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Determination of Vascular Endothelial- and Fibroblast-Growth Factor Receptors in a Mouse Fibrosarcoma Tumor Model Following Photodynamic Therapy

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ABSTRACT

The role of angiogenic molecules, like vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) in tumor angiogenesis was well confirmed. Photodynamic therapy (PDT) action is, to very high degree, based on tumor vasculature damage. Therefore, it seemed to be important to evaluate growth factor receptors after PDT. The extent of receptor expression was studied by immuno-histochemical method. In this study, vascular endothelial growth factor (VEGFR) receptor and fibroblast growth factor (FGFR-1) receptor have been evaluated at different time points after PDT of tumor-bearing BALB/c mice. Two sensitizers: hematoporphyrin derivative (HpD) and 21, 23-dithiaporphyrin (DTP) were given intraperitoneally in doses: 1.25, 2.5 and 5.0 mg/kg followed by light irradiation at total doses: 50 and 100 J/sq.cm 24 hours later. The number of VEGFR and FGFR-1 in control samples did not exceed 40 per one vessel, whereas after PDT, a significant decrease in number of both receptors was observed. No differences between HpD- and DTP-PDT in anti-receptor activities were observed ($p<0.001$ for VEGFR and $p<0.002$ for FGFR-1). The observed decrease in VEGFR and FGFR-1 amount confirms that after PDT, some proteins are inactivated and such a decrease may influence PDT effectiveness. *Iran. Biomed. J. 8 (3): 113-119, 2004*

Keywords: Vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR-1), Angiogenesis, Fibrosarcoma, Photodynamic therapy, Immunohistochemistry

INTRODUCTION

The mechanism of tumor damage after photodynamic therapy (PDT) is mainly attributed to the destruction of tumor blood vessels resulting in, e.g. hypoxia and extravasation of erythrocytes [1, 2]. In contrast to nonproliferating adult endothelium, tumor neo-vasculature is usually highly proliferative [3]. The increase in permeability or disruption of basement membrane is a very common feature of tumor vasculature which plays an important role in the retention of photosensitizers [4]. The mechanism involved in selective tumor retention of these compounds has been studied by Roberts and Hasan [4] with regard to vessel proliferation and permeability. The significance of tumor angiogenesis in human and animals without photodynamic treatment has also been extensively investigated [5-9]. The concept according to which the development of cancer is angiogenesis dependent is generally recognized [10]. Most studies have been completed *in vitro* conditions except for few, like e.g. determination of the most appropriate moment for photodynamic treatment after disulfonated aluminum phthalocyanine-PDT in animals [11]. Many studies evaluated angiogenesis by manual counting and some by computed image analysis [12, 13].

Vascular endothelial growth factor (VEGF) is a member of a family of endothelial cell mitogenic and angiogenic factors which stimulates proliferation of endothelial cells. This activity is apparently

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stimulated by specific VEGF receptors, R1 or R2, which can be found on the surface of endothelial cells. Several members of the VEGF family, namely VEGF-A, B, C, D, E and placenta growth factor (PlGF) described that among them, VEGF-A plays a role of prime importance in angiogenesis [10]. Immunohistochemical staining showed that phosphorylated KDR (VEGFR-2) is present in a wide variety of normal tissues including liver, colon and placenta and is not restricted to endothelium [14]. It was also present in a number of human tumors like breast and colon carcinoma and non-Hodgkin lymphoma [14]. Fibroblast growth factors (FGF) are, in turn, members of a large family of polypeptides that are potent physiological regulators of growth and differentiation of the cells. FGF, which act via cell surface receptors, are also involved in angiogenesis as well as in tumorigenesis and metastasis.

Since the role of biomolecules, such as epidermal-, vascular endothelial-, fibroblast- and transforming growth factor in tumor angiogenesis has been well confirmed, it seemed important to evaluate their receptors after PDT. Former studies substantiated the concept of local growth factor inhibiton by PDT in biologic system [15]. The quantification of growth factors and their receptors after *in vivo* photo-dynamic therapy are still poorly examined. Previous studies have been performed mostly *in vitro* [16-18]. In the present study, two receptors, namely VEGFR -2 and FGFR-1, have been immunohistochemically determined at different time points after photodynamic therapy using specific antibodies.

MATERIALS AND METHODS

Animals. Inbred BALB/c mice, (3.0-3.5 months and weighing between 18-23 g [mean: 20.1 g]) were used in this study. Wroclaw Medical University guidelines for care and use of laboratory animals were followed.

