The histological analysis by transdermal drug delivery on melanoma therapy

L.G. D’Agostino1,2, A. Salomao-Junior3, D.C. Viana2, A.C.L. Luna1,2 and D.A. Maria1

1 Biochemistry and Biophysics Laboratory, Butantan Institute, Av. Vital Brasil 1500, Sao Paulo, Brazil
2 Medical Sciences, Faculty of Medicine, University of São Paulo, Av. Dr. Arnaldo 455, Sao Paulo, Brazil
3 Department of Surgery, Faculty of Veterinary Medicine and Animal Science, University of Sao Paulo, Av. Prof. Dr. Orlando Marques de Paiva 87, São Paulo, Brazil

Melanoma is a type of skin cancer with the worst prognosis of all cancers due to its high potential to produce metastases. Despite the significant scientific advances in the biology and genetics of melanoma, there is currently no effective treatment against metastatic melanoma. In addition to its low efficacy, the chemotherapy traditionally used in the treatment of melanoma has many side effects. In order to make such a substance cross the epidermal barrier, was utilized a method of transdermal drug delivery system (TDDS) and thermal ablation combined with the chemotherapeutic agent etoposide in the treatment of dorsal tumor on mice C57BL/6J bearing B16F10 melanoma. The animals were randomly distributed into 3 experimental groups to observe the therapeutic effect of the different treatment strategies compared with control group. The mice without tumors were used as control. During necropsy, macroscopic evaluations of the tumor followed by sampling of tumor masses and irradiated adjacent tissue were performed. Then histological sections from all organs removed were stained with hematoxylin and eosin (HE) and the slides were scanned into a digital virtual microscope and displayed on an LCD monitor with the help of the program OlyviaTM Olympus Software. The following parameters were evaluated in tumors: 1) size; 2) symmetry; 3) restraint systems; 4) maturation; 5) pagetoid spread; 6) necrosis/ulceration; 7) inflammatory infiltrate; 8) regression; 9) cellular changes; 10) index mitotic; 11) pigmentation; 12) cells proliferation.

Keywords: melanoma; chemotherapy; digital virtual microscope

1. Introduction

1.1 Melanoma

The incidence of melanoma cases is increasing worldwide and despite early detection, appropriate surgical resection and adjuvant therapy, the number of patients dying from metastatic disease continues to rise. According to the World Health Organization (WHO), approximately 80% of all skin cancer-related deaths are attributed to melanoma, although it comprises only 5% of all skin cancers [1]. The prognosis of advanced melanoma remains poor, with the median survival ranging from 6 to 9 months with chemotherapy [2]. Survival outcomes for patients with advanced disease vary depending on the number of adverse prognostic factors that are present such as visceral disease or brain metastases and whether or not serum lactate dehydrogenase (LDH) levels are elevated [3,4,5]. Although the five-year survival for local disease is as high as 98%, it falls to 15% for those patients with distant metastases [5]. Despite extensive clinical research, the treatment options for metastatic disease were limited, with melanoma being considered as one of the most chemotherapy-resistant malignancies. Many agents have been investigated in terms of their antitumor activity in melanoma, but the efficacy of treatment remained poor [6].

1.1.1 Pathogenesis and tumor progression

Primary melanoma is a malignant neoplasm of neural crest–derived melanocytes, specialized pigmented cells predominately found in the basal layer of the epidermis. The normal function of melanocytes is to produce and transfer a dark pigment called melanin to mitotically active keratinocytes, which are also found in the epidermis. The transferred melanin is concentrated in the perinuclear space of keratinocytes and protects the nucleus from UV radiation damage [7].

The transformation of melanocytes to tumor cells occurs in both genetically normal and predisposed patients. Although melanoma pathogenesis is complex and not completely understood, it likely involves interactions between environmental factors, accumulation of sequential genetic alterations, activation of oncogenes, inactivation of tumor suppressor genes, and impaired DNA repair [8,9].

