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Review

Direct and indirect photodynamic therapy effects on the cellular and molecular components of the tumor microenvironment

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ABSTRACT

Photodynamic therapy (PDT) is a novel cancer treatment. It involves the activation of a photosensitizer (PS) with light of specific wavelength, which interacts with molecular oxygen to generate singlet oxygen and other reactive oxygen species (ROS) that lead to tumor cell death. When a tumor is treated with PDT, in addition to affect cancer cells, the extracellular matrix and the other cellular components of the microenvironment are altered and finally this had effects on the tumor cells survival. Furthermore, the heterogeneity in the availability of nutrients and oxygen in the different regions of a tridimensional tumor has a strong impact on the sensitivity of cells to PDT. In this review, we summarize how PDT affects indirectly to the tumor cells, by the alterations on the extracellular matrix, the cell adhesion and the effects over the immune response. Also, we describe direct PDT effects on cancer cells, considering the intratumoral role that autophagy mediated by hypoxia-inducible factor 1 (HIF-1) has on the efficiency of the treatment.

Contents

1. Introduction

PDT is an anti-tumor modality that is approved for clinical use in a number of countries, for the elimination of early-stage malignancies and the palliation of symptoms in patients with late stage tumors [1,2]. The treatment consists of (i) a PS that is administered topically or systemically, (ii) light in the visible range of electromagnetic wave, usually generated by laser sources and (iii) molecular oxygen, which is used in the photodynamic reaction to generate singlet oxygen ($^1O_2$) and other cytotoxic oxygen species, such as superoxide anion radical (O_2-) and hydroxyl radical (OH*) [3].

PSs are localized within cellular organelles, including mitochondrias, lysosomes, endoplasmic reticulum and Golgi apparatus, and/or in the plasmatic membrane, and they have some selectivity for tumor cells; however, selectivity is generally achieved by directed light delivery.

Abbreviations: ALA, aminolevulinic acid; bFGF, basic fibroblast growth factor; BPD-MA, benzoporphyrin derivative monoacid ring A; CHS, contact hypersensitivity; DAMP, damage associated molecular pattern; DC, dendritic cell; EC, endothelial cell; ECM, extracellular matrix; HIF, hypoxia-inducible factor; HSP, heat shock protein; HpE, hematoporphyrin ester; ICAM, intercellular adhesion molecule; Me-ALA, methyl-aminolevulinic acid; mTHPC, meta-tetrahydroxyphenylchlorin; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; PDT, photodynamic therapy; PLGF, placenta-like growth factor; PRR, pattern-recognition receptor; PS, photosensitizer; ROS, reactive oxygen species; SCID, severe combined immunodeficient; TGF-β, transforming growth factor-β; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor

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PSs photo-activation leads to ROS production and tumor cell death [4]. Both lasers and incandescent light sources are used for PDT. Lasers can be coupled into fibers with diffusing tips to treat tumors in the urinary bladder and the digestive tract [5].

PDT offers several advantages over the conventional cancer treatments, such as a minimal systemic toxicity, high selectivity to the tumor, few secondary effects, the possibility of repetitive cycles of treatments and the combination with other therapies, for example chemo and radiotherapy. However, a problem of PDT and other therapies is the apparition of resistant cells. Since an increasing number of PDT treatments are being applied [6–8], it is important to determine how many PDT cycles would be optimal and when the cells begin become resistant to PDT treatment.

The resistance of human tumors to cancer therapies is attributed to mutations, amplifications genetic and epigenetic changes that influence in the take, transport and metabolism of the drug and a great network of survival and proliferation mechanisms. However, a major cause of resistance to therapies arise from the three-dimensional structure present in solid tumors. The efficacy of therapies depends, at least in part on the efficiency of transport of the drug through the tumor vasculature, leakage of the drug in this vasculature and excursion into the tumor tissue. Furthermore, the heterogeneity present in the tumor microenvironment creates a strong gradient in the rate of cell proliferation, and the generation of hypoxic and acidic regions that have a strong impact on the sensitivity of cells to anticancer treatments [9].

PDT on tumor is not only a result of direct tumor cells destruction, beside PDT has effect on tumor stroma. The stroma is composed of extracellular matrix, vasculature and different cellular components, such as fibroblasts, endothelial cells and immune system cells [9]. Emerging evidence has indicated that effective PDT of tumor requires destruction of both tumor cells and stroma [10]. Moreover, there are many studies that shown that application of PDT also develop inflammatory and anti-tumor immune responses [11–13].

Several reviews about the PDT effects describe extensively the most known death [14,15] and resistance cellular mechanisms [16]. However, new knowledge and interrogations continuously sprout, leading to the necessity to compile the available information with the aim to discuss molecular and cellular keys to enhance the efficacy of the treatment.

In this article we summarize how PDT affects indirectly to the tumor cells, including the alterations on the extracellular matrix and the effects over the immune response. Also, we describe PDT effects on cancer cells, considering the intratumoral role that autophagy, mediated by HIF-1, has on the efficiency of the treatment. We consider both positive effects of the treatment, which lead to tumor destruction, and negative effects, such as the induction of resistance mechanisms by the photodynamic treatment.

2. Alterations on the extracellular matrix and cell adhesion induced by photodynamic therapy

The extracellular matrix (ECM) is a dynamic and complex array of glycoproteins, collagens, glycosaminoglycans and proteoglycans secreted by the cells. It provides cells with key signals in a variety of physiological and pathological processes, for example, adhesion, migration, survival, proliferation and differentiation [17].

