Photodynamic therapy potentiates the paracrine endothelial stimulation by colorectal cancer

2014 Laser Phys. 24 115602

(http://iopscience.iop.org/1555-6611/24/11/115602)

IOP Publishing | Astro Ltd Laser Physics
Laser Phys. **24** (2014) 115602 (8pp) doi:10.1088/1054-660X/24/11/115602

Photodynamic therapy potentiates the paracrine endothelial stimulation by colorectal cancer

María Julia Lamberti[1], María Florencia Pansa[1], Renzo Emanuel Vera, Natalia Belén Rumie Vittar[2] and Viviana Alicia Rivarola[2,3]

Departamento de Biología Molecular, Facultad de Ciencias Exactas Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, 5800 Río Cuarto, Córdoba, Argentina

E-mail: vrivarola@exa.unrc.edu.ar

Received 28 March 2014, revised 28 July 2014
Accepted for publication 9 August 2014
Published 10 September 2014

Abstract

Colorectal cancer (CRC) is the third most common cancer and the third leading cause of cancer death worldwide. Recurrence is a major problem and is often the ultimate cause of death. In this context, the tumor microenvironment influences tumor progression and is considered as a new essential feature that clearly impacts on treatment outcome, and must therefore be taken into consideration. Photodynamic therapy (PDT), oxygen, light and drug-dependent, is a novel treatment modality when CRC patients are inoperable. Tumor vasculature and parenchyma cells are both potential targets of PDT damage modulating tumor–stroma interactions. In biological activity assessment in photodynamic research, three-dimensional (3D) cultures are essential to integrate biomechanical, biochemical, and biophysical properties that better predict the outcome of oxygen- and drug-dependent medical therapies. Therefore, the objective of this study was to investigate the antitumor effect of methyl 5-aminolevulinic acid-PDT using a light emitting diode for the treatment of CRC cells in a scenario that mimics targeted tissue complexity, providing a potential bridge for the gap between 2D cultures and animal models. Since photodynamic intervention of the tumor microenvironment can effectively modulate the tumor–stroma interaction, it was proposed to characterize the endothelial response to CRC paracrine communication, if one of these two populations is photosensitized. In conclusion, we demonstrated that the dialogue between endothelial and tumor populations when subjected to lethal PDT conditions induces an increase in angiogenic phenotype, and we think that it should be carefully considered for the development of PDT therapeutic protocols.

Keywords: photodynamic therapy, colorectal cancer, tumor microenvironment, angiogenesis, 3D culture

(Some figures may appear in colour only in the online journal)

1. Introduction

Colorectal cancer (CRC) is the third most common cancer and the third leading cause of cancer death among men and women [1], together with lung, prostate and breast cancer. In recent years, very important advances have been made in the field of treatment of this common disease in order to achieve the best results with low morbidity. Surgery is the primary treatment and results in a cure in approximately 50% of patients [1]. Recurrence following surgery is a major problem and is often the ultimate cause of death. There are factors that clearly influence treatment outcome and these must therefore be taken into consideration. Recent studies have indicated that during the acquisition of genetic and

[1] These authors contributed equally to this project and should be considered co-first authors.

[2] Both authors equally supervised this work.

[3] Author to whom any correspondence should be addressed.

epigenetic alterations that underlie the inherent hallmarks of cancer, transformed cells interact synergistically with their surrounding microenvironment [2]. In this context, the tumor microenvironment (TME) influences tumor progression and is considered to be an essential new feature of neoplastic transformation [3, 4].

The TME is composed of malignant cells (parenchyma) surrounded by a tumor-conditioned stroma that contains extracellular matrix and a variety of nonmalignant populations, including fibroblasts, lymphocytes, and endothelial cells (ECs) that often support tumor growth and survival. Tumor-associated ECs assemble the tree-like tubular network of blood vessels that eventually permits the transport of nutrients and oxygen to the TME. Tumor-derived growth factors and cytokines manipulate ECs moving toward their secreted stimuli, forcing them to invade and adapt to the TME [5]; the growth of blood vessels by the sprouting of new capillaries from pre-existing vessels is a process termed angiogenesis. Tumor neovascularization plays a critical role in CRC progression, and increased angiogenesis has been associated with poor prognosis and relapse of colorectal disease [6, 7].

Photodynamic therapy (PDT) is a novel treatment modality for patients who are not good candidates for an operation. PDT consists of systemic or topical administration of a photosensitizer (PS) or metabolic precursor, photoexcitation of the sensitizer by light in the visible wavelength (400–750 nm), and tumor cell death induced by the release of reactive oxygen species (ROS) [8]. PDT provides better selectivity for the targeting of tumors compared to conventional chemo- and radiotherapy due to the preferential accumulation of PSs in tumors; the cytotoxic effect occurs only in the area exposed to light [8].

Additionally, PDT was proposed as a novel modulator of tumor–stroma interactions [5]. Tumor vasculature and parenchyma cells are both potential targets of PDT damage. Thus, tumor destruction could be achieved directly through tumor cell (TC) damage, or indirectly through microvascular damage and the induction of vascular stasis [8].

