Matrix metalloproteinase-3 induction following photodynamic therapy with liposomal formulations of aminolevulinic acid and its methyl ester

Indukcja metaloproteinazy-3 macierzy pozakomórkowej za pomocą terapii fotodynamicznej z zastosowaniem liposomowych preparatów kwasu aminolewulinowego i jego estru metylowego

Kamil Jurczyszyn[1], Elżbieta Czapińska[2], Elżbieta Gamian[1], Katarzyna Hotowy[2], Grzegorz Terlecki[2], Krzysztof Symonowicz[1], Beata Osiecka[1], Andrzej Bronowicz[1], Piotr Ziółkowski[1]

[1] Department of Pathology, Wrocław Medical University, ul. Marcinkowskiego 1, 50-368 Wrocław, tel. +48 (71) 784 12 25, fax +48 (71) 784 00 57, e-mail: ziolkows@interia.pl
[2] Department of Clinical Biochemistry, Wrocław Medical University, ul. Chałubińskiego, 50-368 Wrocław, Poland

Abstract

Photodynamic therapy (PDT) showed promising results in treatment of malignant and non-malignant disorders. The PDT requires for a therapeutic effect the combined action of photosensitizer and light. PDT causes direct cytotoxicity to malignant cells and may also have both direct and indirect effects upon various non-malignant components of tumor microenvironment. Unfortunately, some photosensitizers reveal low selectivity in pathologic tissues. Previous studies indicated that aminolevulinic acid (ALA) and its methyl ester (metvix) encapsulated in liposomes improved the quality and optimized results of PDT. Matrix metalloproteinases (MMPs) are enzymes implicated in various diseases by enabling the spread of disease. MMP-3 in turn, exerts the protective role in the development of tumors. We report the effect of liposomal formulation of ALA and metvix-based photodynamic therapy on induction of matrix metalloproteinase 3 in animal tumor model. Our results showed strong expression of MMP-3 in tumor-bearing rat tumor cells after PDT with liposomal formulations. The expression of MMP-3 in all tumor-bearing rats treated with PDT was stronger than those observed in animals free of tumors and especially in those treated with free precursor-PDT. The effect of liposomes was found to be important and these formulations elicited stronger intensity of immunohistochemical reactions than ALA- or metvix –PDT without liposomes.

Key words: photodynamic therapy, liposomes, aminolevulinic acid, metvix, metalloproteinase 3

Streszczenie

Terapia fotodynamiczna (*photodynamic therapy* – PDT) daje obiecujące wyniki w leczeniu złośliwych i niezłośliwych chorób. PDT wymaga połączonego działania fotouczulacza i światła dla uzyskania efektu terapeutycznego. PDT wywołuje efekt cytotoksyczny w patologicznych komórkach. Może także działać bezpośrednio i pośrednio na różne składniki mikrośrodowiska nowotworu. Niestety, niektóre fotouczulacze mają niską selektywność w tkankach chorobowych. Poprzednie badania wykazały, że kwas aminolewulinowy (ALA) i jego ester metylowy (metvix) umieszczone w liposomach poprawiały jakość i wyniki PDT. Metaloproteinazy macierzy (MMPs) są enzymami wprzęgniętymi w różne choroby umożliwiającymi ich szerzenie się. Z kolei MMP-3 odgrywa rolę ochronną w rozwoju nowotworów. W pracy przedstawiono wpływ działania preparatów liposomowych ALA i metviksu w warunkach PDT na indukcję MMP-3 w zwierzęcym modelu nowotworowym. Uzyskane wyniki wskazują na silną ekspresję MMP-3 w komórkach nowotworu po PDT z użyciem preparatów liposomowych. Ekspresja MMP-3 u wszystkich zwierząt zaszczepionych nowotworem i traktowanych PDT była silniejsza niż u zwierząt wolnych od nowotworu i szczególnie od tych leczonych PDT z użyciem wolnych prekursorów. Wpływ liposomów okazał się być istotny i przyczyniał się do silniejszej reakcji immunohistochemicznej niż PDT bez tych nośników.