Over the ECM of the tumor there are other cellular components, beside the tumor cells, such as fibroblasts, smooth muscle cells, endothelial cells and immune cells including lymphocytes and macrophages [18]. The cancer cells constitute the parenchyma of the tumor and the other cellular components, together ECM, constitute the stroma. All the cells of the tumor contact with the ECM. Because the ECM regulates diverse cell behavior, any changes in the ECM as a result of cellular activities will in turn influence adjacent cells and modify their behaviors. This feedback regulatory mechanism between cells and the ECM allows cells and tissues to swiftly adapt to their environment. The tumor stroma is highly dynamic with ECM components continuously produced and degraded [19].

The cellular components are adhered over the ECM and among them through cell adhesion proteins which allow cell anchorage, survival, proliferation and migration. There are four main cell adhesion protein superfamilies: integrins, selectins, immunoglobulins and cadherins.

Integrins constitute a group of cell surface heterodimeric receptors composed of α/β subunits. They are ubiquitous glycoproteins that modulate cell adhesion to the ECM via interaction with ECM components, such as collagen, fibronectin, laminin and vitronectin. They form a link between the extracellular environment and the cytoskeleton constituting, together adaptor proteins, focal adhesion contacts. Integrins, in the focal adhesion contacts, also function as signaling nexus and participle in the regulation of survival, proliferation, migration and differentiation. Also, via adaptor molecules, integrin signaling interacts cooperatively with growth factor receptors [17]. Integrin-associated signaling renders cells resistant to genotoxic anti-cancer agents like ionizing radiation and chemotherapeutic substances, through Akt via [20] and the inhibition of caspase-8 [21].

Intercellular adhesion molecules (ICAM) are members of the immunoglobulins superfamily expressed on the luminal surface of EC, on some lymphocytes and monocytes. Integrins on the surface of leukocytes bind to ICAM-1 of EC in order to form more stable adhesions at sites of tissue inflammation. This attachment enables leukocytes to migrate through the EC of capillaries and enter the underlying tissue [22]. Besides ICAM-1, integrins of leukocytes bind to surface adhesion glyproteins of EC called selectins. P-selectin in EC is one of the main adhesion molecules that bind leukocytes and E-selectin is expressed only on inflamed endothelium, after activation by inflammatory cytokines or endotoxin [23]. E-selectin mediated adhesion to EC has been established in inflammatory leucocytes as well as several human cancer cells [24].

Vascular cell adhesion molecule-1 (VCAM-1) is a member of the immunoglobulins superfamily expressed on the luminal surfaces of EC during inflammation and works as an important cell–cell adhesion molecule in white blood cell, platelet or tumor cells. Integrins and selectins of metastatic tumor cells bind to adhesion proteins on EC, for example VCAM-1.

Cadherins are transmembrane adhesion glycoproteins that assemble into adherent junctions to connect neighboring cells, allowing the interaction of cells of the same type. There are multiple classes of cadherin molecules. E-cadherin is the most extensively studied in cancer. However, during cancer progression E-cadherin-mediated adhesion is frequently lost [25]. Down-regulation of E-cadherin expression correlates with a strong invasive potential, resulting in poor prognosis in many human carcinomas [26].

The effect of PDT on the ECM and the cell adhesion is not completely studied yet. So far it is known that PDT produces changes in the ECM and cell adhesion, which are dependent, in large part, of the photosensitizer kind and the treatment doses [27].

There was reported that PDT employing some photosensitizers do not result in extensive collagen damage. Heat shock proteins (HSPs) are highly conserved proteins that are induced by cellular signaling and play a major role in cytoprotection. Several HSPs are induced after PDT injury, in an early cell response. HSP47 is expressed in cells that synthesize collagen, such as fibroblasts. It is involved in collagen type I biosynthesis, and after insult acts as a stress response molecule to sequester abnormal procollagen. An early elevation of HSP47 expression is observed only by modalities affecting collagen or its precursors, such as hyperthermia and riboflavin–PDT. The photoactivation of some photosensitizers, such as hematoporphyrin ester (HpE) and meta-tetrahydroxyphenylchlorin (mTHPC), does not result in extensive collagen damage and the HSP47 is not induced. In contrast, HSP47, at both transcriptional and translational levels, are elevated after hyperthermia and after PDT with riboflavin [28,29].

On 1988 Nelson et al. [[]] indicated that a target of PTD employing hematoporphyrin derivative, chlorin and phthalocyanine was the conjunctive tissue of the subendothelial zone of the tumor capillary wall, which had repercussion over the tumor cells. The effects of PDT leading to rapid necrosis of tumor tissue were not the result of direct tumor cell kill, but were secondary to destruction of the tumor microvasculature. The first observable signs of destruction occur in the subendothelial zone of the tumor capillary wall, composed of dense collagen fibers and other connective tissue elements. However, the ultrastructural changes seen in this zone are different among the three employed photosensitizers. A recent study has demonstrated that local vascular microenvironment is a determinant of tumor response to PDT. Tumor cells were inoculated in mice with basement membrane matrix of collagen (Matrigel) to study the Photofrin-PDT response. Photosensitizer localization to collagen increases vascular damage and improves treatment efficacy in tumors with greater collagen content. The vascular basement membrane is thus identified to be a determinant of therapeutic outcome in PDT of tumors [31].

