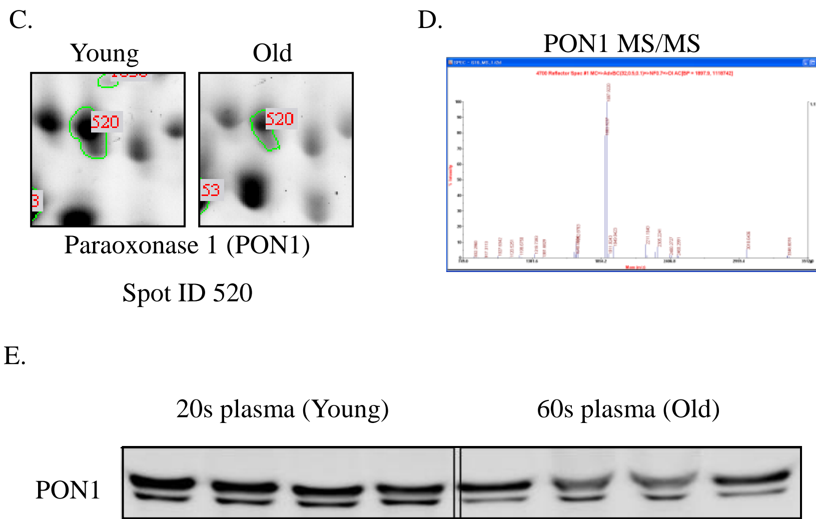


Figure 1. Representative 2-DE images of plasma from young donors (A) and old donors (B). (C) Comparative analysis of differentially expressed protein spots. (D) MS/MS spectra of PON1. Sequences of the selected peptides and their m/z values are denoted at the top of each tandem mass spectra. (E) Western blot analysis of PON1 protein in plasma from young and old healthy donors.

Table1. Proteins undergoing age-related changes as identified by MS or MS/MS

Spot no.	Protein	Accession no. NCBInr	Ratio (~fold)*	MW	PI	Mascot score	Matched peptide no.	Sequence coverage (%)
520	serum paraoxonase/ arylesterase1 precursor	19923106	-2.1	39877	5.08	119	13	51
545	actin, cytoplasmic 2	4501887	-4.6	42108	5.31	96	16	48
553	Chain B, Human Zinc-Alpha-2-Glycoprotein	4699583	-2.5	31854	5.7	71	9	40

620	complement cytolysis inhibitor precursor	180620	-2	52330	5.89	74	10	25
676	Chain A, Tertiary Structures Of Three Amyloidogenic Transthyretin Variants And Implications For Amy	2098257	-2.2	13886	5.35	140	11	92
768	hypothetical protein COPCOM_01102	226323330	-2	40452	4.9	96	12	31
1087	Chain X, Crystal Structure Of Catalytic Subunit Of	224510642	-2	40072	8.7	67	10	33
447	RecName: Full=Zinc finger protein 774	74758196	4.3	55032	8.71	68	10	30
880	unnamed protein product	194378712	2.4	46077	8.96	79	9	29
895	PREDICTED: mediator of RNA polymerase II transcription subunit 29 isoform 2	297277009	2.2	14965	5.84	72	6	28

*Ratio (~fold): old donors/young donors

2. Validation of protein expression level for PON1 in HDMECs

To investigate PON1 expression in HDMECs, we performed Western blot analysis. Mouse liver tissue and HepG2 cell lysate were used as positive controls. Quantitation values were determined relative to HDMECs at passage 8 control. The expression of PON1 protein decreased in HDMECs after passage 20 compared with early passage HDMECs at passage 8.

