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Monitoring of betulin nanoemulsion treatment and molecular changes in mouse skin cancer using surface enhanced Raman spectroscopy

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ABSTRACT

The aim of this study was to obtain in vivo and ex vivo reproducible surface enhanced Raman signal from mouse skin and to use it for the differentiation of skin pathologies. We induced skin carcinoma in mice models using a chemical treatment and tested the chemopreventive activity of a new formulation based on natural compound betulin extracted from the bark of birch trees. Using surface enhanced Raman spectroscopy (SERS) we identified in vivo and ex vivo the spectral signatures characteristic to the healthy skin, melanoma skin induced in mouse models, and to the pathology evolution when the betulin nanoemulsion formulation was topically applied on the cancerous skin of mice. SERS markers associated to each pathology were identified and the signal was distinguished from the classical Raman signal of skin based on several biomarkers, such as the disappearance of the amide I band of proteins, the amplification of the 1574 cm^{-1} band assigned to nucleic acid bases, and the appearance of the highly amplified band at 230 cm^{-1} characteristic to the metal-biomolecules complex. The various skin pathologies were differentiated using principal components analysis and K-means clustering. The effectiveness of the betulin nanoemulsion treatment was validated by the histological examination and the chemometrics methods, which successfully confirmed the direct SERS differentiation between the cancerous and the betulin nanoemulsion treated skin.

1. Introduction

Melanoma is the most dangerous type of skin cancer and the leading cause of death from skin disease [1]. Although treatable in the incipient phase, metastatic malignant melanoma is an extremely aggressive cancer with no current viable treatment. Mouse skin carcinogenesis has become extremely useful for studying the biological modifications involved in tumor promotion, therefore, we induced skin carcinoma in mice using a single application of the chemical initiator mutagen 7,12-dimethylbenzanthracene (DMBA) solution followed by repeated applications of a pro-inflammatory phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA) solution. We employ here a chemical treatment known to produce reactive oxygen species which are the cause for mutagenesis, carcinogenesis, and tumor promotion [2]. According to the literature, papillomas and squamous cell carcinoma should appear starting with week 6 of treatment [3]. Chemoprevention implies the administration of agents with the aim of preventing the mutational and promotional events that occur during the abnormal proliferation of cells. At the moment, there is a constant search for new drugs that can be used as chemopreventive agents in the treatment of cancer. In the present study, the chemopreventive activity of betulin, a natural compound extracted from the outer bark of the Betula birch tree is investigated. Betulin presents a broad field of biological properties, such as anti-cancer, anti-bacterial, anti-inflammatory, anti-fungal, and anti-viral, which were referred to or investigated in multiple reports [4–6] and was shown to be effective on lesions and severe skin damages [7]. The new findings strongly suggest prophylactic (application before carcinogen) properties, but also curative agent (no sign of important damages).

A main concern of biomedical physics is to develop non-invasive diagnosis methods which can detect early stage cancers and image the surgical margins to aid in complete removal of tumors [8,9]. Raman spectroscopy has been used in the biomedical field for cancer diagnosis [10–12] or for the study of cells and tissues with the aim to detect subtle biochemical changes related

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to disease or to the interaction with drugs or toxic agents [13–15]. Raman scattering is a weak effect, however, when coupled to the local optical fields of metallic nanoparticles (NPs), enhanced Raman signals can be observed for the molecules attached to them. Surface enhanced Raman spectroscopy (SERS) based on NPs has been widely used in biomedical applications for in vivo tumor detection [16], diagnosis of cancerous tissues [17,18], for the detection of the molecular structural information in live cells using biosensors [19,20], or for the detection and identification of bacteria [21]. SERS NPs are not approved for human use, therefore systemic administration is employed for in vivo imaging studies of animals [22,23]. NPs can either accumulate non-specifically in the tumor or can bind specifically to cancer biomarker targets [16,24].

In this study we show that SERS effect provides highly sensitive information from in vivo and ex vivo animal tissue and we exploit this effect for differentiating skin pathologies: cancerous, betulin treated, and healthy skin, based on characteristic molecular changes. Acquiring SERS signal from biological tissue samples is a complex task, however the scientific literature in the field presents several methods such as injecting dosages of nanoparticles in the tissue, dropping colloidal solutions on the tissue samples [25], or nitrogen freezing followed by crushing and mixing of the tissue with nanoparticles [26], to name just a few. In this study, a method of acquiring SERS spectra from skin tissues immersed in colloidal NPs is presented. Our group has evidenced before the possibility of acquiring SERS spectra from tissue samples [27,28] and concluded that the Ag colloidal NPs can be incorporated into the interstitial space in tissues and allow the acquisition of high quality SERS signal. Different tissue structures have characteristic features in the SERS spectra making thus possible the SERS based characterization and differentiation of the tissue samples [26]. We also show that the SERS effect can be used to monitor the topically applied treatment using betulin, a natural potential anti-cancer drug candidate.

