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Volatile profiles of human skin cell cultures in different degrees of senescence

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ABSTRACT

It is known that skin releases volatile organic compounds to the environment, and also that its emission pattern changes with aging of the skin. It could be considered, that these compounds are intermediaries in cell metabolism, since many intermediaries of metabolic pathways have a volatile potential. In this work, a simple and non-destructive method consisting of SPME sampling and GC/MS analysis was developed to identify volatile organic emanations from cell cultures. This technique, applied to skin cells culture, indicates that the cells or cell metabolism produce several skin emissions. Chemometric analysis was performed in order to explore the relationship between a volatile profile and the senescence of cell cultures. Volatile profiles were different for cell cultures in different degrees of senescence, indicating that volatile compound patterns could be used to provide information about the age of skin cells.

1. Introduction

It is known that human metabolism synthesizes a range of volatile organic compounds (VOCs) but only recently, has it been noted that several are emanated by the skin [1]. Since the skin is the largest organ in the body, there is great interest in relation to VOCs and their functions. Hundreds of different kinds of organic substances appear in human skin emanations, which can be classified into several groups according to their functional groups such as carboxylic acids, alcohols, aldehydes, aliphatics, esters, ketones, amines, heterocyclics, and others [2].

Studies of body emanations, particularly those associated with perspiration, generate knowledge about the compounds present in skin, but not necessarily about the volatiles that are of importance to cell metabolism. Perspiration, being mainly formed of water (99%), is a dilute solution of compounds containing salts, non-volatile compounds, as well as small amounts of volatiles. Many amino acids (phenylalanine, leucine, valine, and alanine) have been identified in perspiration [3] together with ammonia, urea, lactate, uric acid, creatine polysaccharides, immunoglobulin A, epidermal growth factor, some hormones, vitamins and proteolytic enzymes [4].

It seems that VOC-profiles could be of importance in understanding the metabolism of the skin and other organs: skin emission patterns of VOCs have been found to be different in winter as they are in spring [1], plus it has been reported that alterations of the metabolic balance produced by pathologies, results in modifications in human VOC-profiles [5].

Several studies in humans have suggested that skin emanations are a source of chemical signals containing physiologically active components capable of altering the menstrual cycle in women [6], indicating that volatiles are part of a complex biological system. Several types of skin glands, including apocrine, eccrine, sebaceous and apoeccrine glands, contribute to volatile releases [7]. On the other hand, skin and skin emanations change with aging. The number and function of eccrine sweat glands decreases with age. In contrast, sebaceous glands increase with age, paradoxically accompanied by a marked decreased in sebum production [8], and an increase of senescent skin cells [9].

Senescence is an important characteristic of cells related mainly to the arrest of proliferation after a finite number of divisions. Since it is an irreversible process and the principal cause of tissue aging attracts much attention. The classic method used to identify senescence cells makes use of a biochemical reaction associated with the enzyme β-galactosidase present in the cell. Pre-senescent cells express enzymes with activity only at pH 4, but senescent cells also produce a β-galactosidase with activity at pH 6. This enzyme is called "Senescence-Associated/β-Galactosidase" (SA/β-Gal), and is considered a cellular marker used to identify senescent cells. SA/β-Gal expression can be detected with a stain which produced a blue colour after 16 h of chromogen addition into the cell culture [9]. Other methods have been proposed to measure SA/β-Gal activity [10], such as fluorescent assays [11,12] or differential-pH assays [13], looking after faster analytical methodologies. All of these methods are destructive in relationship to the cell sample. Our approach is based on the use of solid phase microextraction

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(SPME) a technology recently applied to study biological samples with fast and reliable protocols. This technique has been applied to measure VOCs in cell cultures [14,15], human blood [5], human skin [1] and other human samples [16] as their main characteristic is a non-destructive approach.

Aging of the skin is usually accompanied by marked changes in emanation releases [8] and dramatic changes in the synthesis capacity and gene expression of skin-fibroblasts [17,18]. For this reason, we hypothesized that the senescence modifies the VOC-profiles obtained from skin cells. In the present work, the monitoring of these changes by using GC/MS–SPME analysis was done.

2. Materials and methods

2.1. Cell culture

Cells were isolated using standard cell and tissue culture procedures [19,20], adapted to obtain human dermal fibroblasts [21]. Biopsies of human skin were obtained from foreskin of Chilean (mestizo-white) healthy young donors (between 3 and 6 years old), under informed consent from their parents, approval of the surgeon in charge and Ethic Committee, as required by the Helsinki Declaration. Since that circumcision of children is a routine medical procedure, the discarded piece of foreskin was used to obtain primary cell cultures (pre-senescent cells).

The biopsy was washed three times with phosphate buffer saline (PBS) (0.1 M pH 7.4) containing penicillin (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (25 μg/mL). The visible fat was removed. The biopsy was incubated for 1 h at 37 °C in trypsin–EDTA (0.05%–0.53 mM) (Gibco-Invitrogen). The dermal layer was separated from the epidermis, cut in 1 mm^2 pieces and incubated for 1 h in trypsin–EDTA. Cells were recovered by centrifugation and cultured in DMEM/F12 (1:1) (Gibco-Invitrogen) with 10% FBS in 25 cm^2 section flask (Falcon).