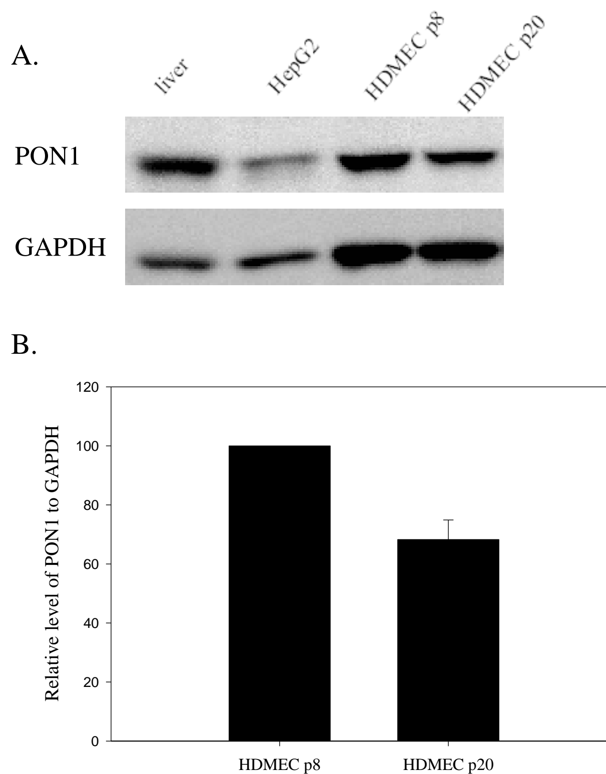


Figure 2. Expression of PON1 protein in HDMECs was analyzed by Western blotting (A) and normalized to GAPDH expression (B). Quantitative values were determined relative to HDMECs at passage 8 control. The expression of PON1 protein was reduced in aged HDMECs at passage 20.

3. Validation of PON1 knockdown by semi-quantitative RT-PCR

We transfected HDMECs at passage 8 with three different duplex siRNAs (Table 2) exhibiting the best likelihood of gene silencing and analyzed the effect at 48h post-transfection. To analyze whether PON1 protein was downregulated at the transcriptional level, we performed mRNA expression analysis by semi-quantitative RT-PCR. mRNA levels of PON1 were dramatically reduced in HDMECs treated with PON1 siRNA #2 (Fig. 3).

Table 2. Duplex RNA sequences for PON1 siRNA

siRNA	Duplex RNA sequences
PON1 siRNA #1	5'-AAGACUGGUGGUUCCUGAAGAGUGC-3'
	5'-GCACUCUUCAGGAACCACCAGUCUU-3'
PON1 siRNA #2	5'-GCUCUGGAUUAAAGUAUCCUGGAAU-3
	5'-AUUCCAGGAUACUUUAAUCCAGAGC-3'
PON1 siRNA #3	5'-GCGUGGUCGUAUGUUGUCUACUAUA-3'
	5'-UAUAGUAGACAACAUACGACCACGC-3'

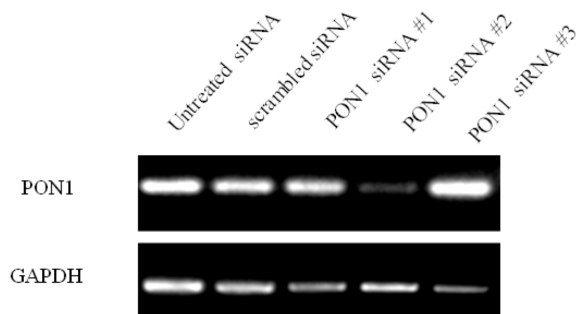


Figure 3. PON1 mRNA expression in HDMECs at passage 8 treated with PON1 siRNA #1, #2 and #3. The levels of PON1 mRNA expression were studied by semi-qRT-PCR. Quantitative data were normalized to the intensity of GAPDH. mRNA levels of PON1 were dramatically reduced in HDMECs treated with PON1 siRNA #2.

4. Confirmation of PON1 knockdown

To analyze whether PON1 protein was downregulated at the translational level, we performed protein expression analysis by Western blotting. Protein levels of PON1 were reduced in HDMECs treated with PON1 siRNA #2 at 24h and 48h post-transfection. The average fold difference of PON1 siRNA-treated cells relative to scrambled siRNA-treated cells using data from all three independent experiments is presented graphically .