2. Materials and methods

2.1. Instrumentation

The Raman and SERS measurements were acquired using a dispersive Bruker Senterra Raman spectrometer with high spatial and spectral resolution, connected to an Olympus MX51 reflected and transmission light microscope. For the excitation of the samples the 785 nm laser line at 100 mW was used. The laser was focused on the mouse skin using the 20× (NA = 0.4) or the 50× (NA = 0.75) Olympus objectives. The backscattered Raman signal was integrated for 3 to 5 s and the signal was acquired in the spectral range from 50 to 4000 cm^{-1}. Two accumulations were averaged for each spectrum to improve the signal-to-noise ratio. The spectral resolution of the instrument was 9 cm^{-1}. The detector used was 1024 × 256 pixels, 16-bit dynamic range Peltier-cooled CCD. Optical images were acquired using a video camera within the system. For the in vivo measurements, the mice were completely anesthetized with xylazine/ketamine. The Raman spectra were acquired by focusing the laser spot on the skin of the anesthetized mice. The increased heart rate and breathing was observed using the Raman microscope video-camera for defocusing reasons. Several measurements were carried out from different positions to account for heterogeneity. Although the spectra presented a fluorescence background, the signal to noise ratio was satisfactory.

The UV–vis spectra of the Ag NPs were collected using a Jasco V630 UV–Vis double-beam spectrophotometer with a silicon photodiode detector. The spectra were recorded in the 190–1100 nm range. The samples were placed in quartz cuvettes with an optical path length of 1 cm. Bidistilled water was used as the reference sample. The UV–vis spectra of the silver colloid were recorded from 3 ml volume of 1:2 ratio of water to silver colloid. The TEM images of the colloidal nanoparticles were recorded with a JEOL JEM 1010 electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan).

2.2. Animal protocol and samples preparation

BALB/c mice were obtained from Charles River laboratories (Köln, Germany). The work protocol followed all NIAH–National Institute of Animal Health rules and regulations at the University of Medicine and Pharmacy Biobase, Timisoara, Romania. The animals were maintained during the experiment in standard conditions: 12 h light-dark cycle, food and water *ad libidum*, temperature 24 °C, humidity above 55%. At the beginning of the experiment the mice specimens were 9 weeks old and the experiment was carried out for 9 weeks. For this experiment 10 BALB/c mice were used. The two-stage model of skin carcinoma was induced using a single application of the chemical initiator mutagen 7,12-dimethylbenzanthracene (DMBA) solution (0,025% – 100 μL) in the first week of experiment followed by repeated applications of a pro-inflammatory phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA) solution (5 nM–100 μL twice a week) [29]. A betulin containing oil-in-water nanoemulsion [30] was applied at 30 min before the carcinogen application in order to test the antitumoral properties of betulin. As control groups, we employed one untreated BALB/c mouse specimen and one specimen treated with acetone (solvent used for the dissolution of the chemicals) 2 times/week. Following the in vivo measurements, skin samples were collected from the immediate regions of the Raman measurement and fixed in 10% formalin solution and in formalin solution mixed with Lee Meisel Ag colloidal nanoparticles. Briefly, the preparation of the Ag NPs consisted of 100 mL of a 1 mM $AgNO_3$ three distilled aqueous solution heated to 93–100 °C to which 2 mL of a 1% trisodium citrate solution was added. The mixture was kept in constant (previously achieved) temperature for about 1 h and then it was allowed to cool down to room temperature. The resultant colloidal mixture was of dark gray color. Finally, the colloidal solution was centrifuged at 5500 rotations/min, for 5 min. For the ex vivo experiment, the skin samples were first immersed in formalin solution mixed with Ag colloidal nanoparticles and thin cut and placed on microscope slides at the time of the measurements.

2.3. Data analysis

The pre-processing of the spectra consisted of baseline correction by fitting and subtracting a first order polynomial from the acquired spectra and normalization at the 230 cm^{-1} band. The mean spectra were calculated by averaging 20 to 30 high quality normalized SERS spectra collected ex vivo from each of the skin pathologies.

Principal component analysis (PCA) and K-means clustering were applied for data analysis. PCA was employed to detect the differences and similarities between the spectra, which could help identify patterns in the Raman data and understand fundamental events in the biological samples. The PCs are expressed as linear combinations of the original variables and describe the maximum possible amount of variance contained in the original data set. K-means clustering, on the other hand, is a partitioning algorithm, which clusters the data into groups, in order to minimize the differences between the data within each cluster and maximize the differences between each cluster. This is done according to some defined distance measure, such as the Euclidean distance which works out satisfactorily for Raman data sets [31]. The algorithm is initiated by choosing the k number of clusters. The initial spectra for the centers of the clusters are taken randomly from the spectral