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"Lipid aldehydes from senescent cells reshape the ECM and propagate senescence."

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1. Introduction

Collagen is the most abundant protein of the extracellular matrix in the skin and its degradation and modification contributes to visible signs of skin aging. Previously, we found that chemically reactive lipids accumulate in senescent dermal fibroblasts [1] and that these lipids have the potential to covalently bind proteins via Schiff Base or Michael Adduction. Our current study aimed to investigate the modifications of collagen by lipids, recently described as part of the senescence-associated secretory phenotype (SASP) [2] and understand their impact on cellular function. We also investigated whether extracts from Camellia sp. could counteract the adduction of SASP lipids to collagen.

2. Materials and Methods

Collagen modification with SASP lipids

The model oxidized phosopholipid OxPAPC was generated by autoxidation of 1-Palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (PAPC, Avanti Polar Lipids, USA) which was dried as a thin film on a 4 ml glass vial and oxidized under compressed air for five days. 4-Hydroxnonenal (HNE, Merck Millipore Corp., USA) was stored as a 10 mg/ml in ethanol at -80°C. The incubation with type 1 collagen(Advanced BioMatrix, Sweden)) was performed using the buffer system reported by [3], where in short the lipids to the desired concentration were suspended in three parts of buffer and incubated with one part of the collagen suspension (of 0.75 mg/ml) for 4h at 37°C. In case where inhibitors or extracts were used, these were added to the buffer at the beginning of the incubation reaction.

Immunoblot for Collagen-HNE adduction

After sham treatment or SASP lipid modification, collagen was harvested using the 4x Laemmli Sample Lysis Buffer (BioRad) containing protease and phosphatase inhibitors. Criterion™

Electrophoresis system (BioRad) was used to perform a sodium dodecyl sulfate polyacrylamide gel electrophoresis (BioRad 4%–12 %), run at 200 Volt for 1h to ensure collagen separation. The proteins were blotted on nitrocellulose membrane (BioRad) using the Trans- Blot® Turbo™ Transfer System (BioRad) and blocked with 5 % dry milk in PBS with 0.1 % Tween®20. The membranes were then incubated with Anti-4-HNE Michael Adducts antibody (Calbiochem 393207, 1:100) overnight at 4 °C, followed by incubation with the corresponding secondary antibody (BioRad 1706515, 1:10000) for 1 h at room temperatureT. After washing, the blots were developed using the Super Signal™ West Dura Extended Duration Substrate (Thermo Fisher, USA).

Fibroblast culture on modified matrix

Collagen (0.75 mg/ml) was modified in a reaction containing either of 100 µg/ml HNE or 600 µg/ml OxPAPC. 300 µl of the mixture was immediately coated per well of a 12-well plate. The coating was incubated for 24h at 37°C to ensure modification and washed two times with Dulbecco´s Phosphate Buffered Saline (DPBS, Gibco, USA) following by washing with the appropriate cell culture medium to wash out unbound lipid aldehyde species

Primary fibroblasts from different donors were seeded at a density of 8,000 cells/cm2 and grown for up to 5 days . Human dermal fibroblasts were isolated from young donors and cultured in Dulbecco's Modified Eagle Medium (DMEM/F-12 + GlutaMAX, Gibco, USA) supplemented with 10% Fetal Bovine Serum, heat inactivated (FBS, Sigma-Aldrich, USA) and 1% Penicillin Streptomycin (Gibco, Thermo Fisher, USA). Primary keratinocytes were obtained from young patient epidermis and cultured in DermaLife growth medium (Lifeline Cell Technology, USA) supplemented with LifeFactorsin an atmosphere with 5% CO2 at 37°C.

qPCR

Reverse transcription of the isolated RNA into cDNA was performed with the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Germany). Quantitative real-time PCR analysis was conducted on a Thermal Cycler (Bio-Rad, CFX96™ Real -Time System) with the use of LightCycler® 480 SYBR Green I Master Kit (Roche, Switzerland). As a housekeeping gene beta-2-microglobulin (B2M) was used. The model of Pfaffl et al. was used for relative quantification and the expression of the target gene was normalized to the housekeeping gene. Significance of differences in gene expression was determined using one-way ANOVA.

3. Results

We have previously characterized the adduction of aldehydic lipid oxidation products, which are derived from the oxidation of polyunsaturated cell membrane lipids and are exposed by senescent cells, to various types of collagen using HPLC-MS/MS adductomics.

