Journal of Chromatography B, 931 (2013) 90–96

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Volatile biomarkers from human melanoma cells

Jae Kwak[a], Michelle Gallagher[a], Mehmet Hakan Ozdener[a], Charles J. Wysocki[a,b], Brett R. Goldsmith[c], Amaka Isamah[a], Adam Faranda[a], Steven S. Fakharzadeh[d], Meenhard Herlyn[e], A.T. Charlie Johnson[c], George Preti[a,d,*]

[a] Monell Chemical Senses Center, 3500 Market Street, Philadelphia, PA 19104, USA
[b] Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA
[c] Department of Astronomy and Physics, University of Pennsylvania, Philadelphia, PA 19104, USA
[d] Department of Dermatology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA
[e] Wistar Institute, Philadelphia, PA 19104, USA

ARTICLE INFO

Article history:
Received 3 December 2012
Accepted 13 May 2013
Available online 22 May 2013

Keywords:
Volatile organic compounds
Gas chromatography–mass spectrometry
Melanoma
Carbon nanotubes
Volatile metabolome

ABSTRACT

Dogs can identify, by olfaction, melanoma on the skin of patients or melanoma samples hidden on healthy subjects, suggesting that volatile organic compounds (VOCs) from melanoma differ from those of normal skin. Studies employing gas chromatography–mass spectrometry (GC–MS) and gas sensors reported that melanoma-related VOCs differed from VOCs from normal skin sources. However, the identities of the VOCs that discriminate melanoma from normal skin were either unknown or likely derived from exogenous sources. We employed solid-phase micro-extraction, GC–MS and single-stranded DNA-coated nanotube (DNACNT) sensors to examine VOCs from melanoma and normal melanocytes. GC–MS revealed dozens of VOCs, but further analyses focused on compounds most likely of endogenous origin. Several compounds differed between cancer and normal cells, *e.g.*, isoamyl alcohol was higher in melanoma cells than in normal melanocytes but isovaleric acid was lower in melanoma cells. These two compounds share the same precursor, *viz.*, leucine. Melanoma cells produce dimethyldi- and trisulfide, compounds not detected in VOCs from normal melanocytes. Furthermore, analyses of the total volatile metabolome from both melanoma cells and normal melanocytes by DNACNT sensors, coupled with the GC–MS results, demonstrate clear differences between these cell systems. Consequently, monitoring of melanoma VOCs has potential as a useful screening methodology.

1. Introduction

Skin cancer is the most common form of cancer in the United States, and it is divided into 3 types: basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and melanoma [1]. BCC is the most frequent skin cancer and rarely spreads ("metastasizes") to other parts of the body. BCC accounts for about 80% of all diagnosed skin cancers, followed by SCC, which accounts for about 16% of skin cancers [2]. SCC requires early treatment to prevent metastasis. More than 2 million cases of BCC and SCC occur annually in the United States alone [3]. Melanoma is the deadliest form of skin cancer because it can rapidly spread to other parts of the body. In the United States, it is estimated that more than 76,000 people will be diagnosed with, and about 9000 patients will die from, melanoma in 2012 [4]. Skin cancer is highly curable when detected early and treated properly. Melanoma is also highly curable if limited to the outermost layers of the skin. Thus, early detection of skin carcinoma is critical and various diagnostic imaging techniques have been proposed [5]. Currently, early detection of skin carcinoma is accomplished primarily through a visual exam, imaging techniques and biopsy of any suspected areas. Biopsy is invasive and usually requires examination by a pathologist. A recent review cites the large increase in the use of reflectance confocal microscopy and dermoscopy *in situ* for diagnosis of primary melanoma and other skin diseases [6]. Sophisticated imaging technologies such as these provide spatial and diagnostic information that will aid the specialist (dermatologist, cosmetic surgeon) in surgery. In addition, the most recent sophisticated versions of these techniques, which have been practiced for many years, are still "emerging" and even after they do mature to a role in the clinic, they may be too expensive and require specialized training for general screening use, such as by primary care physicians or nursing home physicians.