Protoporphyrin IX (PpIX), synthesized from 5-aminolevulinic acid (ALA) in the mitochondria, is an intrinsic and safe PS [8, 9]. PpIX accumulates in several malignant tumors following 5-ALA administration. One of the main advantages of ALA-PDT is that PpIX is cleared from the body within 24–48 h subsequent to systemic ALA administration. Furthermore, 5-ALA is an endogenous agent that is part of the regular diet. Therefore, ALA-PDT has the potential to avoid the risk of prolonged phototoxicity [9]. Chemical modification of the parent ALA molecule is aimed at improving the efficiency of ALA based PDT by increasing ALA delivery, enhancing PpIX accumulation and reducing side effects. Numerous ALA derivatives have been synthesized by various groups worldwide. Lipophilic ALA derivatives, such as FDA approved methyl-ALA (Me-ALA), have been produced in the hope of enhancing tissue penetration, and all of these approaches lead to PpIX accumulation in the target cells sensitizing them to photoinactivation [9].

Early biological-activity assessment in drug discovery has been traditionally based on two-dimensional (2D) cell cultures [10]. Tissue-specific architecture, together with cell–cell and cell–extracellular matrix (ECM) interactions, are reduced when cells grow on flat and adherent substrates. Three-dimensional (3D) cultures are currently being developed as more advanced *in vitro* models for cellular organization in tissues [11]. These milieus provide the third dimension, essential to integrate biomechanical, biochemical, and biophysical properties more similar to a natural microenvironment. In particular, a key advantage of 3D cultures in preclinical research is their capacity to recreate the molecular gradients that exist in living tissues for any soluble component, such as oxygen, nutrients, metabolites, and signaling molecules [10]. Hence, 3D models mimic the hierarchical complexity of tissues more precisely than conventional monolayers. Therefore, the objective of this study was to investigate the antitumor effect of Me-ALA-PDT using LEDs for the treatment of CRC cells in a scenario that mimics targeted tissue complexity, providing a potential bridge for the gap between 2D cultures and animal models and, therefore, show that 3D better predicts the outcome of oxygen- and drug-dependent medical therapies.

Since the photodynamic intervention of the TME can effectively modulate the tumor–stroma interaction, it was proposed to characterize the endothelial response to CRC paracrine communication, if one of these two populations is photosensitized. Specifically, it was demonstrated that proangiogenic phenotype of ECs is enriched when stimulated with parenchyma-derived soluble factors, under lethal but not-sublethal PDT.

2. Materials and methods

2.1. Cell culture

Cell lines derived from primary human Duke's B colorectal tumors with different tumor grades were used: SW480, highly tumorigenic and invasive [12] and its more adherent clonal subline SW480-ADH [13], and the Caco-2 cell line, well-differentiated, less tumorigenic and invasive [14]. The human microvascular endothelial cell line HMEC [15] was also used. All were cultured according to ATCC indications at 37 °C 5% CO_2 in high-glucose Dulbecco's modified Eagles medium (DMEM, Gibco) supplemented with 10% or 20% v/v fetal bovine serum (Gibco) and 1% v/v antibiotic-antimycotic (PenicilinaG 10 μg ml^{-1}, streptomycin 25 μg ml^{-1}, amphotericin B, Gibco).

2.2. 3D cultures

Caco-2 spheroids were grown for 3 d using the liquid overlay technique by seeding 1000 cells onto non-adherent U-bottom tissue culture wells. SW480 spheroids were generated via the hanging drop method incorporating 20 000 cells per 30 μl of culture medium placed on the lid of a non-adhesive PBS-containing Petri dish for 3 d [16].

2.3. PDT treatments

Monolayers and spheroids were subjected to PDT. Me-ALA (Sigma) was added and incubated for 4 h in a non-supplemented

medium. After that, cells were irradiated with different light doses at room temperature with a monochromatic light source (636 nm ± 17 nm) using a MultiLED system (coherent light). The fluence rate was 0.89 mWcm^{-2}, as measured by Radiometer Laser Mate-Q. The drug solution was then removed and replaced with fresh medium.

2.4. Photocytotoxicity evaluation

After 24 h PDT-treatment, cell viability was evaluated by 1-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan (MTT) assay. The ability of viable cells to reduce MTT and convert it to non-water-soluble violet formazan crystals was determined spectrophotometrically using an ELISA reader plate (Thermo) at 540 nm after solubilization of the crystals with dimethylsulfoxide [17]. To determine the migratory capacity of spheroids, they were placed in Petri dishes immediately post-PDT and fixed with methanol 24 h after treatment. They were then stained with toluidine blue and photographed using a Nikon CoolPix 8400 digital camera attached to an inverted microscope Nikon Eclipse TS100.

2.5. Conditioned media generation

Conditioned media (CM) were obtained from CRC cells grown as monolayers or spheroids. Untreated or PDT-treated cells were serum starved for 24 h before collection of the CM. CM were harvested, clarified by centrifugation, and stored at −80 °C [18].

2.6. Endothelial cell proliferation assay

Untreated or PDT-treated HMEC cells (7×10^4 cell ml^{-1}) grown in multiwell-96 plates were starved with a serum-free medium for 4 h and then stimulated for 24 h with CM from tumor cells subjected or not to PDT. Cell viability was determined by an MTT assay. The percentage of proliferation was calculated using the following formula:

$$\text{Untreated endothelial proliferation}(\%) = \frac{\left(\begin{array}{c}\text{absorbance of HMEC stimulated}\\ \text{with PDT-treated tumor CM}\end{array}\right)}{\left(\begin{array}{c}\text{absorbance of HMEC stimulated}\\ \text{with untreated-tumor CM}\end{array}\right)} \times 100$$

$$\text{Untreated endothelial proliferation}\ (\%) = \frac{\left(\begin{array}{c}\text{absorbance of HMEC stimulated}\\ \text{with PDT-treated tumor CM}\end{array}\right)}{\left(\begin{array}{c}\text{absorbance of HMEC stimulated}\\ \text{with untreated-tumor CM}\end{array}\right)} \times 100$$

$$\text{PDT-treated endothelial proliferation}\ (\%) = \frac{\left(\begin{array}{c}\text{absorbance of HMEC stimulated}\\ \text{stimulated with tumor CM}\end{array}\right)}{(\text{absorbance of non-stimulated HMEC})} \times 100.$$