Słowa kluczowe: terapia fotodynamiczna, liposomy, kwas aminolewulinowy, metvix, metaloproteinaza 3

Introduction

Photodynamic therapy (PDT) is a minimally invasive therapeutic modality approved for clinical treatment of malignant and non-malignant disorders. A chemical compound, termed as photosensitizer, with specific photophysical properties is selectively accumulated in pathologic tissues. The activation of the photosensitizer by visible light, preferentially in the red region of spectrum at $\lambda > 600$ nm, results in generation of reactive oxygen species, e.g. a singlet oxygen - $^1O_2$, responsible for cytotoxic damage of pathologic cells and often a tumor regression. Three main mechanisms were described by which singlet oxygen contributes to the destruction of pathologic cells, i.e. vascular occlusion, direct cell damage and activation of immunologic system. Advantages of PDT over other conventional anticancer treatments are low systemic toxicity and selective tumor cell killing. PDT is widely used for the treatment of endoscopically accessible tumors such as bronchial and urinary bladder and in dermatology to treat skin cancer (basal- or squamous-cell carcinoma) and benign lesions (e.g. solar keratosis and acne). PDT is rather promising approach for the treatment of superficially located tumors. ALA and its ester derivatives and photofrin are the ma-

in compounds used in clinical studies. Unfortunately, some photosensitizers reveal low selectivity, e.g. photofrin, and result in long-term skin photosensitivity [1, 2]. A crucial factor in choosing photosensitizer for PDT is its ability to incorporate into tumor cells and this is a real challenge for hydrophilic compounds. Modifications of porphyrins with 2, 3 or 4 meso-substituents were synthesized and indicated that there is lesser binding to liposomes with increased hydrophobicity [3]. Liposomes consist of spherical phospholipid bi-layers with specific properties making them very useful for topical application of drugs. Liposome studies were considerably developed over the last decades and it is now possible to build up a wide range of liposomes with various phospholipid content and size to fit specific applications for which they were built up. Liposomes can be apllied as carriers for hydrophilic as well as lipophilic agents because of their amphiphilic character. They may improve stabilization of drugs by encapsulating them and serve as penetration enhancers facilitating their transport, help in reducing skin irritation by sustaining the release of drugs and by hydration of the tissues. Clinical studies indicated that ALA or glycodendrimeric phenylporphyrins encapsulated in liposomes improved the quality of Fluorescence Diagnosis by ALA-induced Porphyrins (FD) and optimized results of PDT [4].

The hydrophilic nature of the ALA molecule limits to some extent the penetration through the skin and cell membranes. Various attempts were recently investigated to increase ALA penetration, such as the development of new synthetic and more lipophilic compounds derived from ALA and the incorporation of ALA into liposomes. There were ALA esters, ALA aminoacid derivatives and ALA dendrimers among the new synthesized molecules. In general, there is an agreement that the promising results obtained in vitro with ALA esters cannot be reproduced in vivo, however ALA methyl ester was widely used for treatment of skin tumors and ALA hexyl ester proved to be effective in bladder imaging [6].

Egg yolk phosphatidyl choline (PC), phosphatidic acid (PA) and phosphatidyl glycerol (PG) were employed in preparation of liposomes with the ALA-Undecanoyl ester (Und-ALA). Und-ALA and other formulations containing ALA derivatives were stable up to 1 week upon storage at 4o C [7].

The photophysical properties of a silicon derivative of tribenzonaphthoporphyrazinate (Si-tri-PcNc) incorporated into liposomes were studied and that allowed to conclude that Si-tri-PcNc in liposome is a promising agent for PDT applications. In vitro experiments showed that the system was not cytotoxic in darkness and exhibited a substantial phototoxicity at 1 M of photosensitizer concentration, 10.0 J/cm2 of light and was sufficient to kill about 80% of treated cells [8].

The matrix metalloproteinases (MMPs) are enzymes implicated in various diseases. General principles that govern the expression of metalloproteinases in e.g. nervous system were extensively discussed and it is now clear that they are important determinants in enabling recovery from injury to the nervous system [9], however they also enable the spread of disease. MMP-3 was found to be secreted by various cells, e.g. fibrosarcoma cells, trophoblast, astrocytes or macrophages.

