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REVIEW

Developing strategies to predict photodynamic therapy outcome: the role of melanoma microenvironment

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Abstract Melanoma is among the most aggressive and treatment-resistant human skin cancer. Photodynamic therapy (PDT), a minimally invasive therapeutic modality, is a promising approach to treating melanoma. It combines a non-toxic photoactivatable drug called photosensitizer with harmless visible light to generate reactive oxygen species which mediate the antitumor effects. The aim of this review was to compile the available data about PDT on melanoma. Our comparative analysis revealed a disconnection between several hypotheses generated by in vitro therapeutic studies and in vivo and clinical assays. This fact led us to highlight new preclinical experimental platforms that mimic the complexity of tumor biology. The tumor and its stromal microenvironment have a dynamic and reciprocal interaction that plays a critical role in tumor resistance, and these interactions can be exploited for novel therapeutic targets. In this sense, we review two strategies used by photodynamic researchers: (a) developing 3D culture systems which mimic tumor architecture and (b) heterotypic cultures that resemble tumor microenvironment to favor therapeutic regimen design. After this comprehensive review of the literature, we suggest that new complementary preclinical models are required to better optimize the clinical outcome of PDT on skin melanoma.

Keywords Melanoma · Photodynamic therapy · Tumor microenvironment · Monolayer · Spheroids

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"From melanocyte to melanoma": introduction

Melanoma is a type of skin cancer that is characterized by its high aggressiveness and significant therapeutic resistance. Even when it represents 4 % of all diagnosed cutaneous cancer, the death rates account for more than 70 % [1]. Whereas UV exposures were given in increments, associated to changes in fashion regarding very tanned skin tones and socioeconomic factors [2], the number of diagnosed melanoma have increased since the last 30 years [3].

Melanoma arises from cells called melanocytes, whose physiological function is the production of melanin. Melanin is a pigment that absorbs harmful UV light; thus, it protects the skin from sun damage [1]. After UV exposure, melanin synthesis is induced, causing the skin to visibly tan. However, when UV exposure is intense, the sunburn triggers cumulative DNA damage that ultimately leads to genetic alterations in melanocytes [4].

Melanoma can be classified into different categories, considering (a) cell of origin, (b) onset age, (c) ethnic distribution, (d) patient exposure to UV radiation, (e) predisposing hereditary mutations, (f) profile of somatic mutations, (g) mutational processes, (h) clinical and histologic features, and (e) presence of metastatic focus [5, 6]. In addition, according to the degree of melanin accumulation within the tumor lesion, malignant melanomas can be classified in pigmented (melanotic, black) and unpigmented (amelanotic, less differentiated) [4]. Progression of melanoma is a step-by-step process. Initially, in the radial growth phase (RGP), melanoma is characterized by atypia and hyperplasia, clonal proliferation in the epidermis but rarely in the dermis, and decreased differentiation. If they are surgically removed, RGP melanomas are associated with long-term metastasis-free survival. When melanoma cells break the basement membrane and enter into the dermis, it is called vertical growth phase (VGP). Finally, malignant cells acquire the ability

to metastasize preferentially in lymph nodes, lungs, brain, and liver [6]. Interestingly, melanocytes can result in RGP and VGP melanoma cells, and both can progress directly to metastatic melanoma [7, 8].

At a molecular level, this disease is based on genetic and epigenetic alterations and cellular interactions that influence tumor behavior are also important. Genes such as BRAF and N-RAS are usually mutated in melanoma. In addition to mutations, cutaneous malignant melanoma is characterized by chromosomal gains which include the melanoma oncogenes CDK4, cyclin D1 and microphthalmia-associated transcription factor (MITF), and chromosomal losses which encompass the tumor suppressor genes p15INK4b, p16INK4a, p14ARF, and phosphatase and tensin homolog (PTEN), among others [9]. Epigenetic changes that are implicated in melanoma progression include DNA methylation, non-coding RNAs, and histone modifications [10, 11].