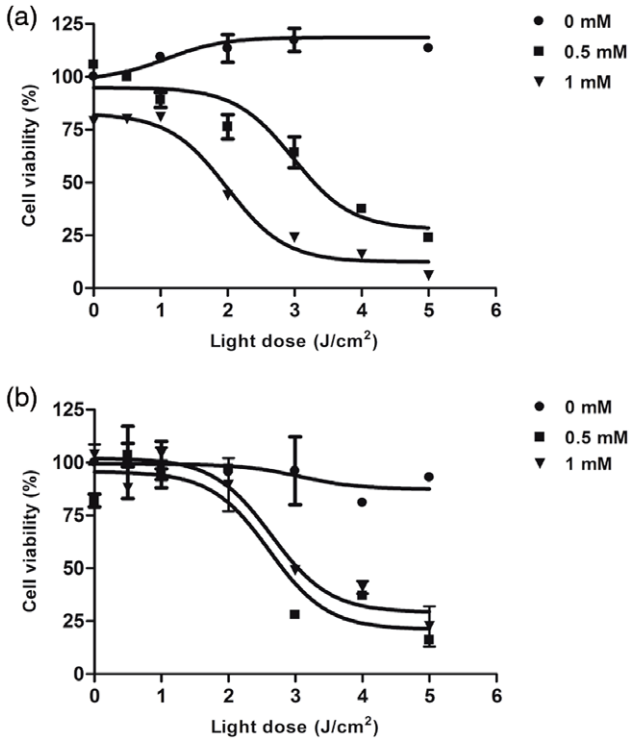

Figure 1. PDT inhibited Caco-2 and SW480 ADH viability. Exponentially growing Caco-2 (*a*) and SW480 ADH (*b*) cells were subjected to PDT. The cell viability was evaluated by an MTT assay and referred to non-irradiated cells in each treatment group. The curves were fitted using a non-linear regression equation (GraphPad Prism); $n = 3$. Treatment groups: • irradiated-only cells; ■ 0.5 mM Me-ALA/PDT-treated cells; ▼ 1 mM Me-ALA/PDT-treated cells.

2.7. Wound healing assay for endothelial migration

Previously starved-monolayers of confluent HMEC cells were wounded by scraping with a plastic pipette tip, rinsed twice with PBS to remove floating cells and then stimulated with CM from tumor cells subjected or not to PDT. Photographs were taken at time 0 and 24 h at the same location. The wound areas were quantified with Image Pro Plus software (version 6.0.0.260). The wound decrease over time was taken as a measure of endothelial migration, using the following formula:

$$\text{Endothelial migration}\ (\%) = \frac{\left(\begin{array}{c}\text{wound area at 0 h} - \text{wound area}\\ \text{at 24 h of HMEC stimulated}\\ \text{with PDT-treated tumor CM}\end{array}\right)}{\left(\begin{array}{c}\text{wound area at 0 h} - \text{wound area}\\ \text{at 24 h of HMEC stimulated}\\ \text{with untreated-tumor CM}\end{array}\right)} \times 100.$$

2.8. Tube formation assay

Growth factor-reduced Matrigel® was thawed overnight at 4 °C on ice. After that, it was put on multiwell-96 plates, and left at 37 °C for 1 h to allow gelification. Starved HMEC (3×10^5 cell ml^{-1}) were seeded on Matrigel and incubated at 37 °C and 5% CO_2 in a humidified atmosphere with CM previously described. After 24 h, tubule formation was examined