Recent study revealed that photodynamic therapy (PDT) with a novel photosensitizer, ATX-S10(Na), (13,17-bis[1-carboxypropionyl] carbamoylethyl-8-etheny-2-hydroxy-3-hydroxyimino-ethylidene-2,7,12,18-tetranethyl 6 porphyrin sodium) showed more potent effects for various skin diseases than ALA-PDT. Using dermal fibroblasts derived from normal and scleroderma patients, and mouse skin in vivo, a comparison was made regarding the effects of ATX-S10(Na)-PDT and ALA-PDT. After the PDT, the expression of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) was assayed using ELISA and RT-PCR and showed that the expression of MMP-3 was slightly decreased in scleroderma fibroblasts compared with normal fibroblasts. Both ATX-S10(Na)-PDT and ALA-PDT increased the expression of MMP-3 in protein and mRNA levels in both normal and scleroderma fibroblasts with more potent effect by ATX-S10(N)-PDT. In mice skin the effect of PDT for MMP-3 was also detected and the effect was more potent in ATX-S10(Na)-PDT [10].

A significant, time-dependent induction of MMP-3 (up to 4.3-fold after 48 h) protein levels was seen after ALA-PDT in normal and scleroderma fibroblasts in a singlet oxygen-dependent manner. The mRNA levels of MMP-3 were significantly increased 12 h after irradiation. These data show that 5-aminolevulinic acid and light induced MMP-3 expression [11].

In this paper we report the effect of liposomal formulation of aminolevulinic acid and its methyl ester, metvix - based photodynamic therapy on induction of matrix metalloproteinase 3. We used animal tumor model to evaluate the above effect specifically for MMP-3, because it was recently reported to exert the protective role in the development of tumors [12].

Materials and methods

Preparation of liposomes

Liposomes containing ALA or metvix were prepared using soya phosphatidylcholine (Phospholipon, Germany). ALA, metvix and all other chemicals (Sigma-Aldrich) were of analytical grade. Weighed out amount of the phosphatidylcholine was placed in the glass test tube or small bulb and dissolved in small volume of chloroform. The solvent was evaporated under a stream of nitrogen to the moment when a lipid film appeared on the tube/bulb wall. Drying of sample was continued under vacuum for at least 2 hours. Dried phospholipid was hydrated with an appropriate volume of 250 mM solution of ALA or 200 mM solution its methyl ester. The final concentration of the phosphatidylcholine in prepared suspention was 100 mg/ ml. The suspension was intensively mixed and shaked for 10 min. at 60 – 62°C to give ALA (or its methyl ester)-containing liposomes. Liposome dispersions were extruded through polycarbonate membrane filter (Corning Costar Corporation, MA, USA). The obtained preparation was dialysed exhaustively against the water at a room temperature. Aminolevulinic acid (or its methyl ester) was determined in liposomes and in water used for dialysis. Usually, 12-15 % of ALA (or its methyl ester) was incapsulated in the liposomes prepared according to above method. Liposomes were homogeneous and 105-130 nm in diameter. Preparations of the liposomes were finally concentrated. Properties of concentrated liposomes were unchanged even after 4-week storage. Preparations were applied in eucerin (free ALA and free metvix) and liposomes (both ALA and metvix).

Light source

All irradiations were performed using halogen lamp (Penta Lamps, Teclas), at the wavelength 630 +/- 20 nm, and total light dose – 100 J/sq.cm.

Animals and tumor model

We used inbred female Wistar rats, age – 3-4 months. They were kept in plastic cages, at room temperature, and moderate humidity, fed with chow for murine and water ad lib.

The local bioethical committee regulations to work with laboratory animals were strictly followed.

Mammary solid adenocarcinoma was used in all in vivo studies. The tumor was obtained from the Institute of Oncology, Gliwice, Poland. The experiments started when the tumor had 1 cm in mean diameter (a mean from width, length and height) , i.e. 7 days after subcutaneous inoculation.