Here we also tested the prototypic lipid aldehyde 4-Hydroxynonenal for its ability to modify cell culture grade collagen and whether this modification could be inhibited by extracts from Camellia sp. Western Blot analysis of the treated but not the untreated collagen preparation showed a characteristic signal with the 4-HNE Michael adduct antibody, which detects the adducts of 4-HNE to various proteins (Fig. 1 a). When Camellia extract was present at the indicated concentrations during the incubation, the 4-HNE-induced signal was significantly reduced (Fig. 1 b,c).

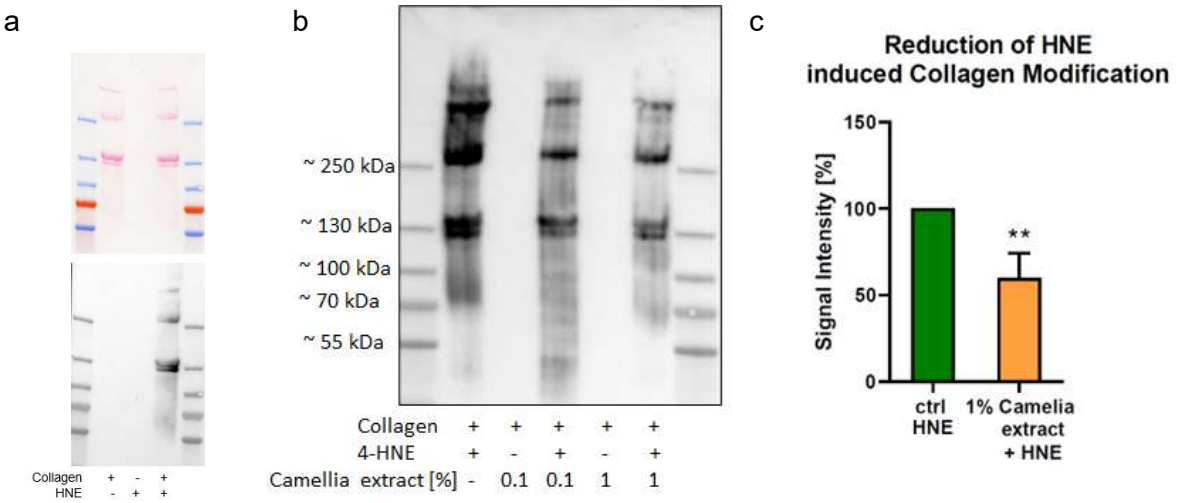
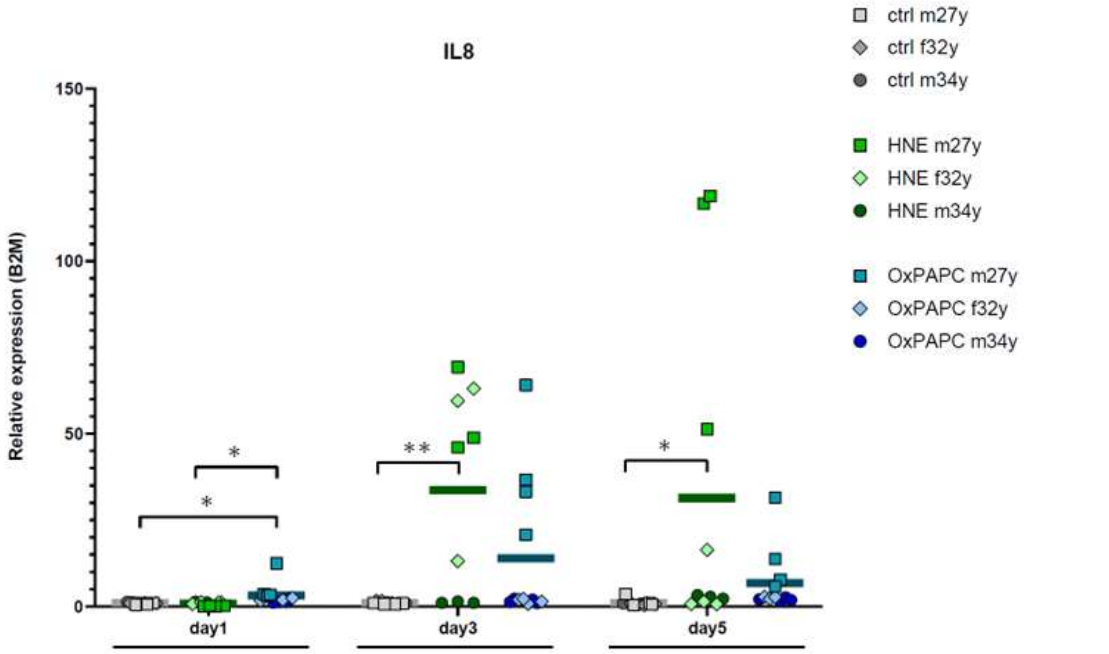
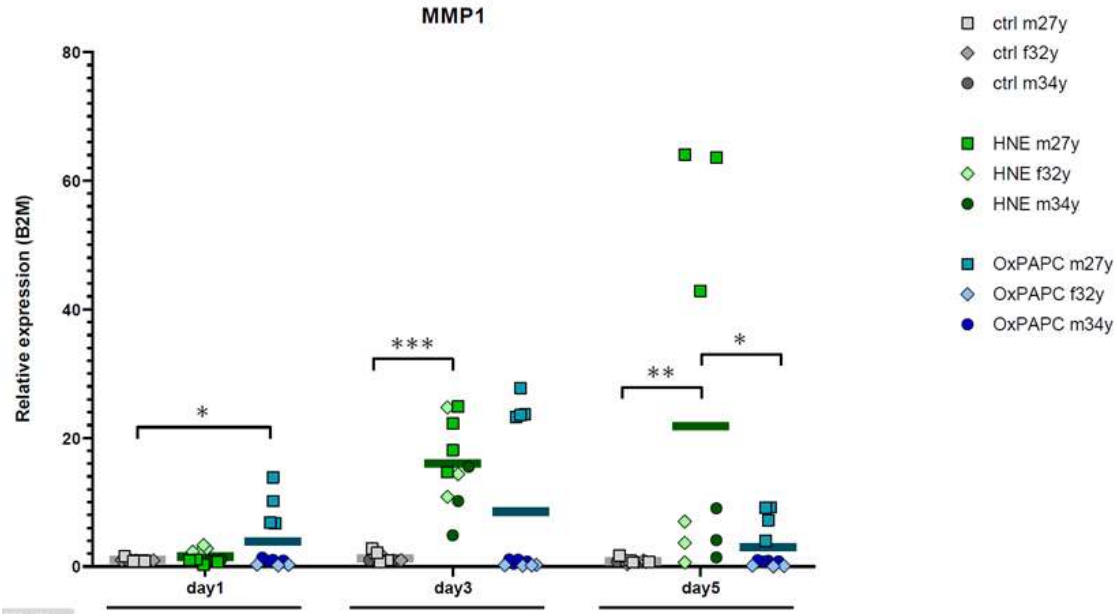


