TP53 Tumor-suppressor Gene Plays a Key Role in IGF1 Signaling Pathway Related to the Aging of Human Melanocytes

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Abstract. *Background/Aim:* The insulin-like growth factor 1 (IGF1) signaling pathway as an aging mechanism related to p53 in human melanogenesis remains unclear. The aim of this study was to investigate the relationship between p53 and IGF1 signaling pathway in young, senescent and H₂O₂-treated cells. *Materials and Methods:* The protein and gene expression in young, senescent and H₂O₂-treated cells were analyzed using western blot and reverse transcription polymerase chain reaction (RT-PCR) assays, respectively. *Results:* The expression levels of (phosphoinositide 3-kinases) PI3K, v-akt murine thymoma viral oncogene homolog 1 (AKT1), mammalian target of rapamycin, β -catenin (CTNNB1), acetylated p53 (ac-p53), p53 and p-p21 proteins, related to IGF1 and p53 signaling pathways, were higher in senescent and H₂O₂-treated cells than those of young cells. Furthermore, AKT reduced melanogenesis through microphthalmia-associated transcription factor (MITF) inactivation by the inhibition of CTNNB1. The gene expression levels of PI3K, TP53 and catalase (CAT) in senescent and H₂O₂-treated cells were increased compared to young cells. *Conclusion:* p53 protein plays a key role in the aging of melanocytes via IGF1 signaling pathways.

The insulin-like growth factor 1 (IGF1) signaling pathway, which is known to be an aging mechanism, is associated with melanogenesis and also affects the antioxidant activity of H₂O₂ (1). IGF1 produced by growth hormone in the liver is responsible for controlling growth hormone action and metabolism (2). The IGF1 signaling pathway is activated by IGF1 binding to insulin-like growth factor 1 receptor (IGF1R), resulting in phosphorylation of the insulin receptor substrate (IRS). Activated IGF1R activates mammalian target

of rapamycin (mTOR) via the phosphoinositide-3 kinase (PI3K)/v-akt murine thymoma viral oncogene homolog (AKT) pathway (3). The IGF1 signaling pathway is associated with p53, which regulates cell longevity. p53 regulates cell survival by regulating transcriptional function and the cell cycle, repairing DNA damage, and inducing apoptosis. Irreversible DNA damage caused by ROS induces p53 activation (4). The activity of p53 is regulated by sirtuin 1 (SIRT1) which induces protein deacetylation, and p300 which carries acetyl groups. The deacetylation of p53 by SIRT1 inhibits apoptosis (5). In addition, p21, which is induced by p53, is involved in cell-cycle arrest (6). These findings suggest that H₂O₂ is closely related to melanocyte senescence, but more research is needed to determine whether H₂O₂ inhibits melanin production and causes hair graying. Therefore, based on the hypothesis that H₂O₂ induces the aging of melanocytes in the graying of hair via IGF1 and p53 signaling pathways, the present study investigated the aging mechanism of melanogenesis using senescent and H₂O₂-treated young melanocytes.

Materials and Methods

Materials. HEMn cells and dermal cell basal medium (DCBM) were purchased from the American Type Culture Collection (Manassas, VA, USA). Trypsin-EDTA, penicillin/streptomycin/amphotericin (10000 U/ml, 10000 g/ml, and 2500 g/ml, respectively) and fetal bovine serum (FBS) were obtained from Gibco BRL, Life Technologies (NY, USA). Citric acid, agarose, and other materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell lines and culture. Cell lines were grown separately as monolayers in a humidified atmosphere with 5% CO₂ at 37°C using appropriate media supplemented with 5% fetal bovine serum, 2 mM glutamine and 100 g/ml penicillin-streptomycin. DCBM was used as the culture medium for HEMn cell line. Cells were passaged three times a week by treating with trypsin-EDTA. Young cells with a population-doubling of 5 were prepared by subculture with HEMn cells purchased from ATCC. The senescent cells with a population-doubling of 30 were prepared by continuous subculture with young cells. Aged cells were prepared by treatment of young cells with H₂O₂ at 250 μ M for 24 h.