Tumor model. BFS1 fibrosarcoma cells were obtained from the Institute of Immunology and Experimental Therapy (Wrocław) and implanted subcutaneously into the left abdominal region in the volume of 1 mm^3. The mice were given sensitizers when the tumors were approximately 6 mm in a mean diameter. The mean doubling time of this tumor is 9 days.

Sensitizers. (a) hematoporphyrin derivative (HpD), [Porphyrin Products, Logan, USA]. (b) 21,23-dithiaporphyrin [5,10,15,20-tetrakis(4-sulfo-phenyl)-21,23-dithiaporphyrin, sodium salt, DTP], synthesized in the Department of Chemistry, University of Wrocław.

Physicochemical and biological properties of DTP were previously published [19]. Both compounds were dissolved in physiological saline and made alkaline with 0.05 NaOH (pH 7.2-7.3). The solutions were given intraperitoneally in the doses 1.25, 2.5 and 5.0 mg/kg of body weight .

Light source. Penta Lamps, Teclas (CH); total light doses - 50 and 100 J/sq.cm, light intensity: 120 mW/sq.cm with no thermal effects, wavelength 630+/-20 nm for HpD and 695+/-20 nm for DTP. Irradiations were performed 24 hours following drug injections.

Glass slide preparation for VEGF and FGF receptors. The mice were sacrified at the following time points. Twenty four hours, 2, 5, 10 and 15 days after irradiation (5 mice were used for each point). The whole tumors were then dissected, frozen, cut into slices, thus providing the cross-section of entire tumor and stained with antibodies for VEGFR-2, KDR/Flk-1, (V4262, Sigma, USA) and for FGFR-1, (F5421, Sigma, USA) using Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Working dilutions were: 1:250 for VEGFR and 1:50 for FGFR-1. Finally, the sections were counter-stained with hematoxylin and mounted on slides. The primary antibodies were omitted from samples to provide negative controls.

Control groups. (a) mice without any treatment (no sensitizer, no light; 5 mice). (b) light only (50 or 100 J/sq.cm; 5 mice for each dose) and (c) sensitizer only (1.25, 2.5 and 5.0 mg/kg; 5 mice for each dose).

The evaluation of positive staining on histopathologic examination. In each tumor sample, the total number of positively stained VEGF or FGF receptors was counted under magnification 400× on the whole specimen under the light microscope (Olympus BX40) and then divided by the total number of vessels, on the same specimen, using computed image analysis system. This system comprised central processing unit with high resolution image monitor, image analysis software
combined with camera (PM-C35B, Olympus,

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Japan). The programme used was MultiScan v. 8.08, CSS Scan Advanced System of Image Analysis (Computer Scanning Systems, Warsaw, Poland).

Statistic analysis. Statistical comparisons were made using the standard Student's *t*-test and a probability $p<0.05$ was considered as significant.

RESULTS

Our studies performed in untreated animals revealed that mouse fibrosarcoma and surrounding tissues (i.e. subcutaneous fat tissue) are moderately rich in blood vessels.

The number of VEGFR and FGFR-1 in control samples (untreated mice or treated with light or sensitizer only) did not exceed 40 per one vessel. Photodynamic therapy resulted in significant decrease in the number of both VEGFR and FGFR in comparison with the control groups. The evaluation of these receptors on histologic examination showed that higher PDT doses (photosensitizer dose × light dose) caused stronger diminution of VEGFR and FGFR-1 than the lower ones. In samples from the tumors treated with 5.0 mg/kg of DTP and 100 J/sq.cm light and excised 24 hours after therapy, no expression of VEGFR could be seen (Table 1). It has to be stressed that no VEGFR expression was seen throughout the whole tumor tissue. Hematoporphyrin derivative (5.0 mg/kg) with equivalent light dose, i.e. 100 J/sq.cm, caused very similar effects and no expression of VEGFR was observed (Table 2). In samples of the tumors treated with 1.25 mg/kg of DTP and subsequently 50 J/sq.cm light (excision 24 h after PDT), VEGFR could be seen only in single vessels, especially at the bottom of tumors, where the total dose of delivered light was not very large (Table 1).