Figure 1. Modification of Collagen by 4-Hydroxynonenal (4-HNE) and its inhibition by Camellia extract. Schemes follow a different format. If there are multiple panels, they should be labeled as follows: (a) Collagen (0.75 mg/ml) was either sham treated or treated with 4-Hydroxynonenal (4-HNE 100 μg/ml) and a western blot detecting the Michael Adduct of 4-HNE to proteins was performed.(b) Camellia extract in the indicated concentrations was added during the modification reaction, and subequently the western blot detecting the Michael Adduct of 4-HNE was performed. (c) Quantification of the Western blot. Camellia extract could significantly inhibit the modification of Collagen – 4-HNE adduction.

To investigate how the structural modification of the matrix would affect the fate of resident cells, we analyzed gene expression of the fibroblasts cultured on modified type 1 collagen. We investigated a possible electrophilic stress response, inflammation or senescence phenotype. We cultured primary fibroblasts of 4 different donors for 24h (day 1), 72h (day 3) and up to 120h (day 5) to see immediate and longer-term effects on the cell fate. We observed significant higher mRNA levels of interleukin-1 (IL-8) and matrix metalloproteinase 1 (MMP-1) in fibroblasts grown on OxPAPC modified collagen already on day 1. (Fig. 2 a-b) After 3 and 5 days, both of the genes shown here were also significantly upregulated in fibroblasts cultured on the 4-HNE modified matrix. Then, we tested whether these changes resulting from the contact with the modified matrix could be inhibited by Camellia sp. incubation during the modification reaction. Indeed, the IL-8 and transcriptional upregulation could be prevented when the 4-HNE-mediated collagen modification was inhibited by Camellia sp. extract (Fig. 2 c-d). Moreover, Camellia sp. tends to decrease MMP-1 mRNA, this result will be confirmed by additional experiments.