Three distinct pathogenic steps have been proposed in melanoma tumor progression. In an early-stage tumor, the melanoma may be confined to the epidermis and displays only radial (or lateral) growth. When melanoma progresses, it can develop into microinvasive melanoma, in which microscopic extensions invade the superficial papillary dermis. More advanced melanomas can progress to the vertical growth phase, which is characterized by invasive growth with discernable involvement deep into the dermis. In this stage of growth, the melanoma has gained the potential to metastasize [10].
1.2 Chemotherapy

Numerous cytotoxic agents, as single agents and in combinations, have been evaluated for the treatment of metastatic melanoma but none has ever demonstrated a survival advantage. Nevertheless, several chemotherapy regimens are often used in advanced disease, including dacarbazine (DTIC) and temozolomide. DTIC is the only chemotherapeutic agent approved by the Food and Drug Administration (FDA) for treatment of melanoma and is often considered “standard” therapy for advanced melanoma, despite its limited activity. DTIC is considered the most active single agent in patients with melanoma; however, relative response (RRs) are <20%, complete responses (CRs) are rare, and a survival advantage has never been demonstrated [11].

The dominant toxicities are myelosuppression, nausea, and emesis. Because it is generally well tolerated and delivers low but consistent RRs, DTIC became the de facto melanoma chemotherapeutic agent and was used in a comparison arm in several pivotal clinical trials. Temozolomide is an oral alkylating agent that breaks down into metabolites similar to those of DTIC. The RRs for temozolomide are similar to DTIC, but temozolomide may be better tolerated. Though not FDA approved for melanoma, tamozolomide differs from DTIC by its ability to penetrate the bloodbrain barrier. A randomized trial comparing DTIC with temozolomide demonstrated similar RRs of 12.1% and 13.5%, respectively [12].

1.2.1 Etoposide

Etoposide is a semi-synthetic derivative of podophyllotoxin poorly soluble in water [13]. At low concentrations (0.3 to 10 mg/ml), this drug inhibits the entry of neoplastic cells in prophase, probably due to its action on topoisomerase II [13].

At high concentrations (10 mg/ml or more), the lysis of cells entering mitosis is observed. The predominant molecular effect is the inhibition of DNA synthesis. The cytotoxicity and apoptosis induced by Etoposide were studied for 72 hours in human melanoma cells. Etoposide initially damages DNA by ATM kinase and by activating p53 and caspase 2 pathways. Consequently, there is an initial increase in the amount and activity of mitochondria of target cells, but which are subsequently suppressed. These changes were not preceded by loss in membrane potential and the release of cytochrome c. After oral administration distribution in CSF (Cerebrospinal Fluid) is variable and weak; Etoposide is mostly distributed in the liver, kidneys, brain, heart and bowels [13].

1.3 Transdermal drug delivery system (TDDS)

Transdermal drug delivery (TDD) employs skin as the administration route for pharmacologically active drug molecules. There are various advantages of TDD over oral delivery, such as the improved bioavailability due to the avoidance of first-pass effect, less fluctuation in plasma drug concentration due to the elimination of variation in pH and gastrointestinal transit time, minimized side effects by circumvention of direct contact with gastrointestinal tract and less frequent administration. Since the first introduction of scopolamine patch for motion sickness in 1981, TDD patches attained high attention as an attractive delivery route for some potent lipophilic drug molecules with low biological half-life. In spite of the enormous effort to develop diverse TDD patches, only about 40 transdermal patch products has been introduced into the market for about 20 drug molecules [14]. We can think of two reasons for this low number of drug molecules developed. One reason is the limitation in the amount of drug that can be delivered through skin for a day using a reasonable size of patch (usually less than 10 mg). The other reason is the barrier property of skin for the penetrating drug molecules. This barrier property is mainly originated from the stratum corneum (SC), the outmost layer of the epidermis. It exhibits multilayer structure of flattened dead cells suspended in hydrophobic lipid matrix. This hierarchical structure is known to provide the barrier property to skin for penetrating substance [15]. There are three major pathways for skin penetration: transcellular, intercellular and appendageal route. The intercellular route through lipid lamellae is known to be the major pathway [16]. The tortuosity of this route elongates the diffusion length substantially and provides significant resistance to the drug permeation [17]. The transcellular route can be a more direct route, however, drug molecules have to pass both hydrophilic and lipophilic domains and face significant resistance to the penetration. Recently, it has been shown that penetration through appendageal route can be an important pathway, though they occupy only 0.1% of the total skin surface area [18]. Appendages (hair follicles and sweat glands) and microlesions in the SC were proposed as vertical pathways for skin penetration [19]. Hence, it is extremely important for TDD to develop new permeation enhancing methods in order to enlarge the number of drug molecules that can be employed in TDD. Various chemical, physical and biological compounds permeation enhancing methods have been developed and applied. Such methods include the use of chemical solvent, iontophoresis, electroporation, sonophoresis, microneedle application, skin ablation, heating and prodrug. Among these methods, incorporation of chemical solvent into the formulation is the simplest way without the use of extra physical devices. Numerous hydrophilic/lipophilic chemical enhancers have been investigated for their capability as the skin permeation enhancer. These enhancers are reported to interact with the SC at various sites in various mode of action.

