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ORAL KERATINOCYTES ARE MORE RESISTANT TO OXIDATIVE STRESS-INDUCED SENESCENCE THAN SKIN KERATINOCYTES

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ABSTRACT

Oral mucosal wounds typically heal faster and with less inflammation and scar formation than skin wounds, suggesting distinct stress-response programs in oral versus skin cells. Cellular senescence is a component of the wound-healing process; however, whether oral and skin keratinocytes undergo senescence differently remains unclear. Here, we used immortalized oral keratinocyte (TIGK) and skin keratinocyte (HaCaT) cell lines to determine whether they respond differently to oxidative stress–induced premature senescence and to evaluate DNA damage, proliferation arrest, and regulation of the senescence-associated secretory phenotype (SASP) that may underlie differential susceptibility to senescence.

Methods
TIGK and HaCaT cells were treated with H_2O_2 to induce oxidative stress–mediated premature senescence. Senescence was evaluated on day 3 post-treatment based on cell morphology and SA-β-gal staining. DNA damage was assessed by immunofluorescence staining for γH2A.X, and cell proliferation arrest was measured using an EdU assay. Expression of senescence-related genes and SASP factors was analyzed by PCR and ELISA. To determine whether secretomes from TIGK influence HaCaT responses to senescence induction, conditioned medium from TIGK cultures was applied to HaCaT cells, and subsequent changes in senescence markers were quantified.

HaCaT cells exhibited a more flattened and enlarged morphology with a β-gal–positive senescent phenotype than TIGK cells following H_2O_2 induction. Oxidative stress also resulted in lower reactive oxygen species (ROS) production, fewer DNA-damaged nuclei, and weaker proliferation arrest in TIGK compared with HaCaT. Both TIGK and HaCaT expressed significantly elevated levels of SASP factors, including IL-6, IL-8, MMP-1, MMP-3, and MMP-9. When HaCaT cells were cultured in TIGK-conditioned medium, senescence was significantly reduced, as demonstrated by decreased morphological changes, fewer β-galactosidase–positive cells, and reduced DNA damage (γH2A.X$^+$) compared with controls.

Conclusion
Oral keratinocytes exhibit greater resistance to oxidative stress–induced premature senescence than skin keratinocytes, and secretomes from senescent TIGK cells can attenuate senescence in skin keratinocytes. These findings suggest that oral keratinocytes possess intrinsic anti-senescence mechanisms that may contribute to the superior wound-healing capacity of oral tissue.

INTRODUCTION

Oral mucosal wounds heal faster with less inflammation and scarring than skin wounds, suggesting intrinsic cellular differences between these tissues. Keratinocytes, the primary epithelial cells in both oral and cutaneous environments, play key roles in wound repair by regulating stress responses. Cellular senescence is an important stress-response mechanism involved in tissue remodeling during healing, but excessive senescence can impair regeneration. Oxidative stress, a common feature of injured tissues, is a major trigger of premature senescence. However, whether oral and skin keratinocytes differ in their susceptibility to oxidative stress–induced senescence and associated molecular responses remains unclear. In this study, we compared immortalized oral and skin keratinocytes exposed to oxidative stress to evaluate differences in senescence phenotypes, DNA damage, proliferation arrest, and SASP expression, and to determine whether secreted factors from oral keratinocytes can modulate senescence responses in skin keratinocytes.

MATERIALS & METHODS

Induction of Premature Senescence
TIGK (an hTERT-immortalized human gingival keratinocyte line) and HaCaT (a spontaneously immortalized human skin keratinocyte line) were seeded into 12-well plates and cultured overnight under standard conditions. Upon reaching approximately 70% confluence, cells were treated with 200 μM or 400 μM H_2O_2 for 2 h to induce senescence, while control wells were processed in parallel without H_2O_2.

SA-β-Galactosidase Staining
Staining was performed using a SA β-Galactosidase Staining Kit (Cell Signaling Technology) according to the manufacturer's instructions. Briefly, after fixation, cells were incubated with β-gal staining solution (pH 6.0) at 37 °C in a dry incubator for 24 h. Stained cells were examined and imaged using an inverted light microscope under identical settings.

Immunofluorescence Staining
TIGK and HaCaT cells were treated with 200 μM H_2O_2 for 2 h. Cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.15% Triton X-100 for 15 min. After blocking with 5% goat serum, cells were incubated overnight at 4 °C with rabbit anti-γH2A.X antibody (Proteintech), followed by incubation with Alexa Fluor 594–conjugated goat anti-rabbit IgG for 60 min. Nuclei were counterstained with DAPI. Fluorescent images were acquired using a ZOE Fluorescent Cell Imager under consistent exposure parameters.

Quantitative Real-Time PCR
Total RNA was extracted using TRIzol (Invitrogen). One microgram of RNA was treated with DNase and reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Invitrogen). Quantitative PCR was performed with SYBR Green PCR Master Mix on a QuantStudio 3 Real-Time PCR System (Applied Biosystems). Gene expression levels were normalized to β-actin using the 2^−ΔΔCt method. Target genes included *CDKN2A, CDKN1A, TP53, IL6, IL8, MMP1, MMP3,* and *MMP9*.

Intracellular ROS Measurement
Intracellular ROS levels were measured using CM-H_2DCFDA. Cells were seeded in black-walled 96-well plates and pre-incubated with 5 μM CM-H_2DCFDA at 37 °C for 30 min in the dark. Cells were then treated with 200 μM H_2O_2 for 3 h. Fluorescence intensity was measured using a microplate reader at excitation/emission wavelengths of 485/528 nm. Stained cells were also visualized using an inverted fluorescence microscope.