As well as other types of tumors, melanoma is composed not only by tumor cells but also by non-transformed keratinocytes, melanocytes, fibroblasts, endothelial cells, infiltrating immunocytes, and others. Normal and malignant cells establish paracrine and physical interactions, which influence tumor behavior in a multifaceted way [12].

Treatment of cutaneous melanoma: PDT as an emergent therapy

Different standard treatments are currently available for patients with cutaneous melanoma: (a) surgery, to remove the tumor; (b) chemotherapy, as adjuvant or therapeutic option; (c) external radiation therapy; (d) biologic therapy, in order to stimulate patients' immune system: ipilimumab, tumor necrosis alpha (TNF-α), interleukin-2 (IL-2), and interferon; and (e) targeted therapy, which involves angiogenesis and signal transduction pathway inhibitors, and monoclonal antibodies- and oncolytic virus-based therapies [1].

When diagnosed early, melanoma is curable by surgery alone, with 80 % of patients relapse-free 10 years after surgery. However, if melanoma has spread to regional lymph nodes or metastasized to distant sites, the 10-year survival rate for patients is less than 10 % [13]. Thus, an improvement in overall survival among patients with metastatic melanoma has been an elusive goal.

Photodynamic therapy (PDT) is considered a promising approach to treat melanoma. PDT is a therapeutic modality which combines a non-toxic drug called photosensitizer (PS) and PS harmless activating visible light. The PS activated generates singlet oxygen and other reactive oxygen species (ROS) (Fig. 1). PDT is recommended by its non-invasive and good cosmetic results.

The tumor regression mechanisms induced for this therapy include (1) direct oxidative stress-associated tumor cell, (2) indirect vascular damage, and (3) host immune system potentiation. An advantage of PDT is that treated area is located, so this therapy causes minimal damage to healthy tissue [14].

A good PS for PDT schedule should be chemically pure with good stability, maximal efficacy upon activation and high quantum yield of singlet oxygen, and minimal toxicity in the absence of light and preferentially accumulated and retained by target tissue [14].

Current guidelines for the use of topical PDT limit its use to precancerous and non-melanoma skin cancers [15]. Regarding cutaneous melanoma, PDT was used as second- or third-line treatment (Table 1). The initial report dates back to the 1970s, when Dougherty and colleagues went on to perform the first PDT trial on patients with skin melanoma using the PS Hematoporphyrin derivative (Hpd). Six patients with pigmented skin melanoma were treated with 5 mg/kg body weight (BW) of Hpd injected intravenously followed by irradiation with xenon lamp (λ=600–700 nm); five of them exhibited total tumor regression [16] (Table 1). Unfortunately, there is no available information about the relapse-free period of those patients.

Despite the promising results from earlier clinical trials of PDT on cutaneous melanoma, no significant survival was observed in patients treated with other photosensitizers. Treatment of 14 patients with pigmented skin melanoma metastases using Chlorin e6 injected intravenously at a dose of 5 mg/kg (body weight) combined with a 250–300 mW/cm^2 laser irradiation (λ=670 nm) showed complete regression after the first PDT (57 %) or after multiple treatments (43 %). However, 11 of 14 patients died due to the progression of the melanoma (pulmonary, cerebral, and hepatic metastases) [17] (Table 1). Similarly, when methyl-aminolevulinic acid (MAL) was topically applied on unpigmented cutaneous melanoma in situ and then photoactivated with 40 J/cm^2 of red light (λ= 633 nm), recurrence at the original tumor site was reported 4 months after PDT [10]. Infusion of Photosan III followed by an argon dye laser exposure (λ=514 nm) in one patient with skin melanoma metastasis reported no meaningful change in overall patient survival [19] (Table 1).

As shown, malignant melanoma and other pigmented tumors are relatively resistant to PDT effect. Researchers have suggested that the presence of the melanin in pigmented tumors will result in inefficient phototoxicity during PDT treatments because melanin may compete with the PS for the absorption of light [20]. The peak absorption of human melanin pigment occurs around 335 nm, and absorption is almost completely attenuated for wavelengths longer than 700 nm [21]. The aforementioned photosensitizers (Hpd, Chlorin e6, Photosan II, MAL) were excited with light sources that emitted visible radiation across the spectrum of wavelengths absorbed by melanin, so this probably interfered with PDT therapeutic efficiency. However, despite the unpigmented melanoma that lacks melanin protection, they develop additional therapeutic resistance mechanisms [10].