Local eradication of vascular cells produced by PDT in vivo is followed by reendothelialization. An in vitro study explored one possible mechanism underlying these findings by investigating the effects of PDT on matrix-associated transforming growth factor-beta (TGF-β), a potent inhibitor of endothelial cell (EC) growth. Increased EC proliferation on PDT-treated matrix is, at least in part, mediated by inactivation of TGF-β. PDT-removal of this EC growth inhibitor in the intima provides a mechanism by which PDT of the vascular wall could potentiate endothelial regrowth, a factor which may promote proper healing and result in the inhibition of intimal hyperplasia [32]. However, a negative effect of the reendothelialization process in cancer treatment arises if not all cancer cells have been removed, contributing to the recidivist event. It is known that many other factors produced by tumor cells and stromal cells, besides the removing of TGF-β, contribute to angiogenesis after PDT, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), angiopoietin, placenta-like growth factor (PLGF) and IL-8 [33]. PDT-induced tissue hypoxia as a result of vascular damage and the photochemical oxygen consumption may limit the efficacy of this treatment. This enhances malignant progression by selection and clonal expansion of altered cells resistant to the deprivation of nutrients and the hypoxia. After photodynamic treatment, hypoxic condition in the surviving tumor cells can cause the stabilization of hypoxia-inducible factor 1α (HIF-1α) and the expression of VEGF by the HIF-1α pathway [34]. Thus, PDT can promote the tumor angiogenesis, enhancing tumor proliferation and survival [35,36].

It was reported that PDT induces cross-linking in collagen matrix. The singlet oxygen generated during PDT interacts with amino acid residues in proteins to produce reactive species. These newly generated free radicals interact with other molecules to form cross-links inactivating matrix-residing growth factors and increased resistance to matrix metalloproteinases (MMPs) degradation [37]. MMPs are key proteinases involved in the degradation of the extracellular matrix and are identified as playing an essential role in tumor angiogenesis, invasion and metastasis. It has been theorized that ECM cross-linking is a major contributor to the inhibition of cell migration following PDT. Thus, cross-linking might contribute with positive effect to the treatment due this produces an increased resistance to protease degradation, hindering invasive cellular migration [38]. Certainly, PDT on a cell-free tridimensional matrix gel using chloroaluminum-sulfonated phthalocyanine induced matrix protein cross-linking, which resulted in resistance to MMP digestion. When PDT was applied over these matrix gels, it led to a reduction of invasive smooth muscle cells and to a reduction of adventitial fibroblast migration, but did not significantly affect secretion of MMPs. Cell detachment was observed, maybe because of induced structural alterations of matrix binding sites. Also, in vivo, PDT altered the vascular wall matrix and led to a durable reduction in pepsin digestion susceptibility of treated arteries and inhibition of periadventitial cell migration. These data suggest that PDT generates a barrier to invasive cell migration [39]. The reduced migration and furthermore the detachment of EC cells of ECM would render the tumor angiogenesis process, altering tumor cells survival. This, together the reduction on the cancer cells migration would hinder the invasion and metastasis. Also, the reduction on the adhesion of fibroblasts would alter their survival or their function on ECM generation. It would generate a circle of reduction of ECM components, reduction of fibroblast survival and of all the other cells of the tumor microenvironment, including cancer cells, by interruption in the integrins connections and inactivating matrix-residing growth factors.

The cell–substrate adhesion mediated by integrins would be interrupted after photodynamic action by damage to ECM and by direct damage on the integrin proteins. In employing benzoporphyrin derivative monoacid ring A (BPD) as photosensitizer, PDT inhibited cell adhesion of human fibroblast and affected integrin signaling without modifying neither the cell membrane integrity nor integrin expression [40]. The photo-activation of this sensitizer in human ovarian cancer cell line decreased markedly the adhesion, which could have caused the cell death. In this case, differing from that cited previously, the loss in adhesiveness was accompanied by a loss of β1-integrin-containing focal adhesion contacts [41]. This may have an impact on long-term effects of PDT, but the topic merits further investigation. Human adenocarcinoma WiDr cells in suspension after 5-aminolevulinic acid (ALA)-PDT were unable to attach to a plastic substratum and showed redistribution of αvβ3 integrin, with no change in E-cadherin expression [42]. Under apoptotic conditions, zinc (II)–phthalocyanine–PDT induced a rapid disorganization of the E-cadherin-mediated cell–cell adhesion, which preceded the detachment of cells from the substratum via β1-integrins [43].

There is evidence indicating that integrins might contribute to PDT cell resistance. Alterations in the cell adhesion caused by PDT have been described for several authors, although the implication of this event in tumor resistance remains to be elucidated. The induction of cellular resistance had been employed to study the resistance mechanisms to antineoplastic treatments, including PDT. One way to obtain cells resistant to the PDT is to apply a high dose of treatment, which allows surviving only to the more resistant cells. Thus, it is possible to obtain resistant cells to PDT increasing drug dose [44], drug exposure time [45] or light dose [46,47]. Repetitive cycles of treatment and cell growth can be performed with the aim to amplify the biochemical changes associated with cell resistance and thus identify potential selective targets on the survival cells. In our laboratory we had obtained human squamous carcinoma cells SCC-13 resistant to photodynamic treatment with methyl-aminolevulinic acid (Me-ALA). The resistant cells were generated employing repetitive cycles of treatment. We had described that SCC-13-resistant cells increased their levels of β1-integrins with the resistant grade [47]. Cell–cell adhesion protein E-cadherin did not change their expression in resistant cells with respect to parental cells. Other researches employing clones of breast cancer murine LM3 resistant to aminolevulinic acid (ALA)–PDT observed that resistant cells had not increased levels neither of β1-integrins [48] nor of E-cadherin [49], indicating that other resistance strategies were involucrated.

Biomedical nanoparticles containing photosensitizer deliver ROS to cancer cells and their microenvironment. Expression of $\alpha_V\beta_3$ integrins is a common feature of tumor vasculatures and nanoparticles surface-coated with an RGD peptide, an $\alpha_V\beta_3$ ligand, were developed to target the vasculature of rats brain tumor. Alexafluor 594 dye was included in the nanoparticle matrix to enable fluorescent detection. Plasma residence time control and specific cell targeting were achieved. The treatment halted, and even reversed in vivo tumor growth [50].