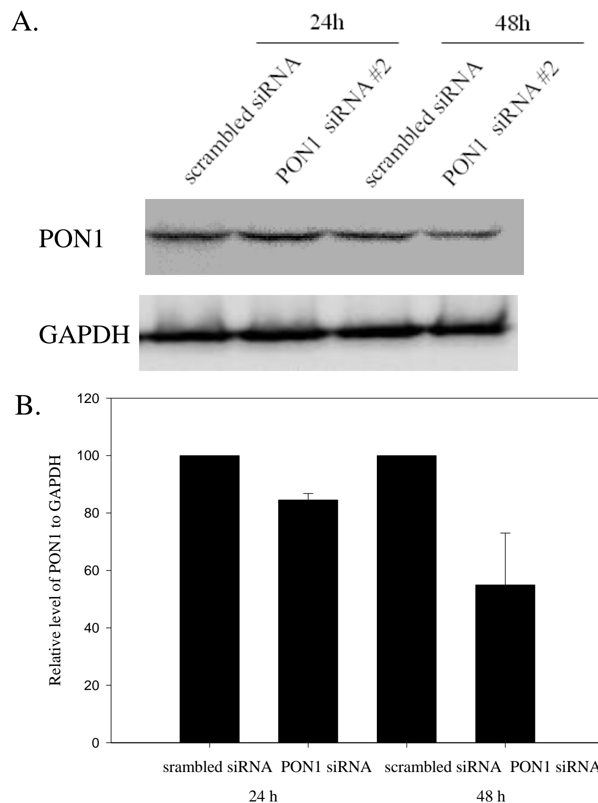


Figure 4. The expression of PON1 protein was analyzed by Western blotting (A) and normalized to GAPDH expression (B). At 24h and 48h post-transfection, the expression of PON1 protein was reduced in PON1 siRNA #2-treated HDMECs.

5. Comparison of the aging level of HDMECs by the SA-β-gal staining

To evaluate the influence of PON1 knockdown on aging of HDMECs, we measured cellular senescence by SA-β-gal staining. Cell senescent morphology, such as an enlarged and flattened shape with an increased diameter, was observed in aged HDMECs at passage 20 and in PON1 siRNA #2-treated HDMECs at 48h post-transfection. The number of cells positive for SA-β-gal staining increased compared to early passage HDMECs at passage 9 and in scrambled siRNA-treated HDMECs.