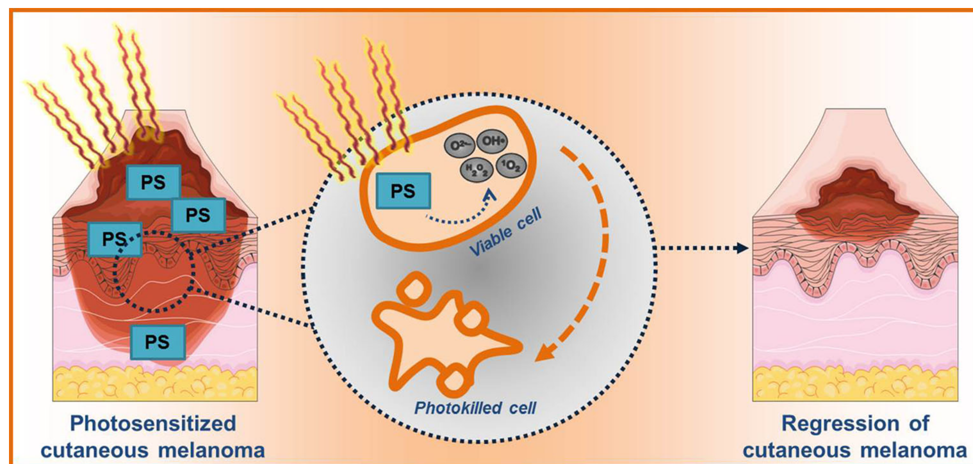

Fig. 1 Photodynamic therapy on cutaneous melanoma. Photodynamic therapy (PDT), an emerging approach to treating skin melanoma, involves the application of a non toxic photoactivatable drug called photosensitizer with visible light irradiation. Singlet oxygen ($^1O_2$) and other highly reactive oxygen species such as superoxide radical anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), or hydroxyl radical ($HO^{\bullet}$) are generated within photosensitized melanoma cells. Tumor regression is predominantly mediated by direct oxidative stress-associated phototoxicity. *PS* photosensitizer, *PDT* photodynamic therapy. Images used under Creative Commons Attribution 3.0 Unported License from http://www.servier.com/Powerpoint-image-bank

Targeting of melanoma has historically focused on the cancer cells, but has failed in effectively treating this cancer. Over the past few years, however, it has become increasingly evident that the tumor and its stromal microenvironment have a dynamic and reciprocal interaction that plays a critical role in tumor initiation, progression, metastasis, and therapeutic resistance. These interactions can be exploited for novel therapeutic targets [22]. In this sense, in situ photoimmunotherapy (ISPI) regimen has been developed in order to improve therapeutic efficiency of photodynamic treatment [23, 24]. ISPI is a promising modality for the treatment of metastatic melanoma that combines local, selective photothermal therapy (PTT) with immunological stimulation. PTT is an extension of PDT, but instead of generating reactive oxygen species, photon energy is converted into heat sufficient to destroy cancer cells. PTT is able to use longer wavelength light (near-infrared) that do not interfere with melanin absorption [25].

The reported ISPI trials on melanoma consisted of three main components applied directly to the cutaneous metastases: (1) local application of topical imiquimod, (2) injection of near infrared absorbing PS: indocyanine green (iCG), and (3) an 805-nm laser for local irradiation. PTT using iCG as PS induced temperature increases in target tissue, killing tumor cells directly and releasing tumor antigens for the generation of antitumor immunity, previously stimulated with the immunoadjuvant imiquimod [23, 24].

Clinical studies where the therapeutic response of ISPI was evaluated showed the effectiveness of PTT on late-stage melanoma patients [24, 25]. The probability of long time survival was 70 %. These promising patient responses demonstrated that ISPI with imiquimod is safe and well tolerated.