There are not many studies about the PDT effect on the cell adhesion between tumor cells and EC. In general, it is postulated that this interaction would be interrupted due damage to EC and cancer cells

adhesion proteins, besides the viability reduction in both cells types. Hematoporphyrin derivative-PDT decreased cancer cell adhesiveness to EC in vitro and it reduced the metastatic potential of cells injected into rats. Photofrin and benzoporphyrin derivative monoacid ring A (BPD-MA) have been evaluated on two colon cancer cell lines with different metastatic properties. Adhesion molecules at the cell surface were significantly decreased and there was a metastatic potential reduction induced by PDT with both drugs on the two cell lines [51].

Immune system cell adhesion is altered too after PDT. Sluiter et al. [52] first observed that neutrophils adhere to the microvascular wall after PDT in vivo and that EC retracted after PDT allowing the adherence of neutrophils by their β2-integrin adhesion receptors to the subendothelial matrix. Activation of the β2-integrin receptors by interaction with the subendothelial matrix is necessary for the increased binding of neutrophils [53]. Volanti et al. [54] observed that the expression levels of the adhesion molecules ICAM-1 and VCAM-1 were down-regulated in EC after PDT. However, a marked up-regulation of the ICAM-1 ligands CD11b and CD11c (integrins), which are found on neutrophils, was also associated with PDT-treated tumors [4]. After PDT in vivo the expression of P-selectin by EC was not stimulated, but the expression of E-selectin was increased in tumors treated with hexylpyropheophorbide–PDT, facilitating neutrophil migration into the tumor area. In contrast, employing Photofrin® PDT only caused polymorphonuclear leukocytes to adhere to the wall of normal vessel but not to those of tumor capillaries.

In solid tumors, MMPs can be secreted by tumor cells, macrophages, EC, smooth muscle cells and fibroblast. MMP-1, -3, -8 and -9 up-regulation have been noticed in Photofrin-mediated PDT and overexpression of MMP-2 was observed after 5-ALA PDT. However, hypericin PDT attenuated MMP-9 and Hexyl-ALA-mediated PDT seemed to suppress MMP-2 concentrations (summarized in [55]). Also, PDT with different photosensitizers impaired MMP-2 and MMP-8 activity in glioma cells [56] and no alterations in the MMPs pattern were observed in some other PDT-treated cells [57,58]. The photosensibilization of fibroblasts produces extracellular matrix components reduction. After several treatments with low doses of topical ALA-PDT, patients with localized scleroderma show a reduction in skin tightness, suggesting that this therapy reduces skin sclerosis. It is generally accepted that dermal fibroblasts are the key in the pathogenesis of skin sclerosis by synthesizing increased amounts of collagen type I and III, whereas collagen degrading enzymes, such as MMP-1, MMP-2 and MMP-3, are decreased. ALA and light induce MMP-1 and MMP-3 expression in normal and scleroderma fibroblasts in a singlet oxygen-dependent way, while reduce collagen type I mRNA expression. Induction of collagen-degrading enzymes together with reduction of collagen production might be responsible for the anti-sclerotic effects of ALA-PDT observed in vivo [59]. PDT can trigger MMP production in dermal fibroblasts not only directly, but also by an indirect paracrine loop mediated by soluble factors released by epidermal keratinocytes. In the PDT resistance model of LM3 clones described by Casas et al. [48], both LM3 and resistant clones exhibit MMP-2 and MMP-9 activities, but no significant differences were found between LM3 and the resistant clones, suggesting that the impaired collagen proteolytic activity observed is not the reason for the different metastatic phenotype. The resistant clones showed lesser invasion in Matrigel and an important decreasing in the number of metastasis when they were injected in mice.

A growing number of studies provide strong evidence of a crucial role for MMP-9 in the process of tumor angiogenesis and growth. MMP-9 is associated with a malignant phenotype in part because of the enzyme ability to degrade type IV collagen. Combining PDT with Prinomastat (AG-3340), a potent synthetic MMP inhibitor with significant affinities for MMP-2 and MMP-9, the therapeutic effectiveness is enhanced. Preclinical studies show that Prinomastat produces growth delays in a variety of tumors including neuroblastoma, as well as in cancers of the lung, breast, colon, brain, and prostate. Prinomastat inhibits tumor angiogenesis and cell proliferation and enhances the efficacy of Carboplatin and Taxol. Infiltrating inflammatory cells and endothelial cells were primary sources of MMP-9 expression after PDT, whereas negligible expression was observed in mouse mammary carcinoma cells [60].

In conclusion, the components of the ECM might favor the photosensitizer accumulation and thus the alteration of the matrix is incremented. It had secondary effect over tumor cells, leading to enhanced death of malignant cells. Also, the cross-linked of the matrix produced by PDT reduced the migration of the malignant and stromal cells. The cell adhesion is altered after photodynamic treatment, by ECM injury or direct damage in the adhesion proteins on the surface of tumor cells and all the cellular components of the tumor microenvironment (Fig. 1).

3. PDT effect on anti-tumor immunity

PDT has a significant effect on the immune system [11,61,62], which can be either immunostimulatory or immunosuppressive. Several recent reviews extensively describe how PDT enhancement anti-tumor immunity [63–65], however there are also reports that PDT can induce various forms of immunosuppression [66]. The effect of PDT on the immune system appears dependent on the treatment regimen, the photosensitizer type and the treatment area [11,67,68].