ALA and metvix doses in in vivo studies (in mM per one animal per one treatment)

Liposomal formulation of ALA or its methyl ester – 13.0 or 13.8; free ALA or free methyl ester of ALA – 12.7; 13.0 or 13.8.

In vivo tumor fluorescence

After application of liposomal formulation of ALA or metvix, or free precursors onto the skin of examined animals we checked whether the fluorescence was present or not. This was confirmed by using visual observation (fluorescence induced by UV lamp) and measurements performed with UV-meter.

Immunohistochemical study of MMP-3 in rat tissues after photodynamic therapy and in control groups

Rats were treated with above doses of liposomal formulation and without liposomes, and light doses – 100 J/sq.cm. Control rats were treated with: a. ALA or metvix without liposomal encapsulation and no light, b. with liposomal formulation of ALA or metvix and no light, c. with light only (100 J/sq.cm), d. with liposomes only, e. with liposomes and light (100 J/sq.cm) and f. without any procedure.

Each group consisted of 5 animals. The above experiment was repeated with healthy animals.

The rats were sacrified at time point 2 hours after light irradiation. The samples of skin, tumor, liver, spleen and kidneys were excised, fixed in formalin, embedded in paraffin and cut in 5 m slices. Then, they were stained using the mouse monoclonal antibodies (Calbiochem-Merck, Poland) for MMP-3 and kit (mouse UniTect ABC, Calbiochem-Merck).

The slides with immunostainings were assessed by two pathologists in light microscope (Olympus BX40) and the positive reactions were documented using digital camera (Olympus DP10). The results were recorded as: (+) – weak; (++) – moderate, and (+ + +) – strong expression as the mean values from 5 samples, i.e. from 5 animals in each studied group.

Table 1. Expression of matrix metalloproteinase 3 in different groups of tumor-bearing rats after photodynamic therapy and in control groups

	Type of treatment	Intensity of reaction for MMP-3
Tumor-bearing rats – experimental groups	Lipo-met ALA+ light	+ + +
	Lipo-ALA + light	+ + +
	metALA + light	+ +
	ALA + light	+ +
Tumor-bearing rats – control groups	Lipo-met ALA; lipo-ALA; ALA; light; no precursor, no light; liposomes; liposomes + light	+

Table 2. Expression of matrix metalloproteinase 3 in different groups of rats free of tumors after photodynamic therapy and in control groups

	Type of treatment	Intensity of reaction for MMP-3
Tumor-free rats – experimental groups	Lipo-met ALA+ light	+ +
	Lipo-ALA + light	+ + +
	metALA + light	+ +
	ALA + light	+
Tumor-free rats – control groups	Lipo-met ALA; lipo-ALA; ALA; light; no precursor, no light; liposomes; liposomes + light	+ or + +

Legend to tables 1 and 2. Expression of MMP-3: (+) – weak; (++) – moderate; (+ + +) – strong expression. Each group consisted of 5 animals. Lipo-met ALA – liposomal formulation of metvix; lipo-ALA – liposomal formulation of ALA; metALA – metvix.

Results

The strong expression of MMP-3 was found in tumor-bearing animals in both PDT groups treated with liposomal formulations of sensitizer precursors, i.e. ALA (fig. 1) and metvix. Clearly weaker expressions were observed with rats treated with free ALA (fig. 2) and free metvix. The expression of MMP-3 in all these above groups treated with PDT was in general stronger than those observed in animals free of tumors and especially in those treated with free ALA-PDT. Positive immunohistochemical reaction was predominantly observed in tumor cells with weaker presentation in inflammatory (fig. 3), connective tissue and endothelial cells.

There was no difference in the expression of MMP-3 between two studied liposomal formulations and there was only a slight difference between two free precursors of protoporphyrin in terms of PDT. A very weak expression of MMP-3 was observed in control groups from tumor-bearing and tumor free rats. Table 1 shows the immunohistochemical records from tumor-bearing rats in all the examined groups including controls, while table 2 from tumor-free animals.