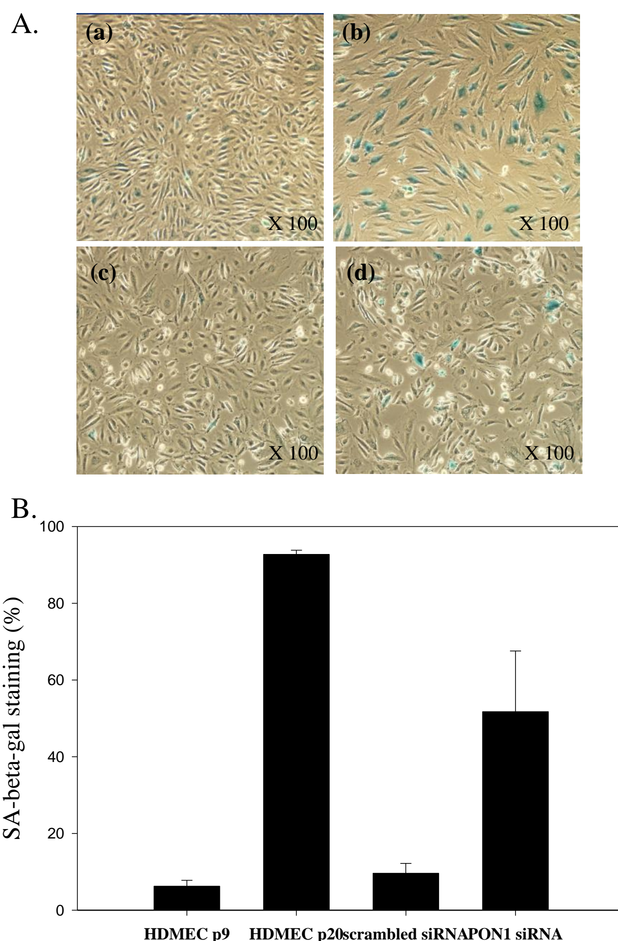


Figure 5. Changes due to aging were observed through SA-β-gal staining (A). Untreated siRNA HDMECs at passage 9 (a) and scrambled siRNA-treated HDMECs (c) were not strongly stained compared with the aged HDMECs at passage 20 (b) and PON1 siRNA-treated HDMECs at passage 9 (d). Percentage of SA-β-gal staining-positive cells (B).

6. Knockdown of PON1 reduces cell viability

To evaluate the influence of PON1 knockdown on aging of HDMECs, we measured cell viability by MTT assay. The MTT assay was performed with HDMECs at passage 9 to compare cell viability. Compared with scrambled siRNA-treated HDMECs, cell viability was decreased in PON1 siRNA #2-treated HDMECs.

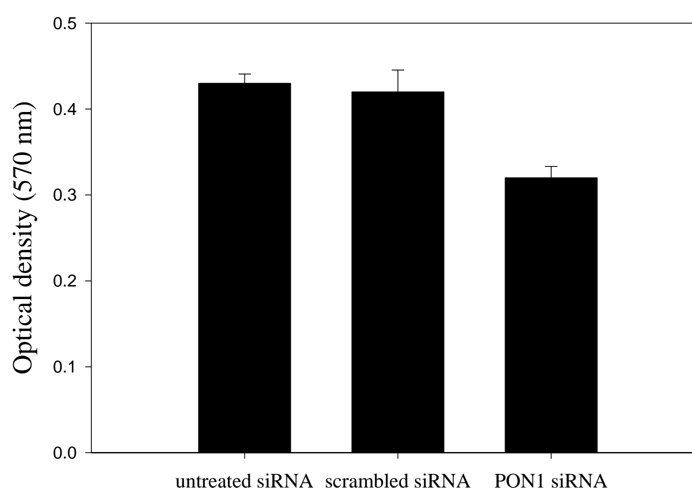


Figure 6. Cell viability was measured by MTT assay in PON1 knock-down HDMECs. At 48h post-transfection, cell viability was decreased more in PON1 siRNA #2-treated HDMECs than scrambled siRNA-treated HDMECs and untreated siRNA HDMECs.

7. Expression of p16 protein in PON1 knockdown HDMECs

The expression of p16 in PON1 knockdown HDMECs was observed using Western blotting. At 48h post-transfection, the protein expression of p16 in PON1 siRNA-treated HDMECs was higher than in scrambled siRNA-treated HDMECs.