In 1994, Canti et al. [69] was the first to demonstrate the induction of anti-tumor immunity by PDT. In this study, cells isolated from the tumor-draining lymph nodes of PDT-treated mice were able to suppress subsequent tumor challenge when transferred to naïve hosts; moreover PDT-treated mice that remained tumor free for 100 days were able to effectively control subsequent tumor challenge suggesting the presence of immune memory [69]. Korbelik and Dougherty [70] later demonstrated the presence of immune memory following PDT. They observed that Photofrin-based PDT at a dose that cured 100% of EMT6 mammary sarcomas in syngeneic BALB/c mice provided only short-term cures in severe combined immunodeficient (SCID) or nude mice. The ability to provide long-term cures was restored when immunodeficient animals were reconstituted with bone marrow cells from BALB/c mice.

At the moment, there is accumulating evidence that describes the mechanism of enhanced anti-tumor immunity by PDT. PDT inflammatory response is considered an important priming event for the development of anti-tumor immunity response. PDT-treated dying cells produce danger signals, these include expression of HSPs and transcription factors such as NF-κB and AP-1 [34,71,72]. These two alone can induce the expression of dozens of cytokines, adhesion molecules, co-stimulatory molecules and immunologically important genes. Additionally, photo-oxidative degradation of membrane lipids and generation of arachidonic acid metabolites are themselves potent inflammatory mediators that precipitate a rapid and strong inflammatory reaction [70]. These processes together with the release of histamine and serotonin from damaged vasculature induce an activation of complement, sequential arrival of neutrophils and other inflammatory cells in large numbers at the treated site and to attack tumor cells [73,74]. In particular, the complement system has emerged as a powerful mediator of the effects of PDT on tumor cells and in vitro studies have indicated that PDT induces fixation of complement C3 protein to tumor cells [75], moreover studies in vivo have demonstrated the importance of the complement C3 in mediating PDT-induced anti-glioma responses in mice [76]. Complement not only acts as a direct mediator of inflammation but also stimulates cells to release secondary inflammatory mediators, including the cytokines IL-1β, TNF-α, IL-6, IL-10, granulocyte colony-stimulating factor, thromboxane, prostaglandins, leukotrienes, histamine and coagulation factors [77].

Stimulation of dendritic cells (DCs) by dying tumor cells appears to be another important event for enhancement of anti-tumor immunity by PDT [78,79]. Induction of acute inflammation by PDT results in

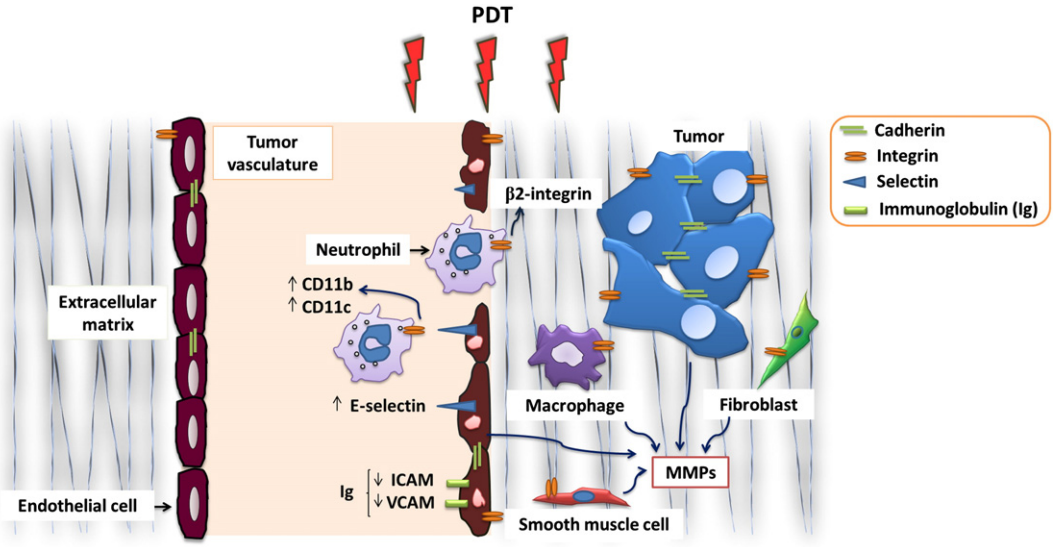

Fig. 1. Photodynamic therapy effects on the extracellular matrix and cell adhesion proteins. PDT produces extracellular matrix alterations and this reduces cell adhesion, migration and survival of all cellular components that constitute the tumor microenvironment. Also, direct injury on the cell surface adhesion proteins is produced by the photodamage; integrin and E-cadherin adhesion is generally reduced. The immunoglobulins ICAM and VCAM expression can be decreased on the surface of the endothelial cells, but E-selectin increments in these cells favoring the adhesion of neutrophils. Also, up-regulation of ICAM-1 ligands CD11b and CD11c (integrins) was observed on neutrophils after PDT. Endothelial retraction induced by PDT allows the adherence of neutrophils by their β2-integrin adhesion receptors to the subendothelial matrix in the way toward the treated tumor area. In solid tumors, MMPs are secreted by macrophages, fibroblasts, endothelial cells, smooth muscle cells and tumor cells. After PDT some MMPs are induced, but others are reduced or no alter in their expression, depending on the photosensitizer and the treatment doses. PDT: photodynamic therapy; ICAM: intercellular adhesion molecule. VCAM: vascular cell adhesion molecule. MMPs: matrix metalloproteinases.