They can interact with intercellular lipid matrix and disrupt the structure (orderness) of the alkyl chain. Azone is known to partition into a bilayer lipid and change the orderness of SC lipid to a more liquid like structure [20]. Azone
molecules may exist dispersed or form a separate domains within lipid matrix [21]. DMSO seems to extract lipids and form aqueous channels [22]. It also has been shown to change the intercellular keratin conformation [23]. Lipid extraction is reported to be the main reason for the enhancement effect of ethanol [24]. ATR-FTIR study using deuterated oleic acid (OA) revealed that OA forms separate domain in the SC lipid matrix and forms permeable defects [25].

Surfactants are reported to act as solubilizer for lipids in the SC [26]. Though the mechanism of action for these penetration enhancers has been widely studied, the mode of action for penetration enhancement is not conclusive yet, and further explanation needs to be provided for better understanding. As a physical skin penetration enhancing method, iontophoresis employs low electric current to enhance the flux of drug molecules. It enhances primarily the delivery of charged molecules, and it also enhances the transport of neutral molecules across skin by lowering the barrier property of skin [27,28]. The typical advantages of iontophoresis are the ease of onset and termination of delivery, the better control over the rate of delivery than other conventional passive patches and programmable drug delivery [29]. Though iontophoresis of ionic species has been used in simple topical treatment for a long time, it has gained significant attention with the progress of biotechnology in 1980’s, because various peptide/protein drugs became available in large quantity for therapeutic usage. However, these drugs are too large and hydrophilic to pass through SC by passive diffusion from the applied patch. As an alternative way to injection, iontophoresis gained particular attention due to its potential to deliver these peptide/protein drugs in a noninvasive way. Currently, iontophoresis is used not only in delivery of various pharmacologically active substances, such as fentanyl, lidocaine and vitamin C, but also in various other areas, such as the treatment of hyperhidrosis, the diagnosis of the cystic fibrosis and the sampling of biological fluids [30].

The total flux (Jt) by iontophoresis can be described by the modified form of the Nernst–Planck equation, which includes the convective solvent flow due to the permselectivity of the skin [31,32]:

\[
J_t = -D_i \left( \frac{\partial C_i}{\partial X} + \frac{C_i Z_i F \partial \phi}{RT} \frac{\partial X}{\partial X} \right) \pm V_w C_i
\]

where J is flux, D is diffusion coefficient, C is the concentration of ionic drug molecule, X is the thickness of skin, Z is the charge of the ion, F is the Faraday constant, R is the Boltzmann constant, T is absolute temperature, u is the potential applied to the skin and Vw is electroosmotic volume flow (EVF). In this equation, iontophoretic flux is described as the sum of three distinct contributions:

\[
J_t = J_{\text{diffusion}} + J_{\text{electromigration}} + J_{\text{electroosmosis}}
\]

\[
= J_D + J_{ER} \pm J_{EO}
\]