However, considering melanoma recurrence and potential resistance, currently PDT is not taken into account to replace surgery as first-line treatment. The research suggests necessary strategies targeting both melanoma and its microenvironment in order to effectively eradicate this cancer. The clinical trials data support the idea that much more work will be necessary in order to optimize preclinical models to enhance the clinical outcome of PDT on skin melanoma.

Preclinical studies on skin melanoma

In PDT for melanoma, some obstacles are important to consider when PSs are selected, such as (1) the presence of melanin, which can absorb radiation over the visible spectrum mainly in the blue region of light, so the photodynamic efficacy is significantly diminished with PS absorbing in this region. The PSs that absorb in near-infrared are much more suitable for PDT of melanoma because this region is most transparent to radiation and the tissues are deeply penetrated by photons; (2) melanin is considered an intracellular antioxidant, thus it neutralizes PDT-induced ROS and decreases PDT success; (3) melanosomes can act as drug trapping or sequestration agents; (4) members of the ATP-binding cassette (ABC) transporters are located in the plasmatic and melanosomal membrane and can reduce the effectiveness of treatment by lowering the concentration of the PSs inside the cell by expelling it into the extracellular space; (5) the depth of tumor infiltration prevents the homogeneous penetration of radiation remaining in areas that lack treatment [26].

Over recent decades, more than 50 PSs have been tested in vitro for melanoma treatment, with promising results [27].

Table 1 Photodynamic therapy clinical trials on skin melanoma: brief description about several features of clinical tested protocols

Tumor type	Pigmentary phenotype	Pretreatment	Photosensitizer				Wavelength, lamp		Outcome of study	Reference
			PS	Dose	Application form	Incubation	Lamp	Dose intensity		
Skin melanoma (7 patients)	Pigmented	–	Hematoporphyrin derivative (Hpd)	5 mg/kg BW	Intravenous injection	3 days	Xenon lamp (λ=600–700 nm)	100–150 mW/cm^2	Complete response in 6/7patients	Dougherty et al., 1978 [16]
Skin melanoma metastasis (1 patient)	ND	–	Photosan III (DHP)	2 mg/kg BW	Infusion	48 h	Argon dye laser	200 J/cm^2	No response	Koderhold et al., 1996 [19]
Skin melanoma metastases (14 patients)	Pigmented	–	Chlorin e6	5 mg/kg BW	Intravenous injection	1 h and 24 h (2 courses of PDT)	LD 680–2000 laser (λ=670 nm)	80–120 J/cm^2 to 250–300 mW/cm^2	Complete regression after the first PDT (8/14) or after multiple treatments (6/14). 11 of 14 patients died due to metastases).	Sheleg et al., 2004 [17]
Skin melanoma metastasis (2 patients)	Pigmented	+ Imiquimod as immune modifier was applied topically 2 weeks before PTT	Indocyanine green	0.5 ml/cm^3	Local injection	–	Diode laser (λ=805 nm)—photothermal effects	1.0 W/cm^2 (applied for 10 min)	Complete response in 2/2 patients	Naylor et al., 2006 [24]
Melanoma in situ (1 patient)	Unpigmented	–	Methyl aminolevulinate acid	–	Topical	3 h	Red light (λ=633 nm)	40 J/cm^2 80 mW/cm^2	Recurrence 4 months after PDT	Chetty et al., 2008 [18]
Skin melanoma metastasis (11 patients)	ND	+ Imiquimod as immune modifier was applied topically 2 weeks before PTT	Indocyanine green	0.5 ml/cm^3	Local injection	–	Diode laser (λ=805 nm)—photothermal effects	1.0 W/cm^2 (applied for 10 min)	Complete response in 6/11 patients	Li et al., 2010 [23]

However, few of them have been shown as relatively successful on melanoma-bearing mice. In the next section of this review, we will focus on those PSs whose therapeutic efficiency was assessed on both in vitro and in vivo preclinical studies (Table 2).