Discussion

By combining FRET (Fluorescence Resonance Energy Transfer) and PDT a concept of photodynamic molecular beacons (PMB) for controlling the photosensitizer's ability to generate singlet oxygen and for controlling its PDT activity, was introduced. The PMB comprises a disease-specific linker, a photosensitizer and a singlet oxygen quencher, so that the photosensitizer photoactivity is silenced until the linker interacts with a target molecule, such as a tumor-associated pro-

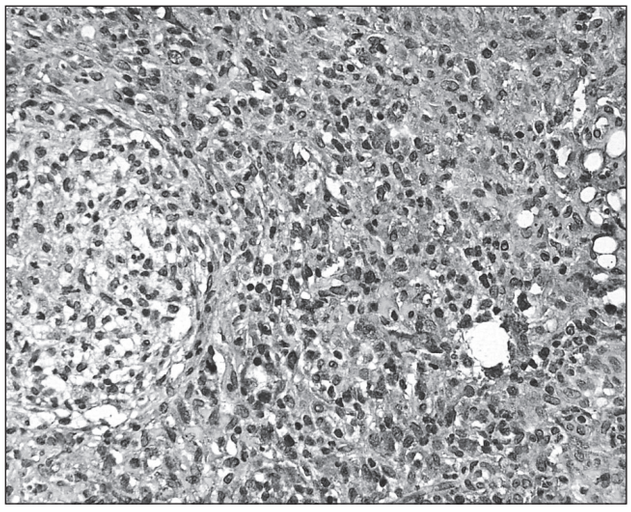

Fig. 1. Strong expression of matrix metalloproteinase 3 in tumor-bearing rat tumor cells after the PDT with liposomal formulation of sensitizer precursor, ALA. Immunostaining for MMP-3, 200x

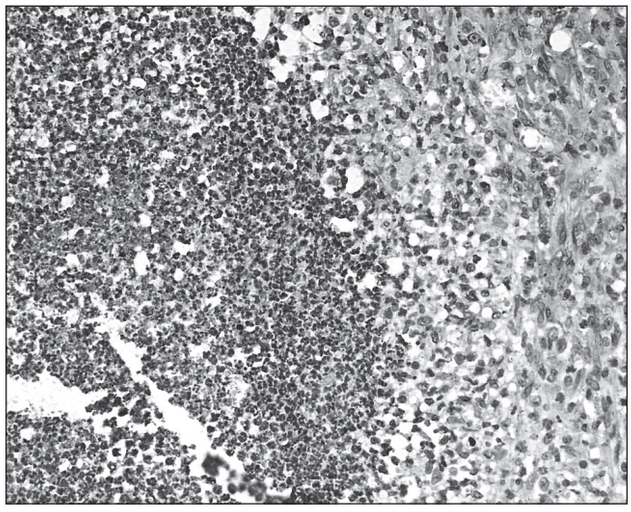

Fig. 3. Positive immunohistochemical reaction for MMP-3 present in inflammatory cells in the tissue adjacent to the tumor. Immunostaining forMMP-3, 200x

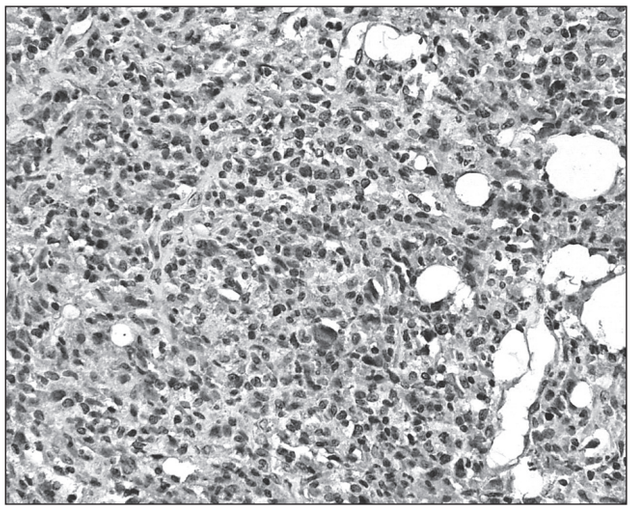

Fig. 2. Expression of matrix metalloproteinase 3 in tumor-bearing rat tumor cells after the PDT with free precursor, ALA. The effect was slightly weaker than that in fig. 1. Immunostaining for MMP--3, 200x