the maturation and activation of dendritic cells [80] and migration to the tumor-draining lymph nodes where they are believed to stimulate T-cell activation [80,81]. Studies have shown that incubation of PDT-treated tumor cells with immature DCs leads to enhanced DC maturation, activation and ability to stimulate T-cell activation [79,82]. A recent report demonstrated that PDT-generated tumor cell lysate induces IL-1α, IL-1β, and IL-6 secretion from DCs, suggesting PDT-enhanced antitumor immunity is due in part to increased DC activation [83]. It is supposed that DC activation by PDT is the result of sensing endogenous danger signals released by dying tumor cells [80,84–87]. These danger signals are referred to as damage-associated molecular patterns (DAMPs) that are immunestimulatory by interacting with pattern-recognition receptors (PRRs) expressed on innate immune cells [88]. Recent studies have shown that PDT effectively induces expression of multiple danger signals capable of activating antigen presenting cells and generating anti-tumor immunity [86,87,89]. HSPs and especially HSP70, are the best characterized DAMPs associated with PDT [86,90]. HSP70 expression is prompted by cellular stress and, when remains intracellular, it chaperones unfolded proteins and inhibits cell death by preventing the aggregation of cellular proteins [91]. This forms stable complexes with cytoplasmic tumor antigens that can then either be displayed at the surface of cellular membrane or escape intact from dying necrotic cells to interact with antigen presenting cells such as DCs and stimulate an anti-tumor immune response [86,92]. The Fig. 2 summarizes the anti-tumor immunity response trigger by PDT.

Paradoxically, considering the discussions above, the effects of PDT on the immune system appear not only to augment immune cell reactivity against tumors, but also to suppress immune cell activation. The immune suppressive effects of PDT have been recognized in mice for more than 20 years. It was reported that skin exposure to the light after a photosensitizer administration resulted in the systemic immunosuppression manifested by the inhibition of contact hypersensitivity (CHS) response [93]. Similar observations were made using different photosensitizers [94,95]. It seems that this suppression involves systemic IL-10 release in cases where the PDT illumination penetrates the skin (red light) [96], but is independent of IL-10 when the PDT is confined to the skin layers (blue light) [97]. Furthermore, it appears that immune suppression generated by PDT is mediated primarily by T cells and this immunosuppression can be adoptively transferred to naïve recipients [98]. Studies in humans demonstrated that topical PDT induced a significant immune suppression [99]; however a recent reported observed that reducing the rate of irradiation prevented this immunosuppression [68].

In contrast to chemotherapy and radiotherapy, which in their current use are inherently immunosuppressive, PDT offers a remarkable advantage of stimulating an immune response. Only in some cases, it has an immunosuppressive effect, but in regulating treatment regimen it is possible to solve this problem. The optimization of PDT regimens that lead to enhanced anti-tumor immunity has been limited due to a lack of mechanistic understanding and the complexity of the effects of PDT on both tumor and host cells. It is entirely possible that the optimal PDT regimen for producing local tumor cures will be different from the optimal PDT regimen for producing inflammation and stimulating immune response. However, understanding the mechanisms used by PDT to augment anti-tumor immunity will permit optimization and exploitation of this aspect of PDT in a clinical setting.

4. Autophagy and photodynamic therapy resistance: implications of a hypoxic tumor microenvironment

The presence of hypoxia in tumors is known to lead to activation of genes associated with angiogenesis and cell survival, and this effect is primarily mediated by HIF-1. The expression of these genes may lead to the expansion of cell populations with altered metabolic pathways and resistant phenotype [100,101] to promote the spread and adaptation to their hostile microenvironment.

HIF-1 promotes transcription of angiogenic factors, such as VEGF and leads to increased glycolysis by inhibition of mitochondrial oxidative phosphorylation [100,102]. Under normoxia, HIF-1 is hydroxylated by Prolyl Hydroxylase Domain-containing proteins (PHD) and targeted for degradation by the proteasome [103]. Hypoxia and a high level of ROS inhibit PHD activity, leading to HIF-1 stabilization and activation [104,105].