The prodrug 5-aminolaevulinic acid (5-ALA) is a widely used photosensitizer, which leads to the intracellular accumulation of the endogenous photosensitizing molecule protoporphyrin IX (PpIX). PpIX absorbs 405-nm light and re-emits 635 and 704 nm fluorescence [46]. 5-ALA-based PDT effectively killed melanoma cells in vitro and triggered apoptotic/necrotic cell death [47, 48]. Unfortunately, when 5-ALA was applied on skin melanoma in vivo, it failed to significantly inhibit tumor progression [49].

Methylene blue (MB) is a cationic dye from the phenothiazinium family, has strong absorption of broad band red light (550–700 nm, maximum at 664 nm), and possesses exceptional affinity to melanocyte containing melanin, which contributes to selective absorption of this compound by cutaneous melanomas. It has been reported that MB localized in mitochondria membranes. This is reflected on an in vitro assay that showed a dose-dependent effect of MB-PDT on cell death, increasing apoptosis by the intrinsic mitochondrial pathway [50]. MB-PDT showed delayed tumor growth in mouse melanoma model regarding MB alone. Similarly, animal survival was higher in MB-PDT with respect to control, but had small differences in relation to MB alone. These facts suggest that MB could be used either as chemotherapeutic or as photosensitizer. However, it is noteworthy that no treatment condition with MB-PDT achieved complete remission of tumors [51].

The porphyrins tetra-meso (*N*-methyl-4-pyridyl) porphine (P4) and C14-alkyl derivative tri-meso (*N*-methyl-4-pyridyl)meso(*N*-tetradecyl-4-pyridyl) porphine (C14) are cationic porphyrins that absorb 620–690-nm light. Both of them were cytoplasmically delivered into melanoma cells, but C14 was preferentially internalized. After irradiation, P4 and C14 inhibited tumor proliferation in a light dose-dependent and dose-independent manner. Cellular response to photosensitization included (a) porphyrin binding to an unusual nucleic acid conformation called G-quadruplex, thus regulating gene expression; and (b) singlet-oxygen-based cell death. Regarding the latter, it was demonstrated that P4 and C14 arrested melanoma proliferation. Apoptotic and necrotic cell death were observed after C14-PDT.On the other hand, P4 was the most efficient porphyrin on in vivo studies. It showed the highest tumor accumulation and promoted a better growth delay and animal survival. However, neither C14 nor P4 photosensitization generated tumor-free animals [52].

Table 2 Photodynamic therapy preclinical studies on different in vitro cultures platforms: summary of photosensitizer applied on melanoma studies according the complexity of experimental models

Cell Culture Model	PDT Assay	Photosensitizers	References
2D Mono-Culture	✓	Phthalocyanines and derivates	[28][29][30][31][32]
		5-aminolevulinic acid (ALA)	[33][34][28][60][50][35]
		Porphyrins and derivates	[53][28][59][36][37][38][39][40]
		Photofrin I y II	[41][37]
		Methylene blue	[51]
		Hypericin	[42]
		Bacteriochlorophylls and derivates	[55][43]
		Chlorins	[54][44]
		Merocyanine	[37]
		Psoralen	[45]
2D Co-Culture	✓	5-aminolevulinic acid (ALA)	[74]
3D Mono-Culture	✓	Rhenium(I) indolato complexes	[64]
3D Co-Culture	✗	--	--
Organotypic Human Skin	✗	--	--

The TPFC is a boronated carboranyl-substituted cytoplasmic membrane-located chlorin, which absorbs light in the far-red region (>650 nm). TPFC was applied in Boron Neutron Capture Therapy (BNCT) and PDT because of its selective tumor accumulation and the chance of being activated by both low-energy neutrons (in BNCT) or red light (in PDT). Necrotic cell death was observed in vitro after TPFC-based PDT. Unfortunately, TPFC-treated mice remained tumor-free only for 5 days [53].