The same pattern of expression was observed in relation to FGFR-1. Higher doses of PDT (5.0 mg/kg of DTP or HpD, and 100 J/sq.cm light) resulted in very strong decrease of FGFR-1, while the lowest doses (1.25 mg/kg of DTP or HpD and 50 J/sq.cm light) caused only partial decrease in the expression of this protein (Tables 1 and 2). In this study, no differences between HpD- and DTP-PDT in anti-receptor activities were observed ($p<0.001$ for VEGFR and $p<0.002$ for FGFR-1). Computed image analysis of both receptor quantities showed significant differences depending on the photo-dynamic doses. Briefly, the higher dose of sensitizer and delivered light, the stronger decrease in the number of receptors. The diminution of receptor quantity occurred very early, i.e. 24 hours after therapy, and showed to be very stable at all the HpD- or DTP-PDT doses used in this study. Within two, five and ten days after PDT no expression of VEGFR and FGFR-1 was still observed. Within fifteen days after treatment with the lowest PDT doses, a weak re-expression of both receptors in newly formed vessels could be seen in survived (non-necrotic) parts of the tumor and in subcutaneous tissue. The pattern of staining was membranous, nuclear and cytoplasmic. Table 1 shows summarized results of the study for DTP-PDT, and Table 2 for HpD-PDT measured at 24 hours after treatment.

Table 1. VEGFR and FGFR-1 number following DTP-PDT of the fibrosarcoma in BALB/c mice. Data present the mean values of 5 mice per each study. The number of receptors was counted on the whole specimen and divided by total number of vessels on the same specimen. Standard deviation is in brackets.

Type of treatment	VEGFR (number per vessel)	FGFR-1 (number per vessel)
DTP - 1.25 mg/kg 50 J/sq.cm	10 (2.2)	11 (3.0)
DTP - 1.25 mg/kg; 100 J/sq.cm	6 (1.8)	6 (1.5)
DTP - 2.5 mg/kg; 50 J/sq.cm	2 (1.0)	1 (0)
DTP - 2.5 mg/kg; 100 J/sq.cm	0	1 (0)
DTP - 5.0 mg/kg 50 J/sq.cm	0	0
DTP - 5.0 mg/kg 100 J/sq.cm	0	0
DTP – 1.25 mg/kg	28 (3.5)	25 (6.0)
DTP – 2.5 mg/kg	29 (2.4)	26 (3.6)
DTP – 5.0 mg/kg	27 (4.0)	20 (1.8)
Light – 50 J/sq.cm	24 (1.6)	22 (3.2)
Light – 100 J/sq.cm	23 (3.8)	26 (5.8)
No sensitizer, no light	31 (2.0)	33 (4.8)

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Table 2. VEGFR and FGFR-1 number following HpD-PDT of the fibrosarcoma in BALB/c mice. Data present the mean values of 5 mice per each study. The number of receptors was counted on the whole specimen and divided by total number of vessels on the same specimen. Standard deviation is in brackets.

Type of treatment	VEGFR (number per vessel)	FGFR-1 (number per vessel)
HpD - 1.25 mg/kg; 50 J/sq.cm	9 (2.0)	10 (2.5)
HpD - 1.25 mg/kg; 100 J/sq.cm	6 (2.1)	5 (1.9)
HpD - 2.5 mg/kg; 50 J/sq.cm	5 (1.5)	2 (1.0)
HpD - 2.5 mg/kg; 100 J/sq.cm	1 (0)	0
HpD - 5.0 mg/kg 50 J/sq.cm	0	0
HpD - 5.0 mg/kg 100 J/sq.cm	0	0
HpD – 1.25 mg/kg	31 (4.5)	24 (1.2)
HpD – 2.5 mg/kg	26 (3.2)	22 (3.6)
HpD – 5.0 mg/kg	28 (2.5)	19 (0.8)
Light – 50 J/sq.cm	30 (4.4)	20 (2.4)
Light – 100 J/sq.cm	25 (2.2)	20 (4.2)
No sensitizer, no light	30 (4.8)	29 (1.5)

DISCUSSION

Tumor angiogenesis may prove to be a prognostic factor in patients with malignant tumors, e.g. ovarian cancer [8]. Quantitation and inhibition of angiogenesis might also be a promising diagnostic and therapeutic approaches [20].

