Pol J Pathol 2003, 54, 2, 117-121 PL ISSN 1233-9687

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Determination of Basic Fibroblast Growth Factor Levels in Serum of Tumor-Bearing BALB/c Mice Treated with Photodynamic Therapy

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In the present study we have checked whether photodynamic therapy (PDT) may influence concentration of basic Fibroblast Growth Factor (bFGF) in *in vivo* conditions. We have implanted malignant tumor, i.e. BFS1 fibrosarcoma into BALB/c mice and have them treated using well established photosensitizer, hematoporphyrin derivative and new compound, hydroxygallium (III) phthalocyanine tetrasulfonic acid tetrasodium salt, BON-6. The administration of those compounds was followed by light irradiation using a halogen lamp at proper wavelengths. Our results indicate that *in vivo* photodynamic therapy may cause a significant decrease in bFGF concentration and this phenomenon is accompanied by prolongation of survival of treated animals.

Introduction

Photodynamic therapy (PDT) is a well established method of cancer therapy which has been developed over the past thirty years or so [2, 17]. In the past, much effort was directed towards the elucidation of the mechanism of PDT, although this has not yet been completely clarified [3, 15].

At present, it is obvious that during photodynamic process some biologically active proteins are induced, like cytokines such as TNF-alpha, IL-alpha [20, 21], growth factors and their receptors, EGFR, and others [22], whereas some proteins become reduced in amount. Angiogenesis is essential to cancer progression [6]. Angiogenesis is also crucial for metastasis [16]. This complex and highly regulated process involves numerous different cell types and mediators [10]. Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) are among the soluble factors that stimulate this process [10]. They are ligands for specific tyrosine kinase receptors that are important in transduction of intracellular signals and induction of angiogenesis [10].

Basic FGF is known to have strong angiogenic activity and has been identified in a wide variety of malignant solid tumors [16] and in hematologic malignancies [9]. The mechanism of bFGF-induced cell death has been investigated in Ewing's sarcoma family of tumors (ESFT) [19]. Basic FGF induces phosphorylation of FGFR-1 and activation of Ras/ERK in such tumors [19]. Elevated bFGF levels were found in serum and urine of patients with head and neck cancer [12].

There is, however, little information about the *in vivo* effects of PDT on growth factor, like basic fibroblast growth factor (bFGF).

In this paper we explore the hypothesis that bFGF levels correlate positively with tumor growth. In order to test this hypothesis we have carried out an *in vivo* study using BALB/c mice transplanted with fibrosarcoma BSF 1. As the photosensitizers we have used well known hematoporphyrin derivative (HpD), and a novel more hydrophilic sensitiser, hydroxygallium(III) phthalocyanine tetrasulfonic acid tetrasodium salt (BON-6). Following drug administration, the tumors were irradiated using a halogen lamp at 630 and 680nm, respectively. Basic FGF levels were measured in sera from the mice, and compared to the time of tumor growth.

Material and Methods

Photosensitizers: hematoporphyrin derivative (HpD) was purchased from Porphyrin Products, Inc., Utah, USA. Hydroxygallium(III) phthalocyanine tetrasulfonic acid tetrasodium salt (BON-6) was synthesized by the urea fusion method from 4-sulfophthalic acid and gallium(III) acetate [1, 4] (Fig. 1), the final purification being dialysis to remove inorganic salts. The structural formula is shown at figure 1: a mixture of Type isomers is expected in this sort of synthesis, and the predominant isomer (Type III) is shown. (HpD is a mixture of porphyrin oligomers, and a representative structure is shown in Figure 6.4 of reference [3]).

Both compounds were dissolved in 0.9% NaCl before administration, the HpD sample additionally being made

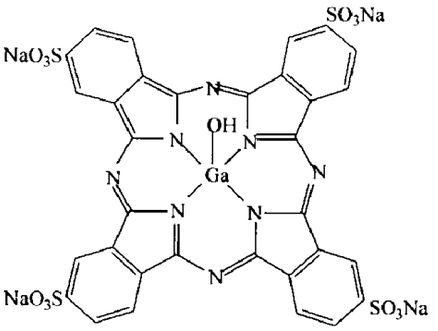

Fig. 1. The structural formula of hydroxygallium (III) phthalocyanine tetrasulfonic acid tetrasodium salt (BON-6).