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Western blot analysis. Western blot analysis was performed according to standard procedures. Young, senescent and H₂O₂-treated cells were lysed with RIPA lysis buffer (Sigma Chemical Co., St. Louis, MO, USA). Cell lysates were resolved on a 4–20% Novex® gradient gel (Invitrogen, Carlsbad, CA, USA), electrotransferred onto a nitrocellulose membrane and blocked with 10% skimmed milk. Primary antibodies to PI3K, AKT1, p-mTOR, mTOR, forkhead box protein O1 (FOXO1), phosphorylated forkhead box protein O1 (p-FOXO1), p53, acetylated p53 (ac-p53), p-p21, extracellular-signal-regulated kinase 1 (ERK1) and phospholated c-Jun N-terminal kinase (p-JNK) β -Catenin and β -actin and their secondary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used to detect respective proteins using a chemiluminescent ECL assay kit (Amersham Pharmacia Biosciences, NJ, USA) according to the manufacturer's instructions. Protein bands were visualized using AlphaEase® gel image analysis software (Alpha Innotech, Hayward CA, USA).

Analyses of gene expression using reverse transcription polymerase chain reaction (RT-PCR). The mRNA was extracted from young and senescent HEMn cells. For that, cells were lysed with Trizol® and centrifuged at 12,000 $\times$ g for 15 min at 25°C following the addition of chloroform. Isopropanol was added to the supernatant at a 1:1 ratio and the RNA pellet was obtained following centrifugation. After washing with ethanol, extracted RNA was solubilized in diethylpyrocarbonate-treated RNase-free water and quantified by measuring the absorbance at 260 nm using the GENios® microplate reader (Tecan Group Ltd., Mannedorf, Switzerland) in a master mix containing 1X reverse transcriptase (RT) buffer, 1 mM dNTPs, 500 ng of oligo (dT) 15 primers, 140 U of murine Moloney leukemia virus (MMLV) reverse transcriptase and 40 U of RNase inhibitor, for 45 min at 42°C. Polymerase chain reaction (PCR) was carried out in an automatic Whatman thermocycler (Biometra Trio, Maidstone, Kent, UK) to amplify *PI3K*, *SIRT1*, *TP53*, *FOXO1*, catalase (*CAT*), glutathione reductase (*GR*) and glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) mRNA. Primer sequences used to amplify the desired cDNA were as follows: *PI3K* (forward primer: ~5'-GGTCTAAGCGGAATTTA T-3' reverse primer: 5'-GGTCTAAGCGGAATTTAT-3'), *SIRT1* (forward primer: ~5'-TCATTGTTATTGGGCTCTCC-3' reverse primer: 5'-ACAGCAAAGTTTGGCATATT-3'), *TP53* (forward primer: ~5'-AATTTGCGTGTGGAGTATTT-3' reverse primer: 5'-CTGGAGTCTTCCAGTG TGAT-3'), *FOXO1* (forward primer: ~5'-CTGGACTCTTGAAGGAGTT G-3' reverse primer: 5'-CTGGGATTCATCATTTTGT-3'), catalase (*CAT*); forward primer: ~5'-CCAATATTCTGGAGAAGTGC-3' reverse primer: 5'-GGA CCTCAGTGAAGTTCTTG-3'), *GR* and *G3PDH* (forward primer: ~5'-TCAGTGGGAAAAAGTACACC-3' reverse primer: 5'-ATCTC CACAGCAATGTAACC-3') PCR products electrophoresed on 2% agarose gels were visualized by Midori green (Nippon Genetics Europe, Dürren, Germany) staining and quantified using Davinch-Chemi™ (Corebiosystem, Seoul, Republic of Korea).