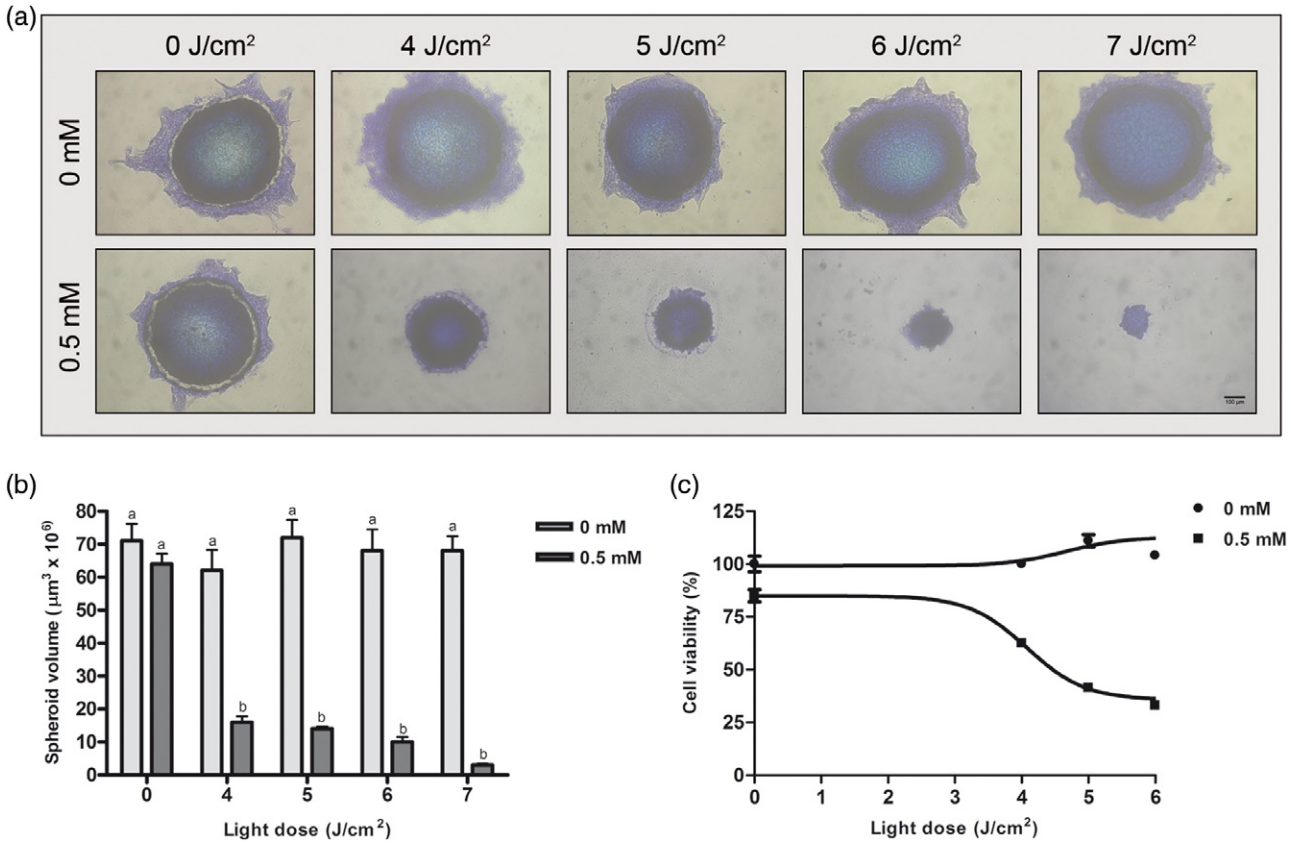

Figure 2. Inhibition of spheroid growth was achieved by PDT. Caco-2 spheroids were subjected to PDT. (*a*) Representative light microscopy images of toluidine blue-stained untreated, irradiated-only, Me-ALA only and PDT-treated spheroids; bar = 100 μm. (*b*) The diameter of each spheroid was measured using ImageJ software and volume calculated using the formula $V = 4/3.\pi.r^3$, where $r = 1/2.\sqrt{d_1}.d_2$ (geometric mean radius). For each irradiation group, bars having letters in common are not statistically different from each other; $n = 30$. (*c*) The cell viability was evaluated by an MTT assay and referred to non-irradiated cells in each treatment group. The curves were fitted using a non-linear regression equation (GraphPad Prism); $n = 3$. Treatment groups: • irradiated only-cells; ■ 0.5 mM Me-ALA/PDT-treated cells.

and digital images were obtained. The loops numbers were measured using a service provided by Wimasis online software.

2.9. Statistical analysis

Differences between groups were tested by 2-way analysis of variance with Bonferroni post-hoc tests using Infostat software. All the results are expressed as mean + standard error (SE) of measurement, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Photodynamic therapy induces death in Caco-2 and SW480 ADH colorectal cancer cells grown as monolayers

The photochemosensivity of Caco-2 and SW480 ADH monolayers was examined in a conventional MTT assay (figure 1). Cell viability was not affected by light or Me-ALA alone, but when cells were exposed to the combination of 0.5 or 1 mM Me-ALA incubation followed by irradiation with light doses ranging from 0.5 to 5 J cm^{-2}, decrease of tumor cells proliferation was observed. The inhibitory effect of PDT was light dose-dependent for both cell lines. Fifty per cent inhibition (LD50, lethal dose 50) of viability was achieved at 0.5 mM & 3.29 ± 0.02 J cm^{-2} and 1 mM & 1.92 ± 0.02 J cm^{-2} in Caco-2 cells (figure 1(*a*)), whereas 0.5 mM & 2.79 ± 0.02 J cm^{-2} and 1 mM & 3.03 ± 0.02 J cm^{-2} was needed to reach the LD50 value in SW480 ADH cells (figure 1(*b*)).

3.2. Irradiation decreased volume and viability of colorectal spheroids sensitized with Me-ALA

Cellular responses to PDT are closely related to oxygen tension. In this sense, 3D cultures, but not monolayers, offer several advantages in PDT research since they have the capacity to modulate the oxygen and other nutrient gradients that exist in tissues [10]. Therefore, Caco-2 3D spheroids were developed and photosensitized with 0.5 mM Me-ALA & 4–7 J cm^{-2} ranging light (figure 2(*a*)). The effect of PDT on the dynamics of tumor spheroid growth was investigated. Caco-2 spheroid dimension after PDT was documented 24 h from treatment when spheroid cultures were stained with toluidine blue (figure 2(*a*)). Untreated spheroids had a mean volume of 71 ± 23 × 10^6 μm^3. The volume of spheroids did not change when spheroids were only irradiated. Me-ALA treatment by itself caused a slight but not significant reduction of Caco-2 spheroids (figures 2(*a*) and (*b*)), with a mean

