Journal of Dermatological Science 86 (2017) 114–122

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Stress-induced premature senescence of dermal papilla cells compromises hair follicle epithelial-mesenchymal interaction

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ARTICLE INFO

Article history:
Received 10 September 2016
Received in revised form 24 November 2016
Accepted 5 January 2017

Keywords:
Premature senescence
Epithelial-mesenchymal interaction
Aging
Alopecia
Stem cell

ABSTRACT

Background: Hair follicle is miniorgan constituted by keratinocytes and its distinctive mesenchyme of dermal papilla. Its aging is characterized by organ atrophy and impaired stem cell activation and differentiation. The contribution of dermal papilla to hair follicle aging change is not well understood.
Objective: This work was aimed at exploring the possible role of premature dermal papilla senescence in the pathogenesis of hair follicle aging.
Methods: Dermal papilla cells were challenged with H_2O_2 to induce premature senescence and the proliferation, apoptosis, gene expression and protein secretion were characterized. Its effect on epithelial-mesenchymal interaction was analyzed by co-culture *in vitro* and implantation of protein-coated beads *in vivo*.
Result: Dermal papilla cells were more resistant to oxidative stress-induced apoptosis than dermal fibroblasts. The surviving dermal papilla cells showed signs of senescence but still preserved key dermal papilla signature gene expression. In addition to the failure to respond to mitogenic stimulation from keratinocytes, they lost the ability to induce hair follicle neogenesis, promoted interfollicular epidermal differentiation, inhibited follicular differentiation and, importantly, suppressed clonal growth of hair follicle stem cells. They produced higher levels of multiple inflammatory cytokines, including IL-6. Functionally, IL-6 inhibited clonal keratinocyte growth *in vitro* and blocked the transition from telogen to anagen *in vivo*.
Conclusion: Stress-induced premature dermal papilla senescence can contribute to hair follicle aging change due to compromised epithelial-mesenchymal interaction.

1. Introduction

Alopecia can be a process of natural aging or prematurely induced by diseases, such as alopecia seen in patients with premature aging, androgenetic alopecia, female pattern hair loss, genotoxic injury, *etc.* [1–5]. Organ aging is often characterized by functional deterioration, reduced size and impaired regenerative ability [3,6–8]. Hair follicle (HF) aging is featured by progressive hair shaft miniaturization, HF atrophy, prolonged telogen, shortened anagen, and even loss of the HFs [1,3,4,9], resulting in diminished hair amount.

HF is constituted by the epithelium of keratinocytes and a group of specified mesenchymal cells, dermal papilla (DP). Both of the developmental morphogenesis and post-natal growth of HFs are regulated by sophisticated epithelial-mesenchymal interaction (EMI) of these two groups of cells [10–12]. DP, derived from local fibroblasts during embryogenesis [13,14], can regulate follicular EMI *via* paracrine effects [12,15–18]. Owing to the cyclic regression and regeneration [19], HFs offer an optimal system to understand

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http://dx.doi.org/10.1016/j.jdermsci.2017.01.003

how epithelial cells, mesenchymal cells and their interaction affect aging.

During telogen to anagen transition, HF stem cells (HFSCs) are activated to fuel the regeneration process [20,21]. HFSC activities are regulated both by its intrinsic regulatory network and the extrinsic environment [21–26]. It has been shown that intrinsic defects in HFSCs contribute to the aging changes. In natural aging, HFSCs are preserved [9,27], but their activation and self-renewal are impaired due to the alterations in both the intracellular regulatory networks and extrafollicular environments [9,28]. Recently, Matsumura et al. found that cumulative DNA damage in HFSCs associated with aging can lead to defective differentiation and transepithelial elimination of HFSCs [4]. In androgenetic alopecia, it was also demonstrated that HFSCs are preserved but their activation is compromised [29], and altered prostaglandin secretion from the HF epithelium inhibits HFSC activation [30]. These results suggest that defective regulation within HF epithelium or HFSCs as well as alteration of the extrafollicular environment can contribute to HF aging.

The role of organ-specific mesenchymal cells in the aging of HFs and other epithelial organs is less well characterized [6,31]. Since HF growth is also dependent on follicular EMI, any pathological changes in DP cells may affect the regeneration of HFs. There is evidence that DP cells from balding scalp exhibit premature senescent changes [32–34]. Whether and how premature senescence of DP contributes to HF aging is not well studied because senescent DP cells cannot be efficiently expanded from the balding scalp for further testing.

In this work, we aimed to investigate how premature senescence in DP affects the functions of HFs. We established an *in vitro* method to stably induce premature senescence in DP and tested how it affects follicular EMI.