Statistics. Data were analyzed using Student's *t*-test for paired data. Data are given as mean values $\pm$ S.D. from three independent experiments, with differences accepted at *p* < 0.05.

Results

Effect of H₂O₂ on mitogen-activated protein kinases (MAPKs), p53 and IGF1 signaling pathway in human melanocytes. The

expressions of proteins such as ERK1 and p-JNK related to melanin synthesis were investigated with western blot analysis in young, senescent and H₂O₂-treated HEMn cells. As shown Figure 1A, the expression level of ERK1 in senescent cells was reduced by 30% compared to that of young cells, and that of aged cells by 18%. Similarly, the expression of p-JNK was reduced by 30% and 21%, respectively. Therefore, these results indicate that H₂O₂ modulated expression of MAPKs related to melanin synthesis.

The expression of p53, ac-p53, p-p21 was investigated with western blot analysis in young, senescent and H₂O₂-treated HEMn cells. As shown Figure 1B, the expression of p53 in senescent cells was increased by 91% compared to that of young cells, and in aged cells by 14%. In addition, the expression of ac-p53 increased by 32% and 58%, respectively. Similarly, the expression level of p-p21 protein was increased by 223% and 163%, respectively. Therefore, this result indicates that H₂O₂ positively modulated the expression of p21 protein through p53 activation.

The expressions of PI3K, AKT1, p-mTOR, mTOR, FOXO1, p-FOXO1 and β -catenin were also investigated by western blot analysis. As shown Figure 1C, the expression of PI3K in senescent cells increased by 20% compared with that of young cells, and by 50% in aged cells, as did that of AKT1 (228% and 259%, respectively). In addition, the expression of p-mTOR in senescent and aged cells was higher by 82% and 170%, respectively. The expression level of mTOR in senescent cells was increased by 79% compared with that of young cells, and by 56% in aged cells. FOXO1 expression was reduced by 28% in senescent cells compared with that of the young cells, and by 21% in aged cells. In contrast, the expression level of p-FOXO1 increased by 204% and by 288%, respectively. Furthermore, the expression of β -catenin correspondingly decreased by 32% and 59%. Therefore, this indicates that H₂O₂ enhanced the expression of PI3K and AKT1 related to the IGF1 signaling pathway.

Effect of H₂O₂ on the expression of genes involved in the aging of human melanocytes. The expression of key genes involved in the aging of human melanocyte was determined with mRNA prepared from young, senescent, and H₂O₂-treated cells using RT-PCR analysis. As shown in Figure 2, the gene expression of FOXO1 was not changed in senescent and H₂O₂-treated cells compared to young cells. In contrast, the levels of *PI3K*, *TP53* and *CAT* gene expression were increased in senescent cells and H₂O₂-treated cells. These results indicate that H₂O₂ increased the expression of genes involved not only in IGF1 signaling pathway but also in p53 signaling pathway.

Discussion

The expression of ERK1 and p-JNK, involved in the MAPK signaling pathway, were reduced in senescent cells and

Figure 1. Comparative analyses of protein expression related to p53 and insulin-like growth factor 1 (IGF1) signaling pathway in young, senescent and H₂O₂-treated (aged) HEMn cells. The cells were treated with H₂O₂ at 250 μ M. The expression of extra-cellular signal regulated protein kinase 1 (ERK1) and p-JNK (A), p53, acetylated p53 (ac-p53), p-p21 (B) and phosphoinositide 3-kinase (PI3K), protein kinase B (AKT1), phosphorylated mammalian target of rapamycin (p-mTOR), mammalian target of rapamycin (mTOR), phosphorylated forkhead box protein O1 (p-FOXO1), forkhead box protein O1 (FOXO1), β -catenin (CTNNB1) (C) were analyzed by western blot. The expression of actin was used as a control for normalization of target proteins. Data are given as mean values $\pm$ S.D. from three independent experiments. Significantly different at ** $p < 0.01$ and *** $p < 0.001$ (Student's *t*-test).