IL8
Relative expression (B2M)
150
100
50
0
day1
day3
day5
*
*
**
*
ctrl m27y
ctrl f32y
ctrl m34y
HNE m27y
HNE f32y
HNE m34y
OxPAPC m27y
OxPAPC f32y
OxPAPC m34y


MMP1
Relative expression (B2M)
80
60
40
20
0
day1
day3
day5
*
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ctrl m27y
ctrl f32y
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HNE m27y
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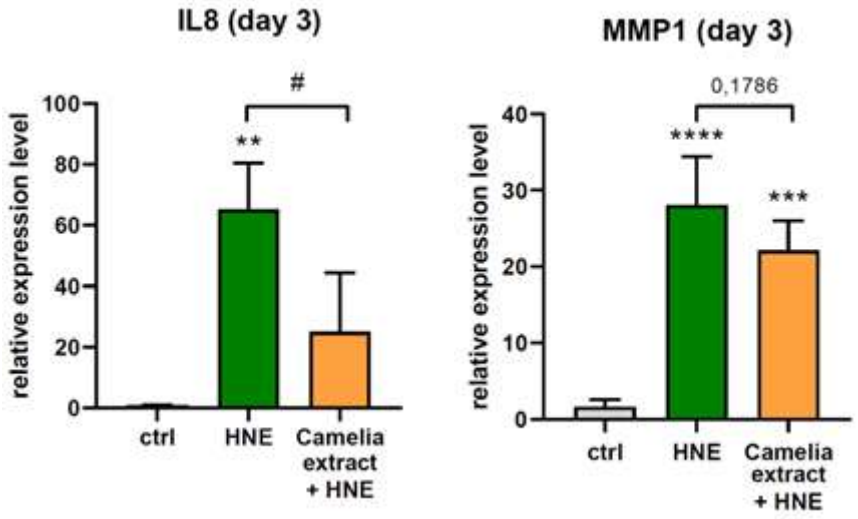

Figure 2. Transcriptional responses to modified matrix. Collagen (0.75 mg/ml) was modified with 4-HNE (100 μg/ml) and OxPAPC (600 μg/ml). The modified matrix was washed, and fibroblasts (3 donors) were grown for 24h, 72h or 120h; n=4, each. (**** p< 0.001) determined by one-way ANOVA. Culture on the modified but not on native matrices elicited a proinflammatory, proteolytic and early senescent phenotype in primary fibroblasts. (c-d) Limitation of the collagen matrix modification by pre-treatment with plant derived substances from Camellia sp. reduced the expression of IL-8 and tends to decrease MMP-1 expression.

4. Discussion

UV- generated lipid peroxidation products which adduct to proteins of the extracellular matrix have been reported in photoaged skin, especially a role in the modification of elastin was reported [3]. Also, we and others reported how ROS-mediatedoxidized lipids (which we have previously identified as SASP factors in fibroblasts) affect the fate and responses of cells exposed to such lipids [5,6]. Here we identified how lipids produced by senescent cells modify the dermal ECM and how this modification elicits an inflammatory phenotype in dermal fibroblasts, impairs the differentiation of keratinocytes and has a dual effect on cells of the immune system. While matrix modification leads to a low grade inflammatory phenotype in macrophages, it also impairs their ability to respond to stimuli via TLR activation. Comparable phenotypes are found in cells exposed to SASP in vivo, and are thought to contribute to the decline of skin structure and functionality in aging [7]. Our findings underline the importance of identifying and characterizing not only the phenotype of senescent cells in vivo [8] but also the physical consequences of the phenotypes, in this case the reactivity of oxidatively generated aldehydolipids found in the SASP. Our work also identifies the lipid SASP itself as a promising and effective target for skin care active ingredients, and we identified Camellia sp. extract as a promising candidate to contain the unwanted effects of SASP lipids on ECM and the downstream effects on cells exposed to such a matrix.

5. Conclusion

Reactive lipids from senescent fibroblasts can adduct to ECM proteins and thereby not only change their structure but also the fate of cells grown on or in such a matrix. Inhibiting the modification of the matrix using a Camellia sp. extract also inhibits the downstream consequences on cell fate that were induced by the modificaiton of the matrix.

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