ELISA
Concentrations of IL-6 and IL-8 in culture media were quantified using ELISA kits (RayBiotech) according to the manufacturer's instructions.

EdU Cell Proliferation Assay
Cell proliferation was evaluated using a Cell Proliferation EdU Imaging Kit (Green Fluorescence; MyBioSource). TIGK and HaCaT cells were seeded in black 96-well plates and treated with 200 μM H_2O_2 for 2 h. Cells were then incubated with EdU for 2 h, fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 15 min, and processed with Click-iT reaction mixture according to the manufacturer's instructions. Nuclei were counterstained with Hoechst 33342. Images were acquired using an inverted fluorescence microscope. Proliferation rate was calculated as the percentage of EdU-positive cells relative to total Hoechst-positive cells.

TIGK Conditioned Medium Treatment of HaCaT
HaCaT cells were treated with medium containing 50% conditioned medium collected from either control TIGK or senescent TIGK during H_2O_2-induced senescence.

Statistical Analysis
Data are presented as mean ± SD. Statistical comparisons were performed using a two-tailed unpaired *t* test. $P < 0.05$ was considered statistically significant.

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RESULTS

Figure 1. HaCaT exhibit more flattened, enlarged cells with a SA β-gal-positive senescent phenotype than TIGK after H_2O_2 induction.

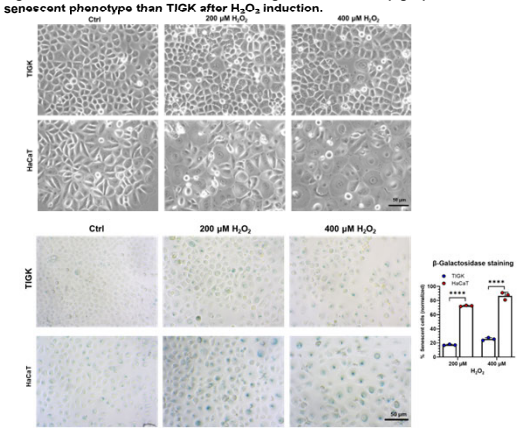


Figure 2. Senescence genes and senescence-associated secretory phenotype (SASP) are elevated after H_2O_2 treatment in TIGK and HACAT.

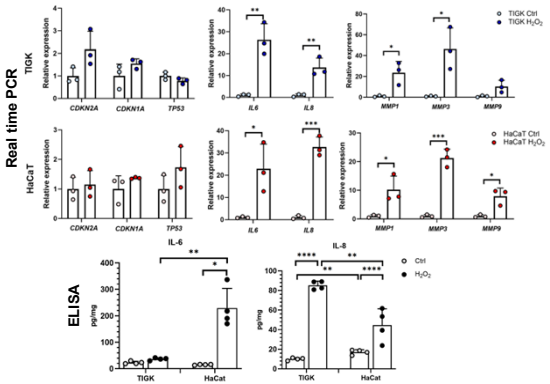


Figure 3. H_2O_2 induces more oxidative stress in HACAT than in TIGK.

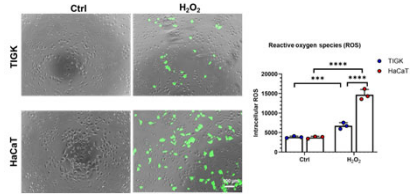


Figure 4. TIGK exhibit lower levels of DNA damage than HACAT following H_2O_2 treatment.

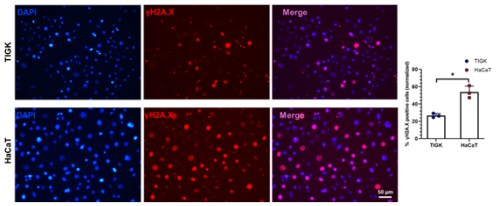


Figure 5. TIGK exhibit less proliferation arrest than HACAT following H_2O_2 treatment.

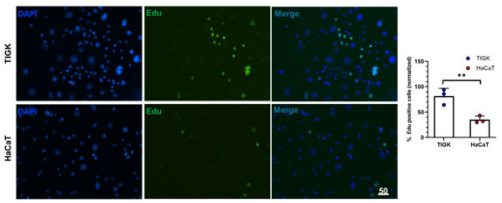


Figure 6. TIGK-conditioned media inhibit senescence in HACAT induced by H_2O_2.

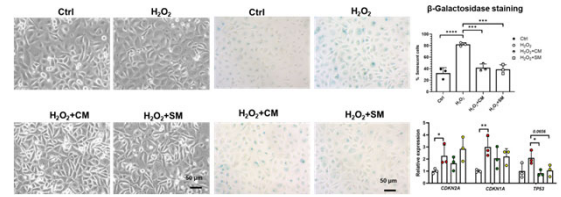
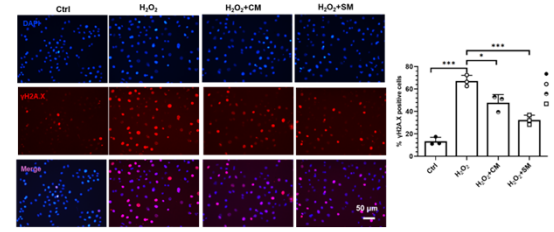


Figure 7. TIGK-conditioned media alleviate DNA damages in HACAT induced by H_2O_2.



CM: TIGK control conditional medium; **SM**: TIGK senescent conditional medium

SUMMARY

1: Oral keratinocytes exhibit greater resistance to oxidative stress–induced premature senescence than skin keratinocytes.

2. Secretomes derived from oral keratinocytes protect skin keratinocytes from senescence.

3: These findings indicate that oral keratinocytes possess intrinsic anti-senescence mechanisms that may contribute to the superior wound-healing capacity of oral tissues.