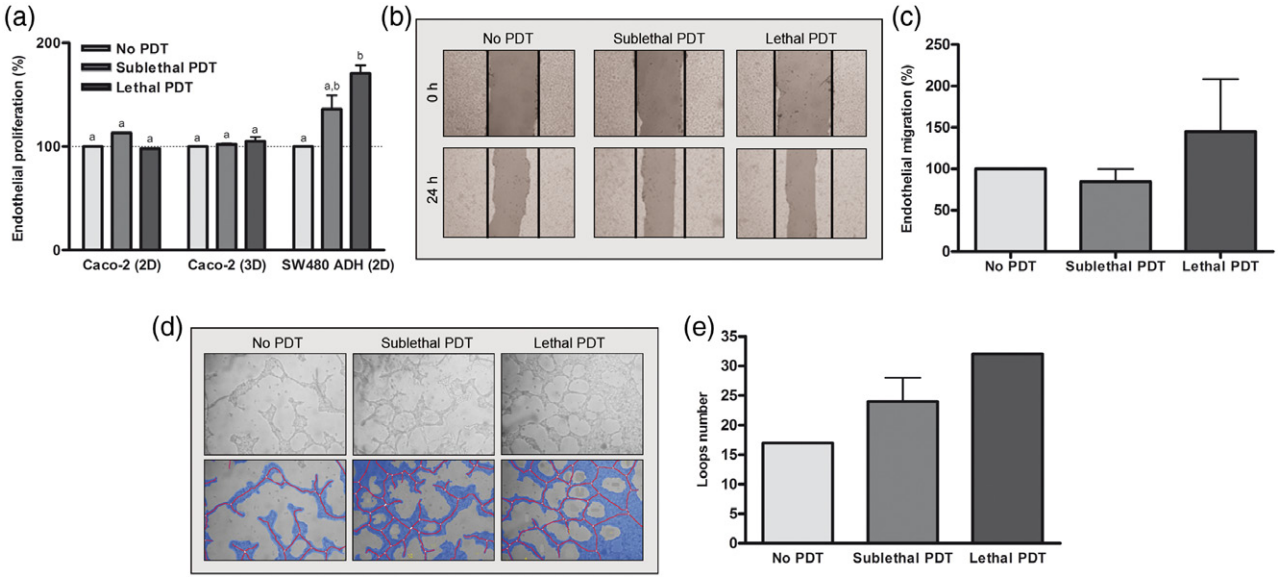

Figure 3. PDT modulated EC behavior during angiogenesis. Exponentially growing HMEC endothelial cells were incubated with conditioned media derived from untreated, sublethal PDT-treated or lethal PDT-treated Caco-2 (2D and 3D) or SW480 ADH (2D) colorectal cancer cells. (*a*) The endothelial proliferation was evaluated by an MTT assay and referred to those stimulated with soluble factors of non-treated tumor cells. For each conditioned media group, bars having letters in common are not statistically different from each other; $n = 3$. (*b*) Representative light microscopy images of the endothelial scratch assay stimulated with SW480 ADH conditioned-media at the start (0 h) and end (24 h) points. (*c*) The wound areas were quantified with Image Pro Plus software. The wound decrease over time was taken as a measure of endothelial migration, referring to those stimulated with soluble factors of non-treated tumor cells; $n = 3$. (*d*) Representative light microscopy images (top panel) of Matrigel tube formation assay were analyzed using a service provided by Wimasis software (bottom panel). (*e*) Loop numbers were quantified using the WimTube algorithm (Wimasis); $n = 3$. No PDT = untreated cells; Sublethal PDT = sublethal PDT-treated cells; Lethal PDT = lethal PDT-treated cells.

volume of $64 \pm 14 \times 10^6\ \mu m^3$. However, after PDT, spheroid mass was inhibited almost entirely, with the mean volume of spheroids after 24 h from $16.0 \pm 8.0\,\mu m^3$ ($4\,J\,cm^{-2}$) to $3.0 \pm 2.0 \times 10^6\,\mu m^3$ ($7\,J\,cm^{-2}$) (figure 2(*b*)). Maximal PDT-exposed spheroids had a 22.5-fold decrease in volume, so these data clearly demonstrated the growth-suppressing effect of PDT. This reduction in volume was due to cell death. Surprisingly, the Caco-2 spheroids showed 50% viability (LD50) at 0.5 mM Me-ALA & $4.45 \pm 0.03\,J\,cm^{-2}$, 1.3-fold more resistant than their 2D counterparts (figure 2(*c*)). As expected, treatment efficacy was proportional to light dose. Spheroids in all three control groups (untreated, Me-ALA only, irradiated-only) demonstrated similar growth throughout the 24 h observation period. A good correlation was found between the trypan blue assay and the MTT test (data not shown).

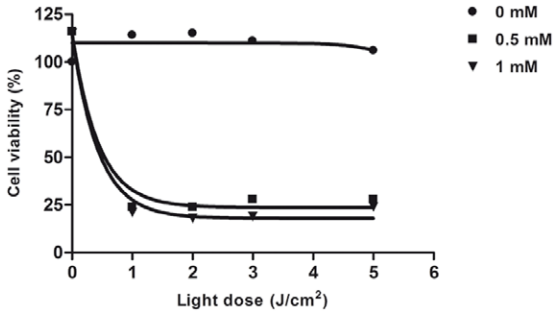

Figure 4. PDT inhibited endothelial viability. Exponentially growing HMEC endothelial cells were subjected to PDT. The cell viability was evaluated by the MTT assay and referred to non-irradiated cells in each treatment group. The curves were fitted using a non-linear regression equation (GraphPad Prism); $n = 3$. Treatment groups: ● irradiated only-cells; ■ 0.5 mM Me-ALA/PDT-treated cells; ▼ 1 mM Me-ALA/PDT-treated cells.