H₂O₂-treated cells compared to young cells. These results suggest that the MAPK signaling pathway is down-regulated by the reduction of expression of microphthalmia-associated transcription factor (MITF), which reduces the synthesis of melanin by down-regulation of tyrosinase, TRP1 and TRP2 as shown in a previous study (7). Investigation of IGF1 signaling pathway regulated by PI3K, AKT1, p-mTOR and p-FOXO1 showed that their expression increased in both senescent cells and H₂O₂-treated, aged cells compared with

young cells. Conversely, the expression of FOXO1 was increased in young cells. Previous studies reported that IGF1 positively regulates p-FOXO1, leading to the inhibition of expression of genes related to antioxidant enzymes (8, 9). In addition, FOXO1 is a transcription factor of antioxidant enzymes, unlike other proteins whose activity is promoted when phosphorylation occurs, its activity is inhibited when phosphorylation occurs. Therefore, the expression of antioxidant enzymes does not seem to be controlled by

Figure 2. Comparison analyses of the gene expression of phosphoinositide 3-kinase (PI3K), TP53, sirtuin 1 (SIRT1), forkhead box protein O1 (FOXO1), catalase, glutathione reductase and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) in young, senescent and H₂O₂-treated (aged) HEMn cells. The cells were treated with H₂O₂ at 250 μ M. Their gene expression was analyzed using reverse transcription polymerase chain reaction (RT-PCR). The expression level of G3PDH was used as a control for normalization of target proteins. Data are given as mean values $\pm$ S.D. from three independent experiments. Significantly different at ** p < 0.01 and *** p < 0.001 (Student's t -test).

Figure 3. Illustration of the role of p53, a tumor-suppressor protein, in insulin-like growth factor1 (IGF1) signaling pathway in H₂O₂-treated human melanocytes.

FOXO1 in melanocytes. Our findings suggest that IGF1 negatively modulates antioxidant enzymes in young cells, but positively modulates them in aged cells through the regulation of FOXO1/p-FOXO1. The IGF1 signaling

pathway is activated by binding of IGF1R with IGF1, and resulting in phosphorylation of IRS1 and IRS2, specifically, IRS2 interacts with SIRT1 to deliver signals to the down-signaling substance in a deacetylated form (10). The higher

expression of SIRT1 in senescent melanocytes than that of young cells in this study demonstrates cell senescence processes *via* the IGF1/IRS2/PI3K/AKT/mTOR pathway is regulated by SIRT1. Moreover, the expression of p-53, ac-p53 and p-p21 in the p53 signaling pathway were increased in senescent cells and H₂O₂-treated cells compared with young cells. p53, a tumor-suppressor protein, is highly expressed in senescent melanocytes, but the expression of SIRT1 and p300, which regulate p53, occurs in an opposite manner in melanocytes unlike general somatic cells (11). These results are consistent with the finding of a previous study showing that AKT-induced senescence is promoted by p53/p21-dependent pathway in human endothelial cells (12). Furthermore, the reduction of β -catenin expression level of was reduced in senescent cells and H₂O₂-treated cells compared to young cells. Those results indicate that the β -Catenin associated with the cyclic AMP/protein kinase A signaling pathway is inhibited by Akt, thus the expression of MITF is decreased, leading to the reduction of melanin production consistent with previous report (13). The above results can be summarised as shown in Figure 3.

In conclusion, these results suggest that p53 protein seems to play a key role in the aging of melanocytes *via* IGF1 signaling pathways.

Conflicts of Interest

None of the Authors have any conflict of interest in regard to this study.

Authors' Contributions

Moon-Moo Kim is a corresponding Author who developed a creative idea regarding this study and as well as western blot and RT-PCR experiments to elucidate the role of TP53 in the IGF1 signaling pathway related to the aging of human melanocytes with Hyeju Ko for the completion of this study.

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