where JD is the flux by passive diffusion, JER is the flux by electrorepulsion and JEO represents the electroosmotic flux. JER plays a major role in transport, and the transport of cationic drugs is enhanced from anode, and that of anionic drugs is promoted from cathode. JD is usually very small, when compared to JER or JEO. Flux by EVF, JEO, occurs due to the net negative charge of the current conducting pathways in skin at physiological pH. Hence the pathways are permselective to cations, and result in the convective solvent flow from anode to cathodal direction [33]. The magnitude of EVF has been estimated to be about 1–3 µl/cm² h [34]. EVF further increases the anodal transport of cations and impedes the cathodal flux of anions. It also increases the anodal transport of neutral drug molecules [27]. Hence, the modulation of EVF may play a significant role in the delivery of drug through skin. EVF can be modulated by the addition of hydrophobic/cationic peptide like nafarelin, or lipophilic/positively charged β-blocking agents like propranolol, timolol, metoprolol and atenolol in the formulation [35, 36].

Various physicochemical factors, such as current density, current application duration, current type, ionic strength and molecular size can change the iontophoretic flux through skin. Flux may also be affected by the formulation factors, such as pH and the addition of organic additives or cosolvents. Sometimes, penetration enhancers are applied in combination with iontophoresis to achieve a synergistic or additive flux enhancement effect. However, these additives and penetration enhancers may partition into the SC and change the dielectric property of lipid domain. They also can modify the lipid structure and thus alter the current conducting pathways in SC. Because these additives and penetration enhancers may change the density and viscosity of the solvent flowing through the current conducting pathways, EVF can also be significantly altered.
1.4 Digital image

The integration of control by the microscope computer with the acquisition and analysis of images has created a new area, called Digital Microscope. Allows accurate degree of automation, enabled real microstructural characterizations [37].

This procedure ensures automatic addition of greater speed, and practicality is reproducible and avoids the occurrence of errors by operator fatigue, as repetition and overlapping fields. Furthermore, correction routines and self-tuning of the microscope make parts of this type of imaging.

To be able to computational processing, an image must be digitized to form a digital image. The digitization of the spatial coordinates of a real image is called image sampling [38].

The digital image is an ordered set of pixels. The pixel acronym of the phrase in English picture element is the smallest unit of digital imaging. A digital image is a matrix where each of its elements is a number that represents the color or intensity of the corresponding pixel position in the real image. Thus, the matrix formed is the digital representation of the image point to point.

The spatial resolution or simply resolution of a digital image is the sampling frequency of the image. It denotes the smallest portion of the image that can give quality; resolution can also be defined as the total number of pixels in each axis of the image. Another important parameter in image quality is the quantization that is the maximum number of levels of intensity or color that this may present. Another inherent characteristic of the image and its analysis are important for brightness and contrast. Are mathematically described respectively as the mean and standard deviation of the intensities of all pixels in the image [39].

Focusing is one of the main characteristics discussed in the case of microscopy, with the advances in this area, the autofocus has become implementable through processing and digital image analysis. This requires the computer control the height of the sample (z-axis), digital image acquisition and image analyzer software, all these integrated. The autofocus is intended to ensure proper focus on each field, even if the sample is not completely flat. To avoid variation in focus, the sample should be placed on the microscope stage so that the surface to be observed is perfectly orthogonal to the optical axis of the microscope.

2. Materials and methods

2.1 Digital microscopy

Our group uses the Olympus VS110 system that allows to combine microscopy to image. The result is an extremely versatile and advanced 'virtual' blade a high-resolution image of the entire specimen. Can be viewed from the overall image in low magnification up to maximum magnification via continuous zoom. Another novelty of this system is the ability to view the entire specimen at different focal planes, allowing for further analysis of the sample to obtain in-depth data. Samples stored electronically can be seen immediately and simultaneously by another controller form. Some features are fundamental to understand the composition of this type of image they are hybrid design, high capacity, automatic productivity, easy operation and global access. The careful integration of all components generates a highly flexible system that allows for quick and effortless digital slides user acquisition. The slides can be scanned in 20x/N.A. 0.75 and 40x/N.A. 0.95 true with automated detection of tissue and ability to scan multiple regions of interest. There is an option for oil 60x/N.A. 100x/N.A 1:35 and 1:40.