3.3. PDT-treated colorectal cells modulated endothelial proliferation, migration and structure-like tube formation

In a clinical situation, application of PDT leads to tumor cells deeply seated within the tumor mass receiving suboptimal light or drug doses. Additionally, it has been well documented that the biological response to PDT depends significantly on the dose applied [19–21]. We therefore decided to evaluate the impact of lethal and sublethal-PDT doses in colorectal cancer cells regarding endothelial-tumor crosstalk. In order to examine the effect of a lethal and sublethal photodynamic schedule on EC stimulation, assays that involve conditioned media (paracrine response) were performed. Soluble ligands secreted by tumor cell cultures grown as spheroids (3D) or monolayers (2D) subjected to lethal (30% tumor survival) or sublethal (80% tumor survival) PDT were collected. PDT, regardless of the scheme used, did not show any effect on EC proliferation when the stimulus came from Caco-2, therefore this cell line was dismissed in further assays. On the other hand, a significant increase in endothelial proliferation was observed with stimulating factors derived from SW480 ADH, even when they were grown as monolayer. Lethal PDT induced significant increase in the proliferation percentage which corresponds to 1.7-fold compared to EC stimulated with soluble factors from untreated TCs (figure 3(*a*)). In addition, lethal

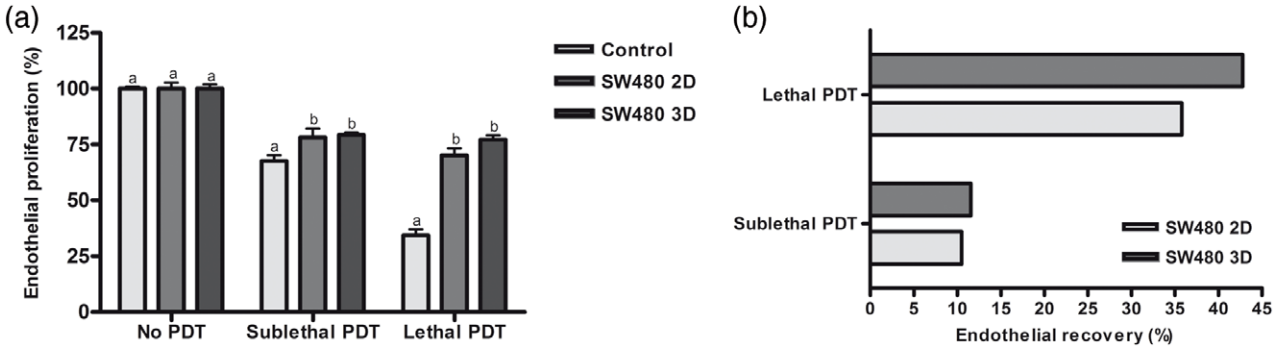

Figure 5. Paracrine soluble factors stimulated endothelial recovery after PDT. Untreated, lethal and sublethal PDT-treated HMEC endothelial cells were stimulated with a non-supplemented medium or with conditioned media (CM) derived from untreated 2D and 3D cultures of SW480 colorectal cancer cells. (*a*) The endothelial proliferation was evaluated by the MTT assay and referred to untreated ECs exposed to the same tumor paracrine stimulus. For each CM group, bars having letters in common are not statistically different from each other; $n = 3$. (*b*) The recovery percentage of the ECs is calculated as the ratio between the proliferation of PDT-treated ECs stimulated with tumor derived-soluble factors and the proliferation rate of non-stimulated PDT-treated ECs. No PDT = untreated cells; Sublethal PDT = sublethal PDT-treated cells; Lethal PDT = lethal PDT-treated cells; Control = non-supplemented medium; SW480 2D = CM from SW480 cells grown as monolayers; SW480 3D = CM from SW480 cells grown as spheroids.

PDT was able to stimulate EC migration 1.5-fold compared to untreated tumor cells (figures 3(*b*) and (*c*)). In order to evaluate the effect of PDT on the morphological differentiation of ECs into capillary-like structures, an *in vitro* tube formation assay was performed (figure 3(*d*)). The result of PDT stimulation showed that the number of vascular loops assisted by the lethal dose was 1.8 times higher compared to untreated cells, whereas the sublethal dose increased 1.4 times (figure 3(*e*)). Lethal PDT induced the greatest increase in interconnections between tubular structures of ECs.

3.4. PDT dramatically reduced HMEC endothelial cell viability

It has been acknowledged that targeting tumor vasculature by PDT proves to be a promising approach in cancer treatment. To further examine the effect of photodynamic intervention on vascular components of the TME, it was proposed to evaluate the HMEC endothelial cell response when subjected to treatment conditions equivalent to those which the TCs were previously treated. Endothelial viability was not affected by light, 0.5 or 1 mM Me-ALA alone (figure 4). Surprisingly, the ECs were shown to be more sensitive to photodynamic treatment compared to the TCs; the LD50 value was reached with only 0.5 mM & 0.54 ± 0.02 J cm^{-2} and 1 mM & 0.47 ± 0.02 J cm^{-2}.

3.5. Tumor paracrine stimulation promoted recovery of photosensitized endothelial cells

TCs have the ability to modulate the behavior of the endothelial population through paracrine secretion of soluble factors [5]. Therefore, it was investigated whether paracrine stimulation of colorectal SW480 cancer cells grown as a monolayer or as spheroids may influence the phototoxicity of PDT on the endothelial population. Proliferation of ECs subjected to PDT significantly increased in response to tumor stimulation regardless of tumoral architecture (figure 5(*a*)). The recovery percentage of the ECs is defined as the proliferation rate of PDT-treated ECs stimulated with tumor derived-soluble factors divided by the proliferation rate of non-stimulated PDT-treated ECs. Surprisingly, paracrine tumor stimulation induced highly significant recovery of ECs photosensitized with lethal PDT. Endothelial proliferation increased by 36% and 43% under the stimulation of SW480 cells grown in 2D and 3D, respectively (figure 5(*b*)).