The bacteriochlorophyll-serine (Bchl-Ser) is derived from the natural pigment bacteriochlorophyll-*a* that was chemically modified at the C-17 propionic residue of its macrocycle and conjugated to a serine molecule. Bchl-Ser is a water-soluble molecule and has strong absorption in the NIR (765–780 nm) enabling large penetration depth (9 mm) in melanoma tumors. In vitro studies showed that cell death triggered by Bchl-Ser was absolutely light and dose dependent [54]. At an in vivo level, this PS had been proposed for vascular photodynamic therapy (vPDT), which is based on massive vascular damage with occlusive thrombi, hemorrhage, and tumor necrosis, eliminating melanoma tumor cells indirectly. This compound showed to be highly successful in generating vascular damage, carrying out complete healing and eradication of the tumor xenograft or an 81 % cure with no signs of tumor recurrence. These findings postulated this compound as a promising PS for melanoma tumor removal. However, in clinical protocols for melanoma treatment, vascular damage is not yet the primary target of PDT [55]. This therapeutic outcome highlights that a successful antitumor should not only be focused in destroying malignant cells.

Complexing culture models to predict treatment outcome by mimicking melanoma microenvironment

A crucial question that scientists always try to answer is about how much we can predict in vivo results from in vitro findings. Toledo and Wahl entitled their review about regulation of tumor suppressor p53 “in vitro hypotheses, in vivo veritas” [56]. We agree with this affirmation in regard to the current research about photodynamic intervention on skin melanoma. Our comparative analysis revealed a disconnect between several hypotheses generated by in vitro therapeutic studies and in vivo assays, which could probably be explained by the ability of mouse models to preserve crucial features of tumor biology [49–51, 53].

Currently, monolayer cell culture models are very useful tools to research and apply molecular and cell biology principles in general and oncology in particular. However, they do not mimic melanoma cells that do not grow in isolation, and in fact they establish interactions with their neighbors and are oriented in a three-dimensional space. Therefore, growing melanoma as monolayers in culture flasks is not always an adequate system to study heterotopic melanoma biology and potential therapies outcome [57].

In this section, we will review two strategies used by photodynamic researchers in order to diminish the discrepancies between in vitro studies and in vivo preclinical and clinical assays: (a) developing 3D culture systems which mimic tumor architecture and (b) targeting tumor microenvironment to favor therapeutic regimen design.

Developing 3D culture systems which mimic tumor architecture

The majority of currently available data about PDT on human melanoma have been obtained from two-dimensional (2D) cultures of melanoma cell lines [50, 58, 59]. However, as we have previously mentioned, most of these studies failed to predict the efficiency of in vivo preclinical studies or clinical trials.

In this sense, three-dimensional (3D) cultures represent a useful in vitro tool to characterize melanoma biology and specifically to prescreen antitumor therapeutics. By applying 3D cultures to experimental design, tumor architecture that was lacking in 2D in vitro culture conditions is preserved, whereas the great complexity of the in vivo animal models is overcame. In addition, 3D cultures are less expensive and less time consuming than animal studies. Besides the experimental benefits, 3D platforms have ethical and economic advantages compared to animal studies because they allow the researcher to narrow down the experiments that need to be done in animals and thus to reduce the number of animals used in preclinical studies [57, 60].

Concerning melanoma, spheroids have been used in order to recapitulate tumor architecture. 3D culture models in melanoma recreate tumor heterogeneity by reconstructing natural gradients such as oxygen and nutrients, so the spheroids have a hypoxic and central necrosis area. Moreover, spheroids certainly allow studies in vitro about cell-cell and ECM-cell (ECM, extracellular matrix) interactions and evaluate the influence of the environment in many tumor processes such as survival, apoptosis, angiogenesis, invasion, metastasis, and others [61]. Furthermore, 3D spheroid model mimics the behavior of melanomas in vivo with regard to radiotherapy response. It was observed that the cellular radiosensitivity is the same in melanoma spheroids with respect to in vivo xenografts, quantified as cell survival or as specific growth delay [62] (Fig. 2).