Some growth factors, such as exogenous EGF, result in decreased toxicity for several glioma cell lines when added to the medium after HpD-PDT [16]. Thus, growth factors may modify cellular response to PDT. Vascular injury results in a smooth muscle cell proliferative response which is in part initiated by release of basic FGF. The injury-induced proliferative response is believed to be a key event in intimal hyperplasia development. PDT produces cytotoxic agent, singlet oxygen, resulting in localized smooth muscle cell (SMC) eradication [18]. In a dose-dependent manner, PDT-generated free radicals reduced cell-associated bFGF levels. After PDT with 100 J/sq.cm and phthalocyanine (CASPC, 5 micrograms/ml), cell-associated bFGF content was reduced by 88%. These results provided a mechanism to explain how, unlike mechanical or other forms of smooth muscle cells injury, optimal doses of PDT can locally eradicate medial vascular SMC without resulting in a bFGF-induced initiation of cell proliferation [18]. In our study, the reduction of FGFR-1 expression was also observed in a dose-dependent manner. The host response to PDT, a form of vascular injury that results in complete vascular wall cell eradication promotes favorable vascular wall healing. These effects do not result in intimal hyperplasia and are suggestive of PDT-induced alterations in the extracellular matrix [21]. In some studies, PDT eliminated detectable levels of bFGF in solution. PDT of extracellular matrix significantly reduced matrix-bound bFGF and this reduction in bFGF after PDT was associated with a decrease in vascular smooth muscle cell mitogenesis when plated on PDT-treated matrix compared with non-treated matrix. In the same experiment, PDT of rat carotid arteries demonstrated a loss of bFGF staining compared with control non-treated arteries [21]. FGF (acidic in that case), TGF and, to a lesser extent, interleukin-1 enhanced the PDT-mediated damage to endothelial cells, whereas for example, tumor necrosis factor, did not significantly modify toxicity [22]. Those results suggested that presence of some tumor secreted cytokines may enhance PDT-mediated toxicity of tumor associated endothelial cells. VEGF is involved in angiogenesis in numerous cancers like human head and neck squamous cell carcinoma [23]. Unfortunately, it is not known which structural features of VEGF and its receptors play a role in high affinity growth factor binding to endothelial cells [24]. Anyhow, VEGF binds to the high affinity tyrosine kinase receptors FLT-1 and KDR/Flk-1 which are expressed on endothelial cells [25]. The KDR/Flk-1 was evaluated in the present study. This high-affinity receptor for VEGF-A mediates most of the endothelial growth and survival signals from VEGF-A [26]. KDR/Flk-1 receptor is the main human receptor responsible for the VEGF activity in both physiological and pathological vascular development, and VEGF-KDR signalling pathway has been validated as a priority target for the development of anti- and pro-angiogenic agents [10]. Nair *et al.* [27] observed that immunization of mice against VEGF or VEGFR-2 stimulated cytotoxic T lymphocyte responses and led to partial inhibition of angiogenesis. At present, there is no information about the role of VEGF and its receptor

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in photodynamic therapy what stays in contrast with the studies on the role of other growth factors and/or their receptors such as bFGF or TGF-β. Similarly to FGFR-1, VEGFR expression was also reduced in a dose-dependent manner. The observed decrease in VEGFR and FGFR-1 expression was expected event and most likely such decrease may influence PDT effectiveness. As it directly upregulates tumor angiogenesis KDR/Flk-1 is an appropriate target for suppression of solid tumor growth using exogenous antibodies, small inhibitory molecules and *in vivo* stimulation of the immune system [26]. Ho *et al.* [28] found positive staining for the receptors VEGFR-1 and -2 in large lymphoid cells in several cases of non-Hodgkin lymphoma concluding that VEGF, but not bFGF, was associated with higher tumor grading of NHL and high-grade transformation of low-grade lymphoma. Giavazzi *et al*. [29] observed that FGF-2 and VEGF stimulate vascularization synergistically but with distinctive effects on vessel functionality and tumor survival. Blockade of either one of the two growth factors results in a decrease in blood vessel density and consequently in tumor burden. However, inhibition of the expression of VEGF, but not of FGF-2, affects also vessel maturation and functionality, leading to tumor hypoxia and necrosis [29]. Recently, an oral potent and selective inhibitor of VEGF-mediated Flt-1 and KDR receptor tyrosine kinases has been shown to reduce growth and microvasculature in subcutaneously implanted human tumor xenografts in nude mice [30]. Phase I studies are under way evaluating the optimum dose and schedule of oral PTK/ZK administered continuously to patients with advanced cancers of types known to overexpress VEGF [30]. In our previous *in vivo* studies, we observed significant decrease in both bFGF and VEGF concentrations in sera of PDT-treated BALB/c mice [31, 32]. We also found that this phenomenon was accompanied by prolongation of survival of treated animals [31, 32].

In our *ex vivo* study, we found that PDT may cause significant decrease in the expression of EGFR in tissue samples derived from human patients with endometrial adenocarcinoma [33]. Such cancer cells may then become less susceptible to mitogens such as EGF. Graeven *et al.* [34] have found significant correlation between elevated preoperative serum VEGF or bFGF levels and tumor mass and histological grading in human patients with soft-tissue sarcomas. They suggested that a consecutive monitoring of both factors in the serum of these patients might be a valuable marker for tumor follow-up [34]. This is probable that such a monitoring of serum levels of different growth factors such as VEGF and bFGF might also be used in PDT.

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