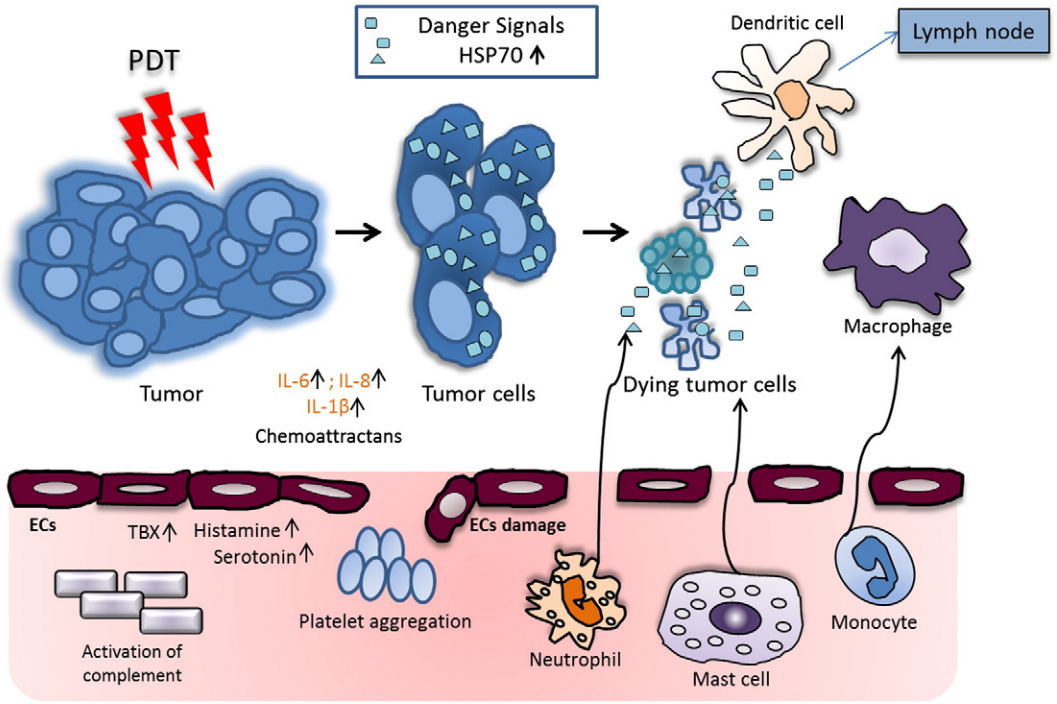

Fig. 2. Photodynamic therapy induces an anti-tumor immunity response. PDT-treated dying cells produce danger signals; these include expression of heat shock proteins and especially HSP70. These induce the expression of dozens of cytokines, adhesion molecules, co-stimulatory molecules and immunologically important genes. These processes together with the release of histamine and serotonin from damaged vasculature induce an activation of complement, sequential arrival of neutrophils and other inflammatory cells in large numbers at the treated site and to attack tumor cells. Dendritic cells are activate by PDT sensing endogenous danger signals release by dying tumor cells, then they migrate to the tumor-draining lymph nodes and stimulate T-cell activation. PDT: photodynamic therapy; ECs: endothelial cells; HSP: heat-shock protein; TBX: thromboxane.

Hypoxia and oxidative stress both induce autophagy. This is the recently recognized most common mechanism of resistance to chemotherapy and other anticancer therapies [106]. The autophagy is defined as a process of programed cell survival that consist of degradation of cellular components in double membrane structures called autophagosomes; these undergo a maturation process that culminates in fusion to lysosomes to form autolysosomes, where cytoplasmatic components are degraded and recycled which provide essential building blocksback to cell, such as amino acids [107]. Autophagy optimizes nutrient utilization in rapidly growing cells when faced with hypoxic or metabolic stresses and, hence it contributes to normal and cancer cell survival [108]. The autophagic response recycling proteins and cellular components, contribute to tumor progression as a protective mechanism against stressful microenvironmental conditions including anti-cancer therapies [109]. It has been suggested that under hypoxia, preservation of cellular fitness by autophagy may be crucial to tumor progression and aid to preventing cancer cell death [110].

One of the problems of PDT [111] and other anticancer therapies is the apparition of resistant cell populations [112]. The incapacity to suffer death in response to treatment consents a selective advantage in the tumor progression and resistance to therapies [113]. Both hypoxia and autophagy pathways are induced by PDT and these have a cytoprotective effect, which would hinder the success of the treatment [16].

ROS produced by the photodynamic treatment can induce autophagy [114]. The first line of defense against ROS can be rapidly overwhelmed during PDT, leading to oxidative stress and progressive failure of cellular machinery. In mammalian cells, the autophagy–lysosomal system represents a major proteolytic system for the clearance of ROS-damaged organelles and irreversibly oxidized cytosolic proteins [115]. Although the molecular mechanisms by which ROS modulate autophagy are not fully understood, the type of ROS, degree of oxidative injury and the involved molecular targets can all affect the outcome of PDT. Mitochondrial and endoplasmic reticulum-localized sensitizers cause selective photodamage to some proteins involved in the autophagic process (i.e., Bcl-2, Bcl-x, mTOR) [116]. The pro-autophagic protein Beclin 1 is known to bind to Bcl-2 [117]. Bcl-2 is photodamaged by many PSs commonly used in investigation and clinical PDT studies [118,119]. Loss of Bcl-2 function could release Beclin 1 protein leading to the initiation of autophagy [120].

Interestingly, it has been demonstrated that PDT results in overexpression of HIF-1α [36,121]. HIF-1 is a master player in the adaptive response to hypoxia and influences the transcription of hundreds of genes, including the BH3-only proteins BNIP3 and BNIP3L, two pro-autophagic proteins. Thus, HIF-1-mediated induction of these BH3-only proteins can free Beclin 1 from Bcl-2/Bcl-XL, hereby stimulating autophagy [122].

The protective role of autophagy involves its ability to repair photodamaged cellular components. It is possible that autophagy is initiated in an attempt to remove organelles damaged by oxidation or to degrade large aggregates of cross-linked proteins, reduced photochemical reactions, which are not removed by the ubiquitin-proteosome system [120]. Silencing of the autophagy gene Atg7 results in the increased photosensitization of cells to photodynamic effects. Hypericin-PDT was also reported to induce a cytoprotective autophagic response in melanoma cells [123]. In addition, Atg7 knockdown of leukemia L1210 cells treated with CPO–PDT was more sensitive to the parental cells [116]. In brief, stimulation of autophagy in apoptosis-competent cells increases cellular resistance to photokilling in PDT protocols.

While PDT is capable to induce both hypoxia and autophagy, these processes are features of solid tumor *per se* [124] and this could have a profound impact on photodynamic effect. The glucose deprivation and hypoxia, produced by ischemia, a common physiological stress in the tumor microenvironment, up-regulate autophagy. Autophagosomes are most prominent in tumor cells that are located in hypoxic tumor regions, and deletion of essential autophagy genes results in tumor cell death specifically in these hypoxic regions [125–127]. This is an indication that

tumors can commandeer the survival function of autophagy to promote tumorigenesis [125–127].