Despite the correlation found between 3D in vitro and in vivo studies in the field of radiotherapy [62], there are only a few reports of PDT on melanoma spheroids. Kastl and colleagues generated 1205 Lu melanoma spheroids and treated them with a rhenium(I) indolato complex followed by LED-mediated irradiation. Previously, they demonstrated that this compound provoked a strong light-induced antiproliferative

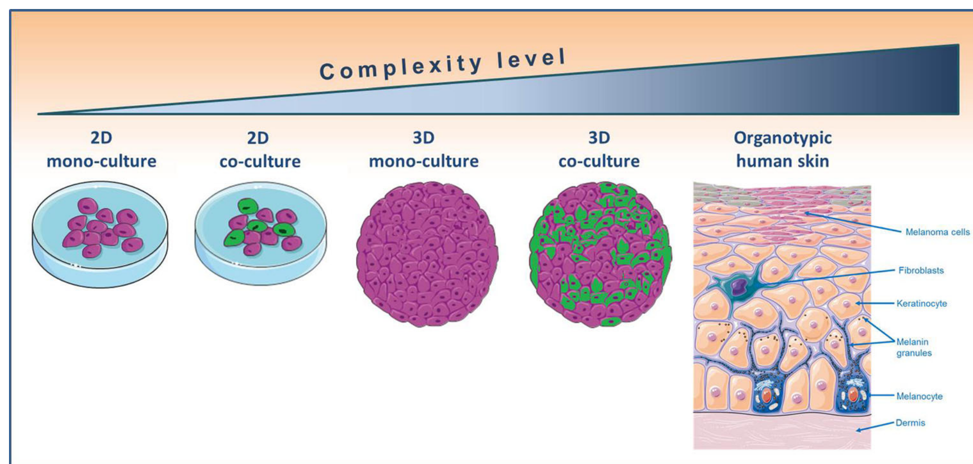

Fig. 2 Evolution of experimental in vitro platforms to mimic melanoma. Several preclinical models have been developed in order to mimic human malignant melanoma. The evolution begins with monoculture cells growing as bidimensional (2D) monolayer and then includes 2D co-culture of stromal and tumor cells. Three-dimensional (3D) spheroids represent both mono- and co-culture retaining tumor architecture and tumor cell-tumor cell and ECM-tumor cell interactions. The most complex platform is the recently designed organotypic human skin which resembles human skin in cellular (melanoma cells, fibroblast, keratinocytes, and melanocytes) and non-cellular composition and the spatial distribution (epidermis, dermis). *ECM* extracellular matrix. Images used under Creative Commons Attribution 3.0 Unported License from http://www.servier.com/Powerpoint-image-bank

activity in cancer cells in vitro. Although drug concentration was five times greater than that for in vitro studies, phototoxicity was suppressed when treatment was applied on spheroids, especially in those layers found in the core of the spheroid [63] (Table 2). In addition, Barbugli and colleagues studied PDT efficiency using chloroaluminum phthalocyanine (ClAlPc) liposomes on 3D cultures. It was shown that PDT treatment on spheroids delays their growth. The effect was observed during 28 days [64]. Despite these results, further studies should be done in order to establish a correlation between melanoma spheroid photodynamic efficiency and xenograft or patient response to PDT.

Targeting tumor microenvironment to favor therapeutic regimen design

In the past decades, researchers noticed that cancer cannot be minimally considered as a collection of mutated cells, but it must be analyzed as part of a global and complex interaction network of cellular and environmental elements [22]. This idea initiated the study of the "tumor microenvironment" (TME) as a potential target in antitumor therapeutics. TME is composed of several cellular populations, classified as parenchyma (tumor cells) and stroma (non-transformed normal cells, immune cells, fibroblasts, fibrocytes, endothelial cells, smooth muscle cells, spindle cells), and non-cellular components, such as the extracellular matrix (ECM) [15].

The composition of the TME varies from tumor to tumor. In cutaneous melanoma, two main types of stroma can be shown. In desmoplastic stroma, fibroblasts and fibrocytes are the predominant population, accompanied by extensive accumulation of fibrillar ECM components. On the other hand, myxoid stroma presents atypical spindle cells with major accumulation of proteoglycan [66]. The presence of melanoma cells in the TME induces stromal reaction that includes proteolysis of collagen, the main component of the dermis, and elastin at the tumor's invasive edge as well as infiltration of lymphocytes and angiogenesis-related endothelial cells [67]. Parenchyma, stroma, and non-cellular components within melanoma TME actively interact through (a) direct cell-cell contact, (b) ECM-cell interactions, and (c) secreted growth factors and cytokines-mediated paracrine dialogue. Melanoma TME is not static, but readapts in response to the needs of malignant cells in all phases of the multi-step process of melanomagenesis, including initiation, progression, maintenance, and metastasis [68, 69]. Recently, we have introduced the term "ecological photodynamic therapy," which identifies the combination of PDT regimen with targeting TME agents as a new and promising opportunity for therapeutic intervention [70].