4. Discussion

PDT holds the potential to promote killing of colorectal tumors [22]. A full response with tumor-free biopsy was achieved after treatment; however, recurrence was later observed [23]. To enhance the therapeutic effect of PDT, a single approach of PDT that targets endothelial or tumor cells was examined here.

Multicellular spheroids may serve as important tools for investigating the performance of medicines because they share several properties with native tumors [24]. Several reports have demonstrated that 3D cultures are more resistant to PDT than 2D cultures, using photophrin [25], HpD [26], hypericin [27], and mTHPC [28] as PSs. In the present study, we compared the 2D and 3D response to Me-ALA/PDT and demonstrated that cells grown as spheroids were considerably more resistant to PDT treatment relative to those cells grown as monolayers. Cell–cell contact-dependent signaling pathways, heterogeneous incorporation of drugs, and hypoxia are factors that may explain the resistance to therapeutic interventions in 3D cultures [10]. In this case, we propose hypoxia as the main photodynamic block because PDT is an oxygen-dependent therapy. SW480 ADH (but not Caco-2 tumor cells) that managed to escape from the lethal effects of irradiation displayed enhanced angiogenic potential. This suggests that the effect of PDT on mitogenic EC event depends on the degree of differentiation of sensitized tumor cells more than the culture mode employed.

As part of the PDT process, hypoxia is induced in tumors and this oxidative stress initiates a variety of responses that could potentially lead to neovascularization. In this context,

an increase in the expression of pro-angiogenic factors and cytokines post-PDT [29] has been reported, such as vascular endothelial growth factor [30], cyclooxygenase-2 [31], and matrix metalloproteinases [32]. Thus, the results from our studies prove that a high-dose irradiated tumor, which caused longer tumor growth delay, failed to produce promising PDT outcomes because this stimulates an unexpected increase in EC recruitment and proliferation necessary to conduct tumor recovery by revascularization. The angiogenic stimulus triggered by soluble factors involves activation of multiple signaling pathways in the ECs as shown with paracrine stimulation from macrophages [33], cancer breast cells [34], glioblastoma [35], and colorectal cancer cells [36]. In this study, we found proliferating EC after exposure to soluble factors from the PDT surviving tumor population, which were also able to lead migration and tube-like structures.

Recent studies have shown that stromal cells may have a strong influence on the responses of tumor cells to therapy [37]. In summary, our data suggest that tumors treated by lethal PDT play an important role in promoting angiogenesis. Thus, the presented modality has a potent antitumor effect but it would not be expected to achieve a clinical outcome. Based on the consideration of a tumor as a communicating ecosystem [5], our findings reinforce the idea that tumor malignancy can be an emergent property of the crosstalk between the ECs and TCs.

On the other hand, ECs have several beneficial characteristics when they are selected as PDT targets [5]. We were able to establish that they are more sensitive to PDT than tumor cells; however, despite these promising results, our data indicated that there is a tendency for ECs to proliferate when they have been exposed to lethal PDT and then tumor stimulated. The reason for tumor-induced EC proliferation may be that the high PDT dose could deprive the cells of oxygen, and this hypoxic condition increases the expression of mitogenic receptors, which at the same time reinforces the EC autocrine proliferative signaling [38–40]. It is worth noting that ECs were more receptive to the tumor stimulus when they were treated with lethal PDT. Overall, the results from our studies proved that lethal PDT makes TCs better donors of angiogenic soluble factors, whereas ECs became more reactive to those stimuli.

Overcoming the unfavorable consequences of PDT and at the same time maximizing its anti-tumor effects should be the goal of future therapeutic interventions that utilize chemoirradiation to control tumor progression. For this reason, we consider that the hypoxic effects that PDT has on the TME and host tissues should be explored and taken into account when new therapeutic protocols are being designed. One strategy could be to divide the required dose of light in lethal PDT into smaller doses, separated and repeated over time to allow tissue oxygenation, so that the genes regulated by hypoxia will not be activated. This is known as metronomic photodynamic therapy (mPDT) [41, 42]. Several studies using mTHPC, ALA and others have reported improvement of therapeutic results with light fractionation compared with single, acute treatments for both clinical and experimental cancers with primarily tumor responses [41]. Based on our evidence, in future we will introduce and establish the novel regimen of mePDT to perform a more successful PDT colorectal cancer clinical intervention.

Our results illustrate that the complexity of the TME and its heterogeneous response to photodynamic treatment with regard to the crosstalk between endothelial and colorectal cancer cells might also play a role in selecting which therapeutic protocol to apply.

Acknowledgments

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica, Secretaría de Ciencia y Técnica (SECyT), Universidad Nacional de Rio Cuarto, Argentina. VR and NBRV are members of the Scientific Researcher Career at CONICET. MJL, MFP and REV hold fellowships from CONICET.