tease. An implementation of this concept by synthesizing a matrix metalloproteinase-7 (MMP7)-triggered PMB and achieving not only MMP7-triggered production of singlet oxygen in solution but also MMP7-mediated photodynamic cytotoxicity in cancer cells was reported. Preliminary in vivo studies also revealed the MMP7-activated PDT efficacy of this PMB. That study validated a principle of the PMB concept that selective PDT-induced cell death can be achieved by exerting precise control of photosensitizer ability to produce singlet oxygen by responding to specific cancer-associated biomarkers. It was suggested that PDT selectivity will no longer depend solely on how selectively photosensitizer can be delivered to cancer cells. Rather, it will depend on how selective a biomarker is to cancer cells, and how selective the interaction of PMB is to this biomarker [13].

Squamous cell carcinomas (SCC) of the head and neck are characterized by their high tendency for invasion and metastasis. Several studies have identified the roles of matrix metalloproteinases (MMPs) and vascular endothelial growth factors (VEGF) in that process. The influence of PDT on the expression of these molecules was in vitro evaluated: a series of human keratinocyte cell lines derived from human oral SCC were used as the PDT targets. Activities of MMP-2, MMP-9, MMP-13 and VEGF were evaluated at the protein levels. Gelatin zymography results revealed that, in control medium, MMP-9 and MMP-2 were secreted in proform. MMP-2 was highly expressed by H376 cells, while VB6 and UP cells relatively show similar MMP-2 with comparatively low expression. For MMP-9, the latent type was highly expressed by VB6 cells and only slightly by H376, while active-MMP-9 was expressed by VB6 cell line only. Following PDT, both active and latent MMP-2 and MMP-9 were down regulated by UP and VB6 cells, while H376 showed an increase in active-MMP-2. These observations were supported by ELISA and demonstrated that PDT causes the suppression of factors responsible for tumor invasion which may be of therapeutic value [14].

Photodynamic therapy causes direct cytotoxicity to malignant cells within a tumor and may also have both direct and indirect effects upon various non-malignant components of the tumor microenvironment. This can lead to PDT-mediated angiogenesis and inflammation, which are emerging as important determinants of PDT responsiveness. Preclinical studies were performed to document how PDT modulates the tumor microenvironment. Photofrin-mediated PDT was shown to be a strong activator of VEGF, MMPs, and COX-2 derived prostaglandins within the tumor microenvironment. Inhibitors that target these angiogenic and pro-survival molecules can enhance the effectiveness of PDT [15].

Photodynamic therapy (PDT) clinical results are promising; however, tumor recurrences can occur and, therefore, methods for improving treatment efficacy are needed. The direct tumor cell death and microvascular injury as well as expression of angiogenic, inflammatory, matrix metalloproteinases and prosurvival molecules after PDT prompted to combination of PDT with specific inhibitors [16, 17]. Administration of inhibitors to these molecules improved PDT responsiveness [18].

Evaluation of the role of Photofrin-mediated PDT in eliciting expression of matrix metalloproteinases (MMPs) and modulators of MMP activity was also carried out. The examination of the efficacy of a synthetic MMP inhibitor, Prinomastat, to enhance tumoricidal activity after PDT, using a mouse mammary tumor model was performed. The results of that study indicated that PDT induces MMPs and that the administration of Prinomastat significantly improved PDT-mediated tumor response without affecting normal skin photosensitization [19].

Our results remain in general agreement with the cited above. The PDT induces metalloproteinase 3 in both tumor-bearing and tumor-free animals in tumor cells as well as in inflammatory and endothelial cells. This induction is stronger, as confirmed by immunohistochemical study, in tissues derived from tumor-bearing animals and from those subjected to the PDT. The effect of liposomal formulations of photosensitizer precursor was found to be important and these formulations elicited stronger intensity of immunohistochemical reaction than ALA- or metvix-PDT without liposomes. ■

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