In vitro co-culture methods are closely related to the in vivo situation and are useful to evaluate the impact of the heterotypic interaction between tumor and stroma cells in several features of melanoma biology. This model allows direct physical contact between different cell types and accounts for the effects of ECM and soluble factors (Fig. 2). Recently, Kästle and colleagues performed for the first time co-culture assays in order to evaluate the impact of ALA-based PDT on melanoma and keratinocyte cells. In melanoma ecology, keratinocytes are the principal epidermal cell population [68], so their influence must be taken into account when designing therapeutic regimens. It is well known that within normal skin, keratinocytes exert tight control of melanocyte proliferation and its deregulation occurs, at least in part, when melanocytes escape from this control [71].

In this sense, a melanoma-keratinocyte co-culture model grown as monolayer was generated to assess photodynamic outcome [72]. The goal of the research was to determine if the presence of immortalized keratinocytes could influence the oxidative-stress-mediated cytotoxicity of PDT when combined with different chemotherapeutic agents. Keratinocytes were more resistant than melanoma cells to photodynamic intervention in monoculture assays. Concomitantly, in co-culture experiments, keratinocytes remained unaffected without visible sign of oxidative stress after photodynamic treatment, and the ratio of keratinocytes to melanoma cells increased after PDT. Interestingly, the crosstalk established between those populations had the ability to modulate the response to PDT and other inhibitors. When PDT was combined with a specific inhibitor of poly(ADP-ribose) polymerase (PARP), PDT outcome could not be supported in co-culture systems due to an effect on keratinocytes [72] (Table 2).

Although this in vitro co-culture platform was able to reproduce, at least in part, melanoma-keratinocyte relationship, it failed to represent tumor spatial distribution. In this sense, spheroids embedded in collagen matrix with fibroblast were generated. It has been shown that PDT response is different when comparing this 3D culture model with PDT outcome of spheroids without matrix [64]. The need for more suitable in vitro culture systems to test the efficiency of antitumor strategies led to the generation of a full-thickness organotypic human skin model (Fig. 2). Artificial skin reconstructs perfectly displayed the different stratus of skin: (a) epidermis with keratinocytes and melanocytes, (b) dermis with fibroblast embedded in collagen, and (c) basement membrane deposited by skin cells [73]. Melanoma cells had been grown in this platform as monolayers [73] and spheroids [74].

Future directions

The diametric differences in responsiveness of melanoma cells in vitro and in vivo to PDT exemplifies the need to develop more complex preclinical model systems in order to mimic human malignant melanoma.

Development of 3D in vitro platform to evaluate the efficiency of photodynamic treatment retained melanoma architecture and tumor cell-tumor cell and tumor ECM-cell interactions [63]. After a comprehensive review of the literature, we suggest that new complementary in vitro tools and platforms are required to better predict the outcome of PDT on patients.

The organotypic reconstructed skin closely resembles human skin in cellular and non-cellular composition and the spatial distribution that significantly represented the physiological properties as in the patients' skin [57]. The organotypic skin-melanoma model would facilitate efforts to improve PDT outcomes for malignant melanoma. This platform will provide photodynamic researchers a novel tool to globally evaluate the influence of abiotic (pH, oxygen, nutrients) and biotic components (keratinocytes, melanocytes, fibroblasts, immune cells, and others) of melanoma microenvironment and better predict clinical outcomes. Most complete and reliable preclinical evaluation would be essential to reduce attrition rates in clinical trials and enhance the benefits of therapeutic interventions.

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Conflicts of interest None

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