The Virtual- Z, a feature of scanning multiple z planes and virtual focus, makes it ideal for scanning thick samples, when the depth of focus is important system. The VS110 also provides a review of the images of the blade, which allows individual expansions of various regions of interest. The advanced software provides experienced users full control of the details of the scanning process. Therefore, various scan settings can be established to the slides, saving significant time scanning and providing the ability to scan only the areas of interest, reducing the size of the data file. The VS110 pictures are stored and transmitted, and a researcher may invite the rest of your group to see the blades of interest, individually or in groups, they are in the same place or not.

2.2 Animals

Fifteen female C57BL/6 J and Balb-c mice aged 8–12 weeks were purchased from the Central Animal Care Unit at Butantan Institute (São Paulo, Brazil). The animals were housed in a temperature-controlled room at 25 °C, with food and water provided ad libitum. All experimental procedures were carried out in accordance with the guidelines for animal experimentation determined by the Butantan Institute Animal Care committee. The study protocol was approved by the Butantan Institute for the Use of Animal (process number 927/12).

2.3 Cancer Melanoma tumor Cells Implantation in C57BL6/J mice

The B16F10 (CRL-6475) melanoma cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). The melanoma cell line was cultured in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% heat-
inactivated fetal calf serum (Cultilab, Campinas, Brazil) 2 mM L-glutamine (Gibco), 5 mM 2-mercaptoethanol (Gibco), penicillin and streptomycin (100 μg/mL; Gibco). Cells were incubated at 37 °C in a humidified incubator containing 5% CO2. To evaluate the time of the melanoma cell growth, the in vivo growth of melanoma cells in C57BL/6 mouse was determined after the subcutaneous (s.c.) inoculation of B16F10 cells. C57BL/6 female mice, 6 weeks of age, were obtained from Butantan Institute and were housed under filter top conditions with water and food *ad libitum*.

The animal model experiments were carried out in accordance with the guidelines for animal experimentation determined by the Institutional Animal Care from Butantan Institute. Mice were s.c. inoculated in flanks with 10⁵ B16F10 cells, the tumor diameters were measured every day with a slide caliper and tumor volume calculated using the formula: volume (mm³) = width (mm²) × length (mm)/0.52.

### 2.4 Treatment of the animals with melanoma

After twenty-eight days after the implantation of the tumor cells, mice were randomly distributed into 3 experimental groups to observe the therapeutic effect of the different treatment strategies. The mice without tumors were used as control. The treatment groups were as follows:

**Group I**: mice with dorsal tumor treated with Etoposide followed by RF (n=05).

**Group II**: mice with dorsal tumor treated with Etoposide followed by RF (n=05).

**Group III**: mice with dorsal tumor no treated (n=05).

The treatment consisted in thermal ablation induced by fractionated RF. The equipment used was Eunsung, Duet RF model, which is heated with bipolar radiofrequency electrodes. The device supplies 300 W power has resistance-type radiofrequency and various densities. The spot used has a density of 100 points/cm². Spots are of metal material to allow electrons to flow freely. The amount of heat energy used was (39 Joules), duration of the pulse was 0.4s, level II on the dorsal area in a single session. The drug was applied immediately before and after radiation, according to the proposed treatment group.

The concentration of drug used was 100mg/5ml. It is available in ampoules, and diluted on saline solution. A thin layer of the drug was applied on the animal’s skin surface without friction.

### 2.5 Necropsy of animals

Forty-eight days after the beginning of the experimental period the animals were sacrificed with the use of anesthetics (Ketamine Hydrochloride 35 mg/ kg and Hydrochloride Xylazine 80 mg/ kg) followed by cervical dislocation (according to article VI of the Brazilian Association for Laboratory Animal Science (SBCAL/COBEA and the Canadian council animal rules). The abdominal cavity and internal organs (heart, lungs, liver, kidneys, lymph nodes, spleen, brain and Peyer plaques) were macroscopically observed in the search for possible metastases. Then, the organs were weighed and placed in buffered formalin (pH=7.4) with identification of the animal and group to which it belonged for subsequent histopathological analysis.