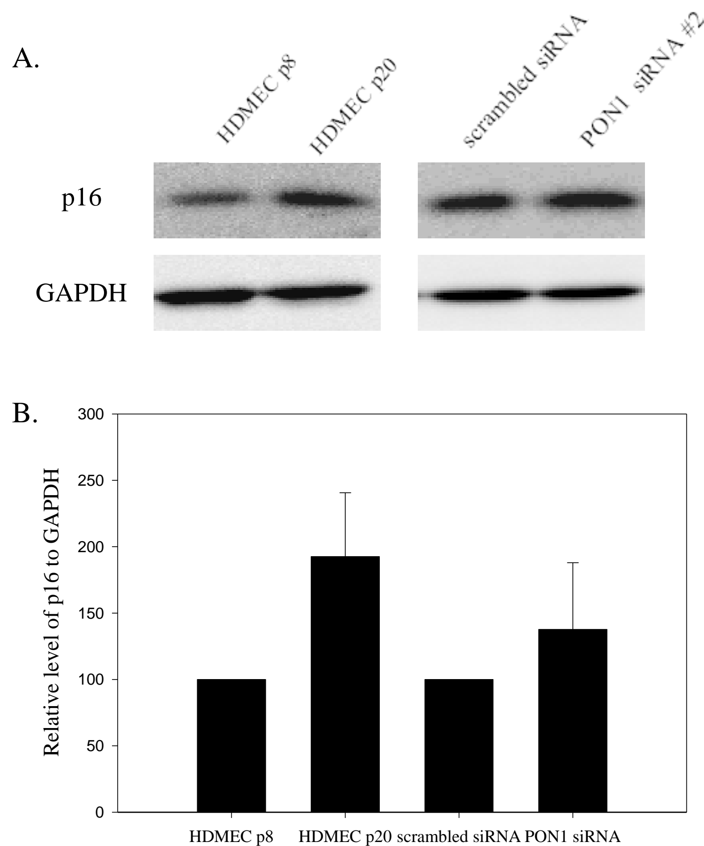


Figure 7. The expression of p16 was analyzed by Western blot (A) and normalized to GAPDH expression (B). The expression of p16 protein was increased in aged HDMECs (p20) and PON1 siRNA #2-treated HDMECs compared to early passage HDMECs (p8) and scrambled siRNA-treated HDMECs.

8. Correlation between aging-related proteins of HDMECs and PON1

To investigate the correlation between the aging-related proteins of HDMECs and PON1, we performed protein expression analysis by Western blotting. At 48h post-transfection, the expressions of moesin and Rho-GDI were decreased more in PON1 siRNA#2-treated HDMECs than in scrambled siRNA-treated HDMECs.

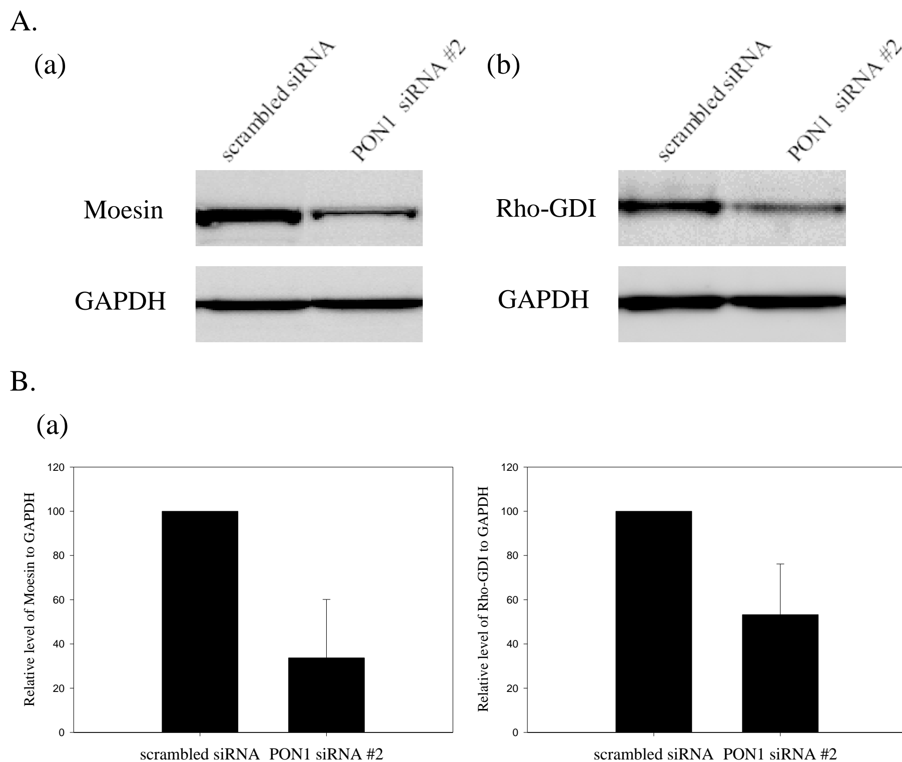


Figure 8. The expression of previously developed candidate aging biomarkers, including moesin and Rho-GDI were analyzed by Western blot in PON1 knock-down HDMECs (A). At 48h post-transfection, the expressions of moesin (a) and Rho-GDI (b) protein were decreased in PON1 knock-down HDMECs. Relative quantitation values were determined relative to scrambled

siRNA-treated control cells, and the ratio of PON1:GAPDH bands were calculated (B).