2. Materials and methods

2.1. Animals

The animal experiments were approved by our Institutional Animal Care and Use Committee (IACUC). Female Wistar rats were from LASCO Company, Taiwan and C57BL/6 and nude mice (BALB/cAnN-Foxn1nu/CrlNarl) were from Taiwan National Laboratory Animal Center. All animals were maintained in our animal facility. Anesthesia was performed with intramuscular injection of Tiletamine-Zolazepam (Telazol®).

2.2. Isolation and culture of rat DP cells, fibroblasts and outer root sheath (ORS) cells

DP cells and dermal fibroblasts were isolated from rat vibrissae and facial dermis, respectively, and were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) containing 10% (v/v) fetal bovine serum (Biological Industries, Israel) and 1% (v/v) antibiotic (Gibco) at 37 °C in a humidified atmosphere of 95% O_2/5% CO_2 as we previously described [67]. DP cells and dermal fibroblasts at the third passage were used for all of the following experiments. For H_2O_2 treatment, DP cells and dermal fibroblasts were seeded at $1.6 \times 10^4/cm^2$ and treated with H_2O_2 for 1.5 h and then the medium was replaced with fresh culture medium. ORS cells were also isolated and cultured from rat vibrissae.

2.3. Co-culture of DP and ORS cells

DP cells and ORS cells were co-cultured together directly or in separation in transwell culture dishes (0.4 μm PET, Millipore, U.S.A.). ORS cells were cultured in serum-free defined KSFM and plated in the lower compartment of transwells at 1×10^5 cells/well. After 24 h, DP cells treated with H_2O_2 or untreated DP cells were added to the upper compartment at the same density. The two types of cells were then co-cultured for 24 h and numbers of ORS cells were quantified.

2.4. Colony forming assay

Viability of primary HF keratinocytes was determined by trypan blue staining. Equal numbers of live cells were co-cultured with feeder cells of H_2O_2-treated or untreated DP cells in E-media supplemented with 15% calcium-free FBS and 0.3 mM calcium as described [68]. For IL-6 treatment, primary HF keratinocytes were co-cultured with mitomycin C-treated Swiss 3T3 fibroblasts in E-medium. After 14 days in culture, cells were fixed and stained with rhodamine B (Sigma) for imaging. The colony numbers were counted using the software ImageJ from NIH (https://imagej.nih.gov/ij/).

2.5. RNA preparation and RT-PCR

Quantitative RT-PCR was performed based on SYBR Green technology and 7900HT High-Throughput Real-Time PCR System (Applied Biosystems) as previously described [67]. The primers used were listed in Supplementary Table S1.

2.6. Senescence-associated β-galactosidase (SA-β-gal) activity

SA-β-gal stain was performed as previously described [69]. Briefly, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, washed twice with PBS and incubated in freshly prepared X-gal staining buffer for 48 h at 37 °C in the incubator. The X-gal staining buffer contained 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl P3-D-galactoside), 40 mM citric acid, 150 mM sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 50 mM $MgCl_2$ and 50 mM NaCl. After 48 h, cells were imaged and quantified under a light microscope.

2.7. Immunoblot

Cells were lysed in 1x RIPA lysis buffer (Millipore, U.S.A.) and the protein was quantified by BCA protein assay (Bio-Rad, U.S.A.). 40 μg protein was resolved by SDS-PAGE and transferred to PVDF membrane (Millipore, U.S.A.). Membranes were probed with primary antibodies as the following: cleaved caspase3 (rabbit, Cell Signaling, #9664), Bcl-2 (rabbit, Cell Signaling, #2876), Bax (rabbit, Cell Signaling, #2772), alpha-tubulin (mouse, GeneTex, GT114), GAPDH (mouse, GeneTex, GT239), γH2A.X (rabbit, Millipore, MABE205), phospho-Rb (rabbit, Bioworld Technology, BS4166), phospho-p53 (rabbit, Cell Signaling, #9284), p16 (rabbit, Abcam, ab81278), p21 (rabbit, Epitomics, 2990). After incubation with secondary antibodies at a 1:5000 dilution, the protein bands were visualized using ECL (Millipore) and was photographed by chemiluminescence (UVP Biospectrum, Level, U.S.A.). The intensity of each protein band was quantified by the ImageJ software. The quantified intensity of the protein band was first normalized to their own internal control and then normalized to that of DP cells or fibroblasts at 0 h, respectively.

2.8. Hanging drop and hair reconstitution assay

DP cells of various cell numbers were forced to aggregate in the hanging drop method on the roof of a 3.5-cm culture dish (Corning, NY, U.S.A.) as described [67]. For patch assays, DP cells were mixed with 1×10^6 keratinocytes from newborn mice (C57BL/6) and injected into the dorsal hypodermis of nude mice at 7–12 weeks of age as previously described [67]. After 3 weeks, the animals were