### 2.6 Histopathological analysis of tumors and internal organs

The specimens were sent to the routine histological processing (Histotec). Five μm histological sections from all organs removed were stained with hematoxylin and eosin (HE) and examined by light microscopy.

Parenchyma of the various organs was evaluated according to the following parameters: presence, intensity and characteristics of inflammatory responses such as predominant cell type in the exudate, fibroblast and vascular proliferation.

The following parameters were examined in the samples of B16F10 murine melanoma tumors: 1) size; 2) symmetry; 3) restraint systems; 4) maturation; 5) pagetoid spread; 6) necrosis/ulceration; 7) inflammatory infiltrate; 8) regression; 9) cellular atypias; 10) mitosis; 11) melanization; 12) isolated cells proliferation. Histological sections were analyzed by a pathologist in a blind procedure.
3. Results

3.1 Microscopic examinations of the tumors

Fig. 1  (A and B) Skin showing epidermis, dermis, skin appendix without histological changes. Note the subcutaneous tissue area showing healing process (increase of fibroblasts and mononuclear inflammatory infiltrate (arrow); (C) Skin showing epidermis, dermis and subcutaneous tissue attachments without histological changes; (D) Subcutaneous cellular tissue showing intense tumor infiltration by mononuclear cells (*). Note the presence of areas of necrosis (arrow); (E) Subcutaneous cellular tissue showing invasion by tumor cells (*). Note the presence of areas of necrosis. These cells are shown in apoptosis and necrosis (arrow); (F) Presence of nuclear pyknosis and cariorgex (*). These cells are shown in apoptosis and necrosis (arrow).

Fig. 2  (A) Skin showing epidermis, dermis, skin appendix without histological changes. Note the subcutaneous tissue area showing healing process (increase of fibroblasts and mononuclear inflammatory infiltrate (arrow); (B) Note the subcutaneous tissue area showing healing process (increase of fibroblasts and mononuclear inflammatory infiltrate and presence of areas of coagulation necrosis (arrow); (C) Skin showing epidermis, dermis and subcutaneous tissue attachments without histological changes.
4. Discussion

TDDF indeed seems to be a method that alters the permeability of the skin to lipo and hydrophilic substances. There are several TDDF methods. However, the latest methods (third generation) seem to be the most effective and with fewer side effects[40]. The choice of the method, as well as the sequence of application plays a key role in the expected clinical outcomes.

Forms of TDDF applications.

The present study showed significant differences in the results of the different sequences of application of transdermal drug infusion. In the group treated first with the drug and then with RF tumor growth was significantly greater. In the group treated first with RF and then the drug, there were lower rates of tumor growth.

This can be an evidence that when the drug is applied before RF, it is destroyed by the heat generated by the technology. Degraded, the drug does not prevent tumor growth even when the skin is permeated by the columns of coagulation.

However, when the drug is applied after RF, its properties remain intact and it produces the desired therapeutic effect, that is, lower tumor growth.

Therefore, it is concluded that if there is a correct sequence of TDDF application, it would be as follows: first RF and then the drug.

Induction of tumor growth by skin aggression by columns of coagulation.

The procedure based on fractional photothermolysis is part of the daily routine of the professionals who work with laser and widespread in the aesthetic area. In recent years, several professionals, many of them not graduated from a medical school and others who are not specialized in this field, have been using this technology.

However, this study found that treatment with RF considerably increased the speed of tumor growth. In other words, there was a significant increase in tumor size after application of RF. This is a strong indication that these technologies can worsen a preexisting condition in the skin of patients, such as melanocytic nevus or dysplastic nevus. Therefore, it is important to clarify that the referred Technologies are not as harmless at it has been reported.

References