The cell cultures were treated with commercial solutions to prevent mycoplasma contamination (BioMyc1/BioMyc2; Biological Industries) and tested for the presence of mycoplasma using a PCR-assay (EZ-PCR Mycoplasma Test Kit; Biological Industries).

Lactate concentration in the culture supernatant was measured with a commercial enzymatic kit (Lactate oxidase/Peroxidase; Sentinel Diagnostic).

2.2. Generation of senescent cell cultures

To evaluate the aging of the fibroblast cultures, the senescent cell assay was made using the conventional protocol for Senescence-Associated/Beta-Galactosidase (SA/β-Gal) staining [9] in non-confluent cultures [17].

Senescence in human skin cells was induced by serial passage. In each passage, the cells were grown up to approximately 80% of confluence and trypsinized (trypsin–EDTA 0.05%–0.53 mM; Gibco-Invitrogen). The senescence status was verified by SA/β-Gal staining in each passage of the cell cultures. When cells reached 10, 15, 20, 30, 50 and 70%SA/β-Gal, they were used in VOCs experiments.

2.3. Selection of the solid phase microextraction fiber

Three commercial solid phase microextraction (SPME) fibers were used together with a SPME manual holder: 100 μm polydimethylsiloxane (PDMS), 65 μm polydimethylsiloxane–divinylbenzene (PDMS-DVB) and 75 μm carboxen–polydimethylsiloxane (Car-PDMS). The SPME fibers were conditioned as recommended by the manufacturer (Supelco) at some degrees below each fiber's maximum temperature before they were used for the first time.

Conditioned media was used as a sample [22,23], prepared by mixing cell culture supernatants and fresh medium (DMEM/F12, 10% FBS), in a 3:1 ratio. Supernatants were obtained from five cell cultures, each one prepared from different donors.

The SPME fiber was introduced inside a headspace-vial of 20 ml, through a silicon septum (Supelco). Previously, the headspace-vial was loaded with 1 ml of sample (for 25 cm^2 culture flasks, no more than 5 ml conditioned media were available, obtaining a sample of 1 ml per quintuplicate). The loaded vial was stabilized for 15 min at extraction temperature (60 °C). Since the culture media contain blood serum, extraction was performed at the optimal extraction temperature to human blood (60 °C) for 60 min, therefore optimizing VOCs extraction and preventing protein denaturation [5].

2.4. Gas chromatography parameters

Targeted analytes loaded in the fiber were analyzed in a Hewlett-Packard (HP) 6890 gas chromatography (with a HP MD5973 quadrupole mass spectrometer) in splitless mode (5 min). Desorption was carried out at 250 °C for 5 min [5]. Helium at a constant flow (1.5 mL/min, 32 cm/s) served as a carrier gas. Separation was conducted in a 60 m length × 0.32 mm i.d. × 1.8 μm film thickness HP-VOC column (Agilent Scientific, USA). The oven was programmed as follows [1]: initial temperature of the column at 60 °C (2 min hold) followed by a ramp of 8 °C/min until 200 °C (15 min hold) and a second ramp of 5 °C/min to reach 260 °C, finally ramped to 270 °C with a post-run for 3 min. The parameters of detector were: Ion mass/charge ratio 20-550 m/z; quadrupole temperature 150 °C; electron impact ionization (EI); EI source temperature 230 °C; interface temperature 280 °C; electron multiplier voltage 1780 eV; scan mode.

2.5. Blanks

Two sorts of blanks were required: Blanks associated with the equipment (to discard contaminant related to the SPME fiber and chromatographic column, like siloxanes) and environmental blanks (to discard possible contaminants present in the laboratory environment). For an equipment blank, a SPME fiber was injected into the GC/MS without extraction samples. For an environment blank, a sterile PBS was used, in the same extracted condition of the respective sample.

The called "compensed-chromatogram", used to compare different volatile profiles, is the result of balancing the sample chromatogram to the related blank chromatogram.

2.6. VOCs identifications

Potential emanations were analyzed by matching sample mass spectrums with those of the National Institute of Standards and Technology (NIST) MS spectral library (98/02) for peaks presented in the chromatograms. Emanations were considered "unknown", when their mass spectral fit values were <90%, and discarded of this identification process. Putative emanations with mass spectral fit value ≥90% were compared with their respective chemical standard (Sigma–Aldrich), and informed as "identified", if retention times and mass spectra fitted those of standard compounds.

2.7. Comparative analysis of VOC-profiles of different cell cultures

Three cell cultures replicates of each senescence degrees evaluated by SA/β-Gal (10, 15, 20, 30, 50 and 70%SA/β-Gal) were seeded in six well plates at 2×10^4 cell/cm^2. The selected SPME fiber associated process was applied to detect the VOC-profiles. Supernatant of these 18 cell cultures after 72 h of seeded were analyzed per triplicate (generating 54 chromatograms). One ml of sample