9. Immunohistochemical staining of PON1 in skin tissues

The expression of PON1 in young and old skin tissues was observed by immunohistochemical staining. Specifically, immunohistochemical staining of PON1 was performed with samples of skin from 34-year-old (young) and 80-year-old (old) donors. Young skin tissue was strongly stained with PON1 antibodies, whereas old skin tissue was only weakly positive.

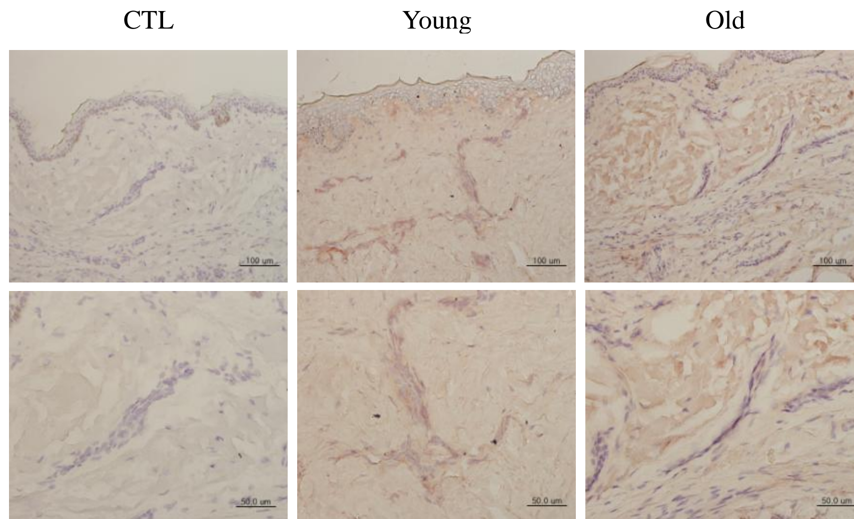


Figure 9. The expression of PON1 in young and old skin tissues was observed using immunohistochemical staining. Samples were incubated with anti-human PON1 overnight at 4℃. Young skin tissue was strongly stained with PON1 antibodies, whereas old skin tissue was only weakly positive.

Ⅳ. DISSCUSSION

PON1 activity was found to be significantly decreased with age, though its arylesterase activity and concentration in the serum exhibited no significant changes. Decreased PON1 activity may contribute to the increased susceptibility of HDL to oxidation modification that is observed with aging.[31-33] The function of organs decreases with cellular senescence. Indeed, the replicative senescence of cells and changes in the function and morphology of cells may be key for studying the pathophysiology of aging.[34]

We depleted PON1 in HDMECs using siRNA to determine its effects on senescence and proliferation. RNA interference (RNAi) has become a widely used technique for silencing gene expression. Successful application of RNAi in mammalian cells depends on knocking-down targeted transcripts by the effective intracellular delivery of small interfering RNAs (siRNA). Chemically synthesized siRNAs are incorporated into the RNA-induced silencing complex, and the delivery results in sequence-specific silencing of the expression of the corresponding gene.[35-37] Using this effective technique, we attempted to elucidate the role of PON1 in the aging of endothelial cells and to understand the correlation between aging-related proteins of HDMECs and PON1.

Senescence-associated β-galactosidase (SA-β-gal) is a widely used biochemical marker for assessing senescence in cultured cells.[38, 39] Our study showed that there were significant changes in SA-β-gal staining in PON1 siRNA-treated HDMECs and late passage HDMECs. These results imply that transfection of PON1 siRNA affected the senescence of HDMECs.