In this sense, the cells in the hypoxic core of the tumor contain the features related with PDT resistance. The tumor hypoxic region contains a poor vasculature, which could hinder the inclusion of PSs. The photodynamic effect is decreed in poorly oxygenated regions [128]. Moreover, the autophagic activity [127] contributes to tumor cells adaptation in a hostile hypoxic environment, permits tumor cells survive after destruction of tumor vasculature by PDT and enhances the resistance of these cells to a subsequent application of PDT [116]. This population is the result of a strong selection pressure and it is capable to generate a novel tumor (tumor relapse) with a phenotype more aggressive and resistant. The role of hypoxic microenvironment in the PDT-resistance is schematized in Fig. 3.

Summarizing, the tumor cells alter numerous metabolic pathways to maintain homeostasis. The increased glycolysis, by inhibition of mitochondrial oxidative phosphorylation, is necessary to the cell adaptation to a hypoxic microenvironment [101], which is associated with both nutrient deprivation and ROS production. All of these alterations result in a stimulation of autophagy [129].

Thus, the therapeutic inhibition of autophagy would result in cancer cells unable to survive in their metabolic stressed microenvironment and promote their regression as well as sensitize tumor cells to photodynamic treatment.

5. Conclusions

PDT is a promising anticancer treatment modality. The evident advantages of PDT over other conventional cancer treatments encourage the investigation in this area. The analysis of the PDT effects is difficult due to the complex of the tumor macro and microenvironments and the numerous and intricate death and resistance cellular signaling and because it can combine many photosensitizers, light doses, light sources and different application protocols.

Enhancing the efficacy of this treatment is a great challenge and numerous investigations are necessary. In this review, we describe same fundamental cellular and molecular mechanisms that would be considering in the applications of PDT to provide with a more effective treatment and a better future for oncologic patients. Modulating cell adhesion, destructing the extracellular matrix, interrupting autophagic tumor way and optimizing clinical setting to enhance anti-tumor immunity, would be some keys to improve the efficacy of the treatment. In addition, the possibility of combine PDT with other treatment regimens, such as chemo and radiotherapy, is an interesting tool to consider to improve the photodynamic action on tumors.

Conflict of interest

The authors declare no conflict of interest.

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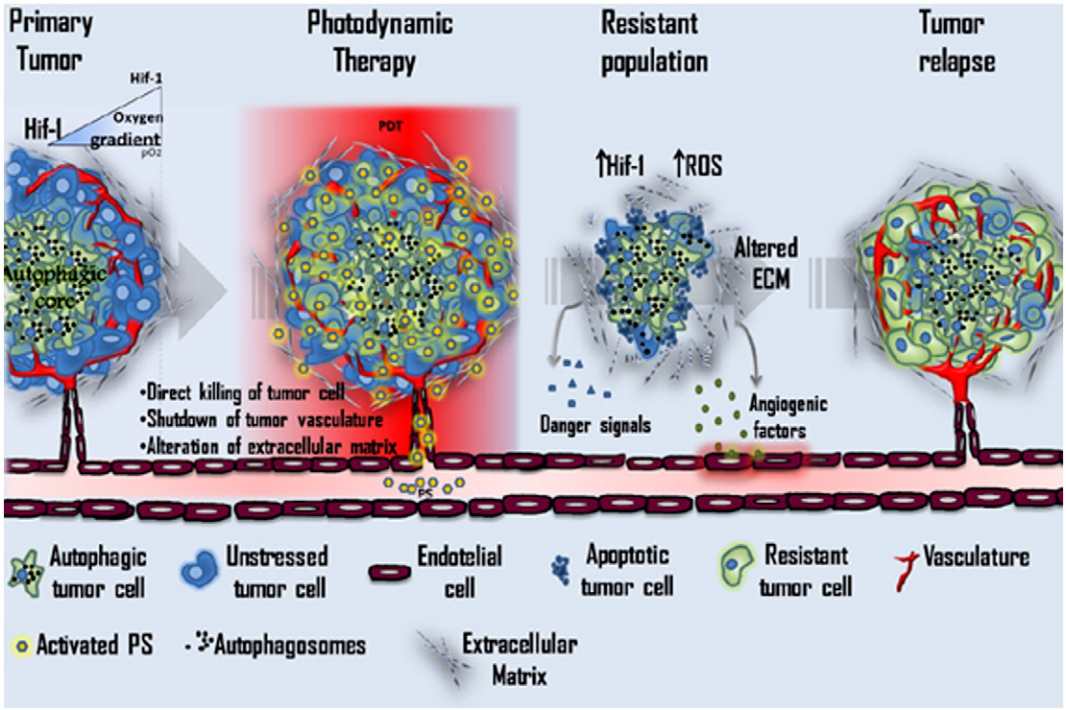

Fig. 3. Hypoxic microenvironment induces PDT resistance mediated by autophagy. The primary tumor is characterized by present an oxygen pressure gradient decreasing toward regions poorly vascularized. These regions contain metabolically stressed cells with autophagic activity ("Autophagic Core") mediated by high expression of Hif-1. PDT removes the tumor by direct killing of tumor cells, shutdown of tumor vasculature and alterations of extracellular matrix. Followed of PDT, the tumor cells activate cell death pathways, such as apoptosis, as well as surviving pathways, such as autophagy. Although autophagy is active in the untreated tumor, after PDT it is exacerbated by Hif-1 and ROS induced by the therapy. Moreover, the damage on vasculature causes nutrient depletion and hypoxia, both inducers of autophagy. The surviving cells represent the resistant population that uses autophagy to adapt to stress microenvironment and to induce expression of surviving and angiogenic signals. In consequence, the recovery of these cells result in a tumor relapse adapted to resist to subsequent PDT application. PDT: photodynamic therapy; ROS: reactive oxygen species; Hif-1: hypoxia-inducible factor 1.

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