Abbreviations: VOCs, volatile organic compounds; GC–MS, gas chromatography–mass spectrometry; DNACNT, DNA-coated nanotube; BCC, basal cell carcinoma; SCC, squamous cell carcinoma; SPME, solid phase micro-extraction; e-nose, electronic nose; CNT, carbon nanotube; CNT FET, carbon nanotube field effect transistors; ssDNA, single strand DNA; RGP, radial growth phase; VGP, vertical growth phase; Mm, metastatic melanoma; TIC, total ion chromatogram.

* Corresponding author at: Monell Chemical Senses Center, 3500 Market Street, Philadelphia, PA 19104, USA. Tel.: +1 267 519 4920; fax: +1 215 898 2084.
E-mail addresses: preti@monell.org, preti@pobox.upenn.edu (G. Preti).

1570-0232/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2013.05.007

In addition to the traditional reliance upon visualization and optical techniques, there has been an effort to employ proteomic techniques to discover biomarkers [7,8]. The discovery of cancer-related biomarkers using proteomic techniques has primarily focused on prognostic indicators of melanoma, *i.e.*, examining the serum and plasma proteome for biomarkers indicative of metastases to distant sites. There has not been, however, a concerted effort to exploit the evidence that volatile cues are released from melanoma tissues that can be differentiated from those of normal skin. Several anecdotal reports suggest that dogs can sniff out skin carcinoma. Williams and Pembroke reported a patient whose dog constantly sniffed at a mole on her leg, which turned out to be histologically confirmed melanoma [9]. Similar cases have been reported since then [10]. Pickel et al. [11] demonstrated that dogs can be trained to localize melanoma tissue samples hidden on the skin of healthy volunteers and even the region of the skin of patients with melanoma, suggesting that odorous volatile organic compounds (VOCs) released from melanoma cells on the skin are recognizable as different from those of normal skin.

D'Amico et al. [12] employed gas chromatography/mass spectrometry (GC–MS) and a gas sensor array to investigate whether skin lesions of melanoma and nevi can be differentiated. The gas sensor array discriminated the different lesions with about 80% accuracy. The authors were able to find a number of peaks in the gas chromatograms present only in the melanoma lesion. They suggested that one of them is probably propanal; however, they did not report the identity of any other VOCs. More recently, the same research group analyzed the VOCs released from 3 different human melanoma cells cultured *in vitro*, but the majority of the identified compounds do not appear to be from cell metabolism [13]; notably, the synthetic antioxidant butylated hydroxytoluene was the most abundant peak in the melanoma cells. It seems to be an additive of the growth medium. Abaffy et al. [14] compared the volatile fingerprints released from melanoma tissues with those from nevi or normal skin tissues using solid phase micro-extraction (SPME)–GC–MS and reported dozens of potential VOCs putatively derived from melanoma. However, the majority of them were environmental contaminants such as isopropyl palmitate (a cosmetic ingredient), limonene, propylene glycol, methoxy-phenyl oxime (a SPME fiber-derived compound), phthalates, 2-ethyl-1-hexanol (a phthalate metabolite), styrene, propofol (an anesthetic), *o*-hydroxybiphenyl (a disinfectant used in hospitals), alkyl benzenes and hydrocarbons. The use of small amounts of skin samples (3 mm diameter) may have contributed to the detection of many compounds from the environment; unfortunately, the authors do not appear to have realized that they were not identifying compounds from tissue metabolism.