Senescence-associated cell cycle arrest becomes irreversible and is no longer revoked by subsequent inactivation of pRb, suggesting that the $p16^{INK4a}$/ Rb-pathway activates an alternative mechanism to irreversibly block the cell cycle in senescent human cells. The induction of $p16^{INK4a}$ collaborates with

p21$^{Cip1/Waf1/Sdi1}$ to prevent the phosphorylation of pRb, leading to a stable G1 arrest in senescent cells. Consequently, p16 expression induces cell cycle arrest.[40-42] Since p16 expression is directly correlated with the chronological aging of human skin and is a biomarker for human aging, we evaluated p16 protein expression analysis by Western blotting. The level of p16 expression was increased in both the PON1 knock-down HDMECs and aged HDMECs at passage 20. At the cellular level, aging is associated with a decrease in the proliferative ability of most cell types.[43] We measured proliferation rates by MTT assay. As a result, the cell viability decreased more in PON1 knock-down HDMECs than in scrambled siRNA-treated HDMECs and untreated siRNA HDMECs. Based on these outcomes, we assumed that PON1 knock-down might control not only cell morphology, but also the progress of cellular senescence.

Previously, we performed a high-throughput screening of a variety of aging-related proteins as determined from proteomics analysis. Based on these results, we identified several major candidates for aging including moesin and Rho GTP dissociation inhibitor using bioinformatics. Moesin was the most promising protein for aging because its expression was the most significantly changed after aging and was recovered rapidly by anti-aging agents. The expression of moesin was found to be decreased in elderly HDMECs, and an association between moesin and senescence has been suggested.[1, 3, 13] Moesin is a member of the ezrin/ radixin/ moesin (ERM) cytoskeletal protein family, which connects cell membrane proteins and actin located underneath cell membranes. Moesin has a signal transduction function in addition to its function of remodeling the cytoskeleton.[44] Rho GTP dissociation inhibitor (Rho-GDI) controls the GDP/ GTP cycle and the formation of the cytoskeleton. The expression of Rho-GDI was decreased in HDMECs at late passage, whereas the expression was increased in the cytoplasm of HDMECs treated with anti-aging agents.[3]

To investigate the correlation between aging-related proteins of HDMECs and PON1, we performed protein expression analysis by Western blotting. At 48h post-transfection, the expressions of moesin and Rho-GDI protein decreased in PON1 knock-down HDMECs. Thus, the results of this study suggest the presence of aging-related PON1 protein in the plasma as well as a functional role of PON1 in cellular senescence in HDMECs. Our results also showed that the knock-down of PON1 expression triggered the aging of HDMECs. Taken together, these results suggest that this fundamental discovery may be useful as a basis for understanding the mechanism of aging and age-related diseases.

Ⅴ. CONCLUSION

This study was carried out to elucidate the function of a novel protein as an aging-related biomarker of endothelial cells and to determine the function of PON1 in HDMECs. We knocked-down the expression of endogenous PON1 in HDMECs using siRNA and determined the effects on senescence and cell proliferation. Knock-down of PON1 decreased cell viability, and the expressions of moesin and Rho-GDI in HDMECs. These findings were similarly observed in high passage number HDMECs.

These results suggest that PON1 influences cell senescence and could be a novel biomarker for cell senescence as well as for evaluating the level of aging. The effect of PON1 over-expression and its physiological relevance should be the focus of further studies.

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ABSTRACT (IN KOREAN)

Paraoxonase 1의 발현 감소가 혈관내피세포의 노화에 미치는 영향

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혈관내피세포는 혈액과 조직 사이에 있는 세포로서 세포와 그 주변 혈장의 상호작용을 통해 항상성을 유지하고, 새로운 혈관을 생성시키는 능력을 가지고 있다. 피부 노화는 피부를 구성하고 있는 세포들의 노화에 의한 것이며, 피부 노화를 이해하기 위해서는 피부조직의 주요 구성 세포인 각질형성세포, 섬유아세포, 혈관내피세포 등의 세포 노화에 대한 연구가 필요하다. 특히, 혈관내피세포의 기능 이상은 노화와 여러 노화 관련 질병들과 연관성이 있으며 피부 혈관 기능은 심장 혈관과도 관련되어 있다. 그러므로, 혈관내피세포의 기능 연구는 피부 노화에서 매우 중요한 역할을 할 것이다.