References

[1] Labianca R *et al* 2010 *Crit. Rev. Oncol. Hematol.* **74** 106
[2] Gout S and Huot J 2007 *Cancer Microenviron* **1** 69
[3] Solinas G, Marchesi F, Garlanda C, Mantovani A and Allavena P 2010 *Cancer Metastasis. Rev.* **29** 243
[4] Colotta F, Allavena P, Sica A, Garlanda C and Mantovani A 2009 *Carcinogenesis* **30** 1073
[5] Rumie Vittar N, Lamberti M, Pansa M, Vera R, Rodriguez M, Cogno I, Milla Sanabria L and Rivarola V 2013 *Biochim. Biophys. Acta* **1835** 86
[6] Rmali K, Puntis M and Jiang W 2007 *Colorectal Dis.* **9** 3
[7] Martins S, Reis R, Rodrigues A, Baltazar F and Filho A 2011 *World. J. Clin. Oncol.* **2** 272
[8] Agostinis P *et al* 2011 *CA Cancer J. Clin.* **61** 250
[9] Donnelly R, McCarron P and Woolfson A 2008 *Perspect Med. Chem.* **1** 49
[10] Alemany-Ribes M, García-Díaz M, Busom M, Nonell S and Semino C 2013 *Tissue Eng.* A **19** 1665
[11] Alemany-Ribes M *et al* 2013 *J. Anal. Bioanal. Tech.* **S1** 004
[12] Leibovitz A, Stinson J C, McCombs W B III, McCoy C E, Mazur K C and Mabry N D 1976 *Cancer Res.* **36** 4562
[13] Palmer H G 2001 *J. Cell. Biol.* **154** 369
[14] Fogh J, Fogh J and Orfeo T 1977 *J. Natl Cancer Inst.* **59** 221
[15] Ades E, Candal F, Swerlick R, George V, Summers S, Bosse D and Lawley T 1992 *J. Invest. Dermatol.* **99** 683
[16] Metzger W, Sossong D, Bächle A, Pütz N, Wennemuth G, Pohlemann T and Oberringer M 2011 *Cytotherapy* **13** 1000
[17] Lamberti M, Rumie Vittar N, De Carvalho da Silva F, Ferreira V and Rivarola V 2013 *Phytomedicine* **20** 1007
[18] Valcárcel M, Arteta B, Jaureguibeitia A, Lopategi A, Martínez I, Mendoza L, Muruzabal F, Salado C, and Vidal-Vanaclocha F 2008 *J. Transl. Med.* **6** 57
[19] Yoo J, Lim Y, Kim Y and Ha K 2011 *J. Cell Biochem.* **112** 3061
[20] Mackenzie G, Dunn J, Selvasekar C, Mosse C, Thorpe S, Novelli M, Bown S and Lovat L 2009 *Lasers Med. Sci.* **24** 729
[21] Bissonnette R, Sharfaei S, Viau G and Liu Y 2004 *Br. J. Dermatol.* **151** 653
[22] Barr H, Krasner N, Boulos P, Chatlani P and Bown S 1990 *Br. J. Surg.* **77** 93

[23] Kashtan H, Haddad R, Yossiphov Y, Bar-On S and Skornick Y 1996 *Dis. Colon. Rectum.* **39** 379
[24] Friedrich J, Seidel C, Ebner R and Kunz-Schughart L 2009 *Nat. Protoc.* **4** 309
[25] West C 1989 *Br. J. Cancer* **59** 510
[26] West C and Moore J 1989 *Photochem. Photobiol.* **505** 665
[27] Kamuhabwa A, Huygens A and De Witte P 2003 *Int. J. Oncol.* **23** 1445
[28] Marchal S, Fadloun A, Maugain E, D'Hallewin M, Guillemin F and Bezdetnaya L 2005 *Biochem. Pharmacol.* **69** 1167
[29] Bhuvaneswari R, Gan Y Y, Soo K C and Olivo M 2009 *Cell. Mol. Life Sci.* **66** 2275
[30] Ferrario A, Von Tiehl K F, Rucker N, Schwarz M A, Gill P S and Gomer C J 2000 *Cancer Res.* **60** 4066
[31] Luna M, Wong S, Ferrario A and Gomer C J 2008 *Photochem. Photobiol.* **84** 509
[32] Filip A, Clichici S, Muresan A, Daicoviciu D, Tatomir C, Login C, Dreve S and Gherman C 2008 *Exp. Oncol.* **30** 212
[33] Lamagna C, Aurrand-Lions M and Imhof B A 2006 *J. Leukoc. Biol.* **80** 705
[34] Ma J, Xue Y, Liu W, Yue C, Bi F, Xu J, Zhang J and Li Y 2013 *PloS One* **8** e66275
[35] Kenig S, Alonso M B D, Mueller M M and Lah T T 2010 *Cancer Lett.* **289** 53
[36] Hong B S *et al* 2009 *BMC Genomics* **13** 1
[37] Pietras K and Ostman A 2010 *Exp. Cell. Res.* **316** 1324
[38] Nilsson I, Shibuya M and Wennström S 2004 *Exp. Cell. Res.* **299** 476
[39] Gallagher-Colombo S, Maas A, Yuan M and Busch T 2012 *Isr. J. Chem.* **52** 681
[40] Svensson K J, Kucharzewska P, Christianson H C, Sköld S, Löfstedt T, Johansson M C, Mörgelin M, Bengzon J, Ruf W and Belting M 2011 *Proc. Natl Acad. Sci. USA* **108** 13147
[41] Bisland S, Lilge L, Lin A, Rusnov R and Wilson B 2004 *Photochem. Photobiol.* **80** 22
[42] Togashi H, Uehara M, Ikeda H and Inokuchi T 2006 *Oral Oncol.* **42** 526