Although the previous chemical analyses failed to identify any meaningful, characteristic VOC profiles from melanoma, the above cited studies using dogs and a gas sensor array, suggest that the volatile metabolome emanating from melanoma may be different from that of normal skin. While the discovery of differences between normal skin *vs.* melanoma is best accomplished using GC–MS techniques, translation of these results to point-of-care diagnostics requires an easily-used, hand-held device. For this ultimate goal we propose the utilization of an "electronic nose" (e-nose) device employing functionalized DNA-coated carbon nanotube sensors, capable of sensitive and selective detection of compounds emitted from skin. Single walled carbon nanotube field effect transistors (CNT FET's), functionalized with single stranded DNA (DNACNT), have been shown to respond through a change in source drain current when exposed to VOCs [15]. By varying the sequence of the adsorbed single stranded DNA the response of the DNACNT system can be tuned for desired volatile markers. We have demonstrated that this sensor class has a unique set of properties making them ideal for use in a very large sensor array as part of a nanotechnology-enabled e-nose system [16–18]. The sensors show rapid response and recovery (seconds), very low signal drift, and chemical responses that are single strand DNA (ss-DNA) base sequence dependent. Single stranded-DNA is chosen for functionalization of the CNTs because it displays recognition for chemical vapors [17–19], and it binds *via* a *non-covalent* π–π stacking interaction to the CNT that preserves the latter's ideal electronic readout properties. The large number of distinct sequences, even for short oligomers ($>10^{12}$ for strands of 20 bases), will make it possible to generate hundreds of sensors with uncorrelated odor responses as required for an e-nose with computational power approaching that of mammalian olfactory systems. The CNT FET functions as an array-able electronic read-out element [20] that is exquisitely sensitive to variation in the electrostatic environment [21].

For biomarker discovery, we employed SPME and GC–MS to identify the VOCs that differentiate between human melanoma and normal melanocyte cells cultured *in vitro*, which may provide a model for *in vivo* human melanomas. Our analyses, employing GC–MS and DNACNT, demonstrate reliable and significant differences between VOCs emanating from cultures of normal skin cells *vs.* melanoma cells.

2. Materials and methods

2.1. Cell cultures

Human melanoma cells and normal melanocytes were obtained from Dr. Herlyn's laboratory (The Wistar Institute, Philadelphia, PA) and maintained as described in Hess et al. [22]. Three types of melanoma cells, radial growth phase (RGP) primary melanoma cells (WM35, WM3211 and Sbcl2), vertical growth phase (VGP) primary melanoma cells (WM115 and WM983A) and metastatic melanoma (Mm) cells (WM983B and WM1158), as well as 2 normal neonatal foreskin melanocytes (FOM136 and FOM191) were examined (see Ref. [23] for detailed information regarding the cells). RGP cells have neither tumorigenic nor metastatic potential. They reside predominantly in the epidermis. VGP, however, can be tumorigenic and have metastatic potential. Melanoma cells were maintained in plastic "T25" plates containing 4 ml of Tu 2% medium (MCDB153, Sigma–Aldrich, St. Louis, MO) supplemented with Leibovitz's L-15 Medium (Invitrogen Co., Carlsbad, CA), 2% fetal bovine serum, 5 μg/ml bovine insulin (Sigma–Aldrich), and 1.68 mM $CaCl_2$. Normal melanocyte cells were maintained in the T25 plates containing 4 ml of 254 medium with human melanocyte growth supplement (Cascade Biologics, Portland, OR). All cells were incubated at 37 °C in a humidified environment containing 5% CO_2. Each cell line was cultured in triplicate. Media were changed 2 times a week and cells were harvested once a week. T25 containers holding only media were treated in an identical fashion.

2.2. Collection of VOCs from T25 containers

After growth to maximum confluence was achieved, the T25 vessel was fitted with a screw cap containing a 18 mm septum cap (Supelco, Bellefonte, PA, USA) through which we inserted the SPME syringe and fiber described below. The fiber was left exposed to the headspace above the cells and media fluid for 30 min. Collection was performed at room temperature; no stirring or agitation was performed. We also examined the VOCs expressed from empty T25 containers and T25 containers containing media to determine their contribution to background.