노화에 영향을 주는 물질을 규명하기 위해 프로테오믹스 기법을 이용하여 정상인 20대, 60대 군의 혈장에서 차이가 나는 단백질을 비교해 본 결과, paraoxonase 1 (PON1)과 transthyretin을 포함한 45개의 단백질이 20대 군에 비해 60대 군의 혈장에서 2배 이상 감소하였고, zinc finger protein 774를 포함한 34개의 단백질이 60대 군의 혈장에서 2배 이상 증가되었다. 이 중에 PON1은 혈액 내에서 HDL

(high density lipoprotein)과 결합하는 효소로서 간에서 합성되어 혈액으로 분비되고, 항 산화 효소로써 LDL (low density lipoprotein)의 산화를 억제하는 역할을 한다. PON1의 활성이 떨어지면 산화적 스트레스와 관련이 있는 질병인 죽상동맥경화증(atherosclerosis)을 포함한 여러 혈관 질환들과 연관이 있다고 알려져 있으며, PON1 locus에서의 유전적 변이성 (genetic variability)은 특히 고령의 사람들의 생존에 영향을 줄 것으로 보고되고 있다.

따라서 피부 노화와의 상관성을 보기 위해 PON1의 발현 감소가 혈관내피세포의 노화에 미치는 영향을 살펴보고자 하였다. 본 연구에서는 PON1의 기능 검증을 위해 siRNA를 이용하여 혈관내피세포에 PON1을 knockdown시킨 후, 세포의 노화 정도를 관찰하였다. 먼저 PON1 siRNA transfection 후, RT-PCR 과 Western blot으로 혈관내피세포에서의 PON1 발현감소를 확인하였다. PON1의 발현감소가 세포 노화에 미치는 영향을 알아보기 위해 SA-β-gal 염색을 시행한 결과 배양 초기의 HDMECs과 동일 passage인 scrambled siRNA를 처리한 HDMECs과 비교하여, 노화된 배양말기 HDMECs과 PON1의 발현이 감소된 HDMECs에서 염색된 노화 세포가 많이 관찰되었다. 따라서 PON1의 발현이 감소된 혈관내피세포에서는 일반적인 혈관내피세포와 비교할 때 노화의 진행 속도가 증가하였음을 알 수 있었다. 세포의 노화와 세포 주기는 밀접한 관련이 있는데 혈관내피세포에서 노화가 일어나면 세포 주기 중 G1기가 중단되고, 노화가 진행된 혈관내피세포에서는 p16 단백이 증가되어 있는데, PON1의 발현이 감소된 HDMECs에서 p16의 발현량을 확인해 본 결과, 배양 초기의 HDMECs과 동일 passage인 scrambled siRNA를 처리한 HDMECs과 비교하여, 노화된 배양말기 HDMECs과 PON1의 발현이 감소된 HDMECs에서 p16 단백이 증가하였다. 이러한 결과들을 볼 때, PON1의 발현 감소가 세포의 형태뿐만 아니라 기능적으로 세포 노화의 진행도 억제하는 역할을 할 것으로 생각된다.

또한, 이전 연구에서 발견된 노화 관련 후보 biomarker인 moesin과 Rho-GDI의 발현양을 Western blot으로 관찰한 결과, PON1 knockdown HDMECs에서 moesin과 Rho-GDI 단백이 감소한 것으로 나타났다. 본 연구에서는 siRNA를 이용해 PON1을 knockdown시킨 혈관내피세포의 노화의 정도를 관찰 함으로써 PON1이 피부노화에서 중요한 역할을 할 가능성을 보여주었다.

핵심되는 말: 노화, paraoxonase 1, 혈관내피세포, 혈장