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PRELIMINARY STUDIES REGARDING AN EARLY DETECTION METHOD APPLIED FOR MALIGNANT MELANOMA ON MICE

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ABSTRACT. One of the most devastating types of cancer is represented by cutaneous melanoma. In order to study this pathology models have been developed that recapitulate the condition of melanoma invasion in vivo. In this study B16 mouse model for melanoma has been used on C57BL/6J mice. This work reports a comparative study on cutaneous melanoma evolution investigated by histopathology and UV-Vis-NIR spectroscopy. After housing for four weeks, four C57BL6 female, mice were inoculated with 0.5 ml B16 melanoma cell suspension prepared in the moment of using with saline solution. After three weeks mice with most representative skin lesions were chosen for analyses. Tissue samples (skin) were fixed in 10% formalin solution and were embedded in paraffin and cut at 4 microns. Finally after deparaffinized the samples were stained with H&E (hematoxylin-eosin) and microscopically analyzed. The biopsy for sample 1 was obtained in the day 7 after inoculation and for sample 10 after 20 days. UV-Vis-NIR absorption spectroscopy analyze were also made with a Lambda 950 UV-Vis-NIR double-beam spectrometer (Perkin Elmer) by transmission method. After 3 weeks from the inoculation phase the damages on the skin level are obvious. B16 cells are invasive and determine a metastatic process. In the NIR wavelength range 350-800 nm it can also be observed the evolution of melanoma. The conclusion of this study is that even if spectral data are not enough to make a diagnosis on skin pathology, their qualitative agreement with the histological evaluation results indicate that the method is promising for the development of techniques for the study and characterization of pigmented skin lesions. Further research is needed for the improvement of sample preparation method and development of UV-Vis-NIR diffuse reflectance spectroscopy techniques for this purpose.

Keywords: cutaneous melanoma, mouse, histopathology, spectroscopy

INTRODUCTION

One of the most devastating types of cancer is represented by cutaneous melanoma. Melanoma is a malignant tumor originating from melanocytes, cells mainly present in the skin in the basal layer of epidermis, hair follicle, inner ear, meninges and uveal tract of the eye. Transformation of skin melanocytes to cutaneous melanoma is a multistep process also called melanomagenesis (Okot et al., 2003; Gaggioli et al., 2007).

The first phase, considerate as benign, is represented by aberrant proliferation of melanocytes. It gives rise to melanocytic naevi within the epidermis that can show varying degrees of dysplasia. The majority of these do not give rise to malignant disease but a subset will begin to spread. The initial spread of dysplastic melanocytes is almost always lateral and within or very near to the epidermis. This step is called the Radial Growth Phase (RGP) and is associated with a good prognostic (Bar-Eli, 1997; Gaggioli et al., 2007). In this step melanocytes are clustered and have lost their appropriate contact with keratinocytes. During RGP melanocytes tend to proliferate superficially to the basement membrane of the epidermis. During the next phase, cells proliferate actively in a vertical manner in the dermis, crossing the basement membrane. This step is called VGP-Vertical Growth Phase. At this stage cells migrate into the dermis and become clearly invasive. Clinically it is more dangerous. Acquisition by the cells of metastatic characteristics: the cells are able to enter the bloodstream or lymphatic vessels from which they colonize tissues and organs is the ultimate tumor progression stage (Okot et al., 2003 ; Chin, 2003; Gaggioli et al., 2007). In order to study this pathology over the past 15 years many attempts have been made to use transgenic approaches in the mouse to model melanoma (Teicher, 2002; Yoshiura et al., 2005).In recent years new models have been developed that more closely recapitulate the condition of melanoma invasion in vivo. The B16 mouse model for melanoma has been extensively used mainly because it has the benefits of being metastatic and syngeneic with the C57BL6 background. As an in vivo model for solid tumor formation and metastasis the model has been instrumental in the dissection of the many steps now

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associated with tumor establishment and the metastatic cascade (Teicher, 2002). Because of the incidence and gravity of this pathology a rapid, new technique that could be utilized for characterization of skin lesions prior to biopsy would be useful.

Over the last decade, optical spectroscopy has rapidly evolved, as an in vivo tool for diagnosis and characterization of human skin (Zhiwei et al., 2006). Like all biological tissues, the skin is composed of biomolecules with specific biophysical properties that are determined by their chemical structure and microscopic environments. The biophysical properties of cutaneous chromophores such as melanin, hemoglobin, collagen, keratin, and lipids can be studied noninvasively and in vivo using a variety of optical spectroscopic techniques such as diffuse reflectance, fluorescence, and Raman scattering (Alaluf et al., 2002; Kollias et al., 2002; Zhiwei et al., 2006). Recently, Zonios and coworkers demonstrated that the histological transition from dysplastic nevi to melanoma in situ and then to malignant melanoma is reflected in the melanin absorption spectra. The optical absorption spectrum of melanin exhibits an exponential dependence on wavelength, characterized by a decay slope. For different pigmented skin lesion types, the value of the decay slope varies in a wide range and can be used for their relative identification (Zonios et al., 2008).

This work reports our comparative study on cutaneous melanoma evolution investigated by histopathology and UV-Vis-NIR spectroscopy metastatic and syngeneic with the C57BL6 background. As an in vivo model for solid tumor formation and metastasis the model has been instrumental in the dissection of the many steps now associated with tumor establishment and the metastatic cascade (Teicher, 2002). Because of the incidence and gravity of this pathology a rapid, new technique that could be utilized for characterization of skin lesions prior to biopsy would be useful.

Over the last decade, optical spectroscopy has rapidly evolved, as an in vivo tool for diagnosis and characterization of human skin (Zhiwei et al., 2006). Like all biological tissues, the skin is composed of biomolecules with specific biophysical properties that are determined by their chemical structure and microscopic environments. The biophysical properties of cutaneous chromophores such as melanin, hemoglobin, collagen, keratin, and lipids can be studied noninvasively and in vivo using a variety of optical spectroscopic techniques such as diffuse reflectance, fluorescence, and Raman scattering (Alaluf et al., 2002; Kollias et al., 2002; Zhiwei et al., 2006).

Recently, Zonios and coworkers demonstrated that the histological transition from dysplastic nevi to melanoma in situ and then to malignant melanoma is reflected in the melanin absorption spectra. The optical absorption spectrum of melanin exhibits an exponential dependence on wavelength, characterized by a decay slope. For different pigmented skin lesion types, the value of the decay slope varies in a wide range and can be used for their relative identification (Zonios et al., 2008). This work reports our comparative study on cutaneous melanoma evolution investigated by histopathology and UV-Vis-NIR spectroscopy.

MATERIALS AND METHODS

Animal models

C57BL/6J mice of eight weeks were purchased from Charles River (Germany). The work protocol followed all NIAH-National Institute of Animal Health rules: animals were maintained during the experiment in standard conditions: 12h light-dark cycle, food and water *ad libidum*, temperature 24 º C, humidity above 55%. The number of mice taken into study was eight. Mice were divided into two groups: group A, blank group formed by four mice, received nothing but food and water and group B formed by four mice received food , water and B16 melanoma cells. After housing for four weeks the mice from group B were inoculated with 0.5 ml B16 melanoma cell suspension prepared in the moment of using with saline solution as is described in cell culture protocol. From group B, mice with most representative skin lesions were chosen for analyses.

Cell culture

B16 melanoma 4A5 (ECACC and Sigma Aldrich, origin Japan stored UK) cells were grown in DME media, supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) in specific cells culture plates. Cells were incubated with 1 ml of media at 37°C in an incubator for cell culture with 95% air and 5% CO2. For the subcutaneous injection with B16 cells on mice were used 0.5 ml of cells without media, in saline solution.

Histology

Tissue samples (skin) were fixed in 10% formalin solution and were embedded in paraffin and cut at 4 microns. Finally after deparaffinized the samples were stained with H&E (hematoxylin-eosin) and microscopically analyzed. The biopsy for sample 1 was obtained in the day 7 after inoculation and for sample 10 after 20 days.

UV-Vis-NIR absorption spectroscopy

The tissue samples embedded in paraffin and cut to a thickness of 4 microns were placed on glass slides (1.1 mm thick) and subjected to UV-Vis-NIR absorption measurements. A glass slide of the same type was used as reference. The investigations were carried out on a Lambda 950 UV-Vis-NIR double-beam spectrometer (Perkin Elmer) by transmission method. Absorption spectra were acquired in the 330-1700 nm wavelength range with a resolution of 1 nm and the data were processed using UV Win Lab Standard v6.0.2 software.

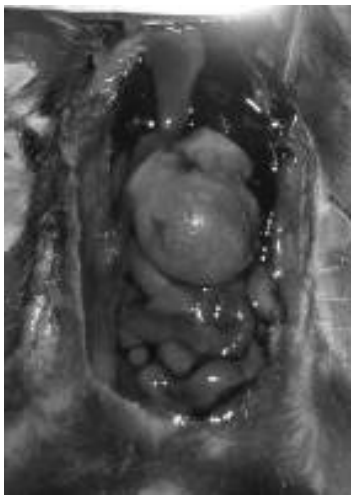

Fig. 1 Aspect of internal organs from a mouse in the blank group

RESULTS AND DISCUSSIONS

Figure 1 and Figure 2 presents images representing the evolution of melanoma after three weeks from inoculation. It can be observed that in the first case no organ was affected by cancerous cells while in the second picture it can be clearly seen the process of metastasis to different organs: liver, kidneys, lungs, gastrointestinal tract.

Results for the histological evaluation of the skin are presented in Figure 3 and Figure 4.

In the Figure 3 is represented the normal structure of the skin. The two cases of pathology presented in are very different. The second case represents the pathology in evolution by apparition of melanoma on dermal level. The cases analyzed in present work represent the beginning and the preliminary phase of the experiment. After 3 weeks from the inoculation phase the damages on the skin level are obvious. B16 melanoma cells are invasive and determine a metastatic process. Between the initial and final phase is a clear difference of pathologic signs. As a comparing method for early detection of malignant melanoma, UV-Vis-NIR technique was used (Figure 5).

The UV-Vis-NIR absorption spectra in the 330-1700 nm wavelength range for sample 1 and sample 10 are presented in Figure 5.

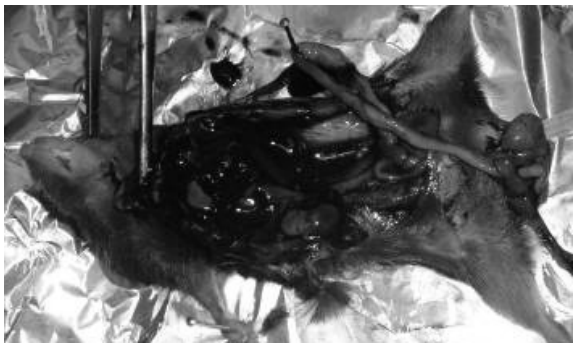

Fig. 2 Evolution of melanoma after three weeks from inoculation on a inoculated mouse

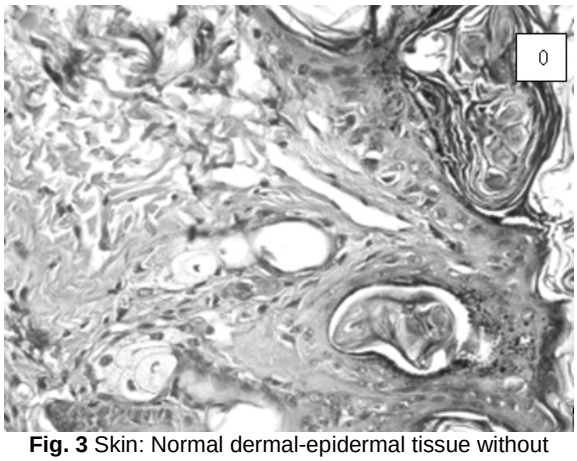

Fig. 3 Skin: Normal dermal-epidermal tissue without histological changes on a non-inoculated mouse (HEx400)

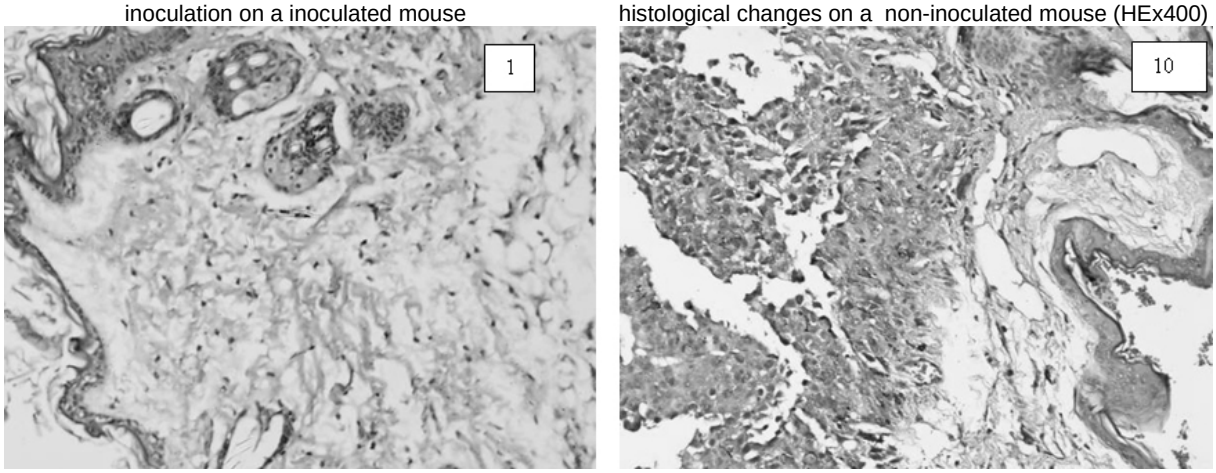

Fig. 4 Histological evaluations for tested skin on an inoculated mouse A (sample 1) a dermal-epidermal tissue with an atrophic epithelium and a dermal edema (HEx200). B (sample 10) Dermal-epidermal area with a tumor proliferation on dermal level and pigmented cells (HEx200)

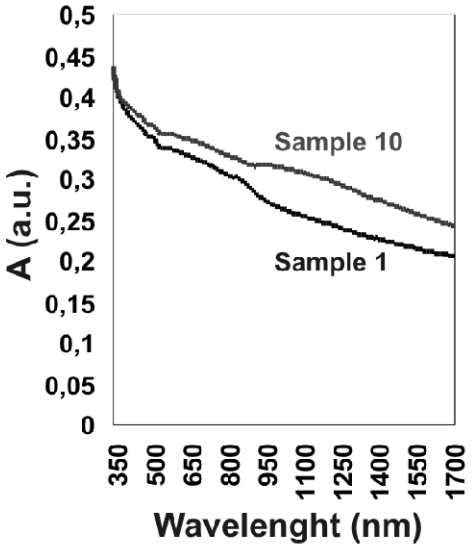

Fig. 5 Normalized UV-Vis-NIR absorption spectra of skin sample 1 and sample 10

As observed in Figure 5 in the 350-800 nm range one can see that the absorption curve decay slope of sample 10 is lower than that corresponding to sample 1 suggesting an evolution to melanoma. This is in accordance with our histological evaluation results: in the sample 10 one can observe the apparition of melanoma on dermal level indicating the evolution of the pathology.

The standard tool for the diagnostic of pigmented lesions is the histological examination, which allows assessment of the level of dysplasia, separation of premalignant from malignant lesions, and prediction of the behavior of melanoma (Elenitsas et al., 1998). In melanoma one of the most important criteria for prognosis is the type of growth: in situ, RGP- Radial Growth Phase of invasive superficial spreading melanoma or VGP- Vertical Growth Phase of invasive superficial spreading melanoma (Nickoloff, 2001).

As observing in Figure1 the second case represents the pathology in evolution by apparition of melanoma on dermal level. The cases analyzed in present work represent the beginning and the preliminary phase of the experiment. After 3 weeks from the inoculation phase the damages on the skin level are obvious. B16 melanoma cells are invasive and determine a metastatic process. Between the initial and final phase is a clear difference of pathologic signs. Even if the inoculation of the tumor cells was on the skin surface the spread ability along the body is clear and affects also the cutaneous organ. This could indicate a vertical distribution of pathology. The histological analyze of sample ten confirm the diagnostic: cells grow in form of expansible nodules in the dermis, whose cytology is different from the melanoma cells in the overlying epidermis. The intraepidermal melanocytes are of epitheloid shape with very fine melanin granularity, whereas the corresponding dermal nodules consist of spindle cells and small epithelioid cells. Yoshiura and coworkers underline that the appearance of on evident tumor after the inoculation of $1x10^5$ B16 tumor cells especially in tail vein is clear after 14 days from the injection point and all mice from the tested group are affected (Yoshiura et al., 2005) Roomi MW and coworkers confirm the metastatic capacity of B16F0 cells even if the inoculation was applied in the intrasplenic region. Any treatment applied on the studies was helpful in reducing the tumor weight but was not able to avoid the tumor cells spreadability (Roomi et al., 2008).

As a comparing method for early detection of malignant melanoma UV-Vis-NIR technique was used.

The in vitro spectral absorption characteristics of melanin have been extensively studied for various types of melanin, such as synthetic melanin produced by oxidation of tyrosine, melanin from *Sepia officinalis*, and melanin extracted from human and animal skin and hair by employing various chemical processing methods (Liu et al., 2003).

One way to assess melanin in vivo noninvasively is through the study of its optical properties. It can be measured using diffuse reflectance spectroscopy (Zonios et al., 2008).

As mentioned in the introduction, the optical absorption spectrum of melanin exhibits an exponential dependence on wavelength, characterized by a decay slope which varies in a wide range for different pigmented skin lesion types and this fact can be used for their identification (Zonios et al., 2008). In their diffuse reflectance studies, Zonios and coworkers concluded that the decay slope of the absorption spectra of melanin decreases with the evolution to melanomas. In our study (see Figure 2), similarly, in the 350-800 nm range one can observe that the absorption curve decay slope of sample 10 is lower than that corresponding to sample 1, suggesting an evolution to melanoma. This is in accordance with our histological evaluation results: in the sample 10 one can observe the apparition of melanoma on dermal level indicating the evolution of the pathology.

In the NIR wavelength range one can also observe (Figure 2) differences between the shapes of the two spectra, which are difficult to interpret in this preliminary stage of our experiments.

CONCLUSIONS

Although these preliminary spectral data are not enough to make a diagnosis on skin pathology, their qualitative agreement with the histological evaluation results indicate that the method is promising for the development of techniques for the study and characterization of pigmented skin lesions. The model that applied the inoculation of B16 cells on C57BL/6J mice is proper for an experimental analysis of melanoma. Further research is needed for the improvement of sample preparation method and

development of UV-Vis-NIR diffuse reflectance spectroscopy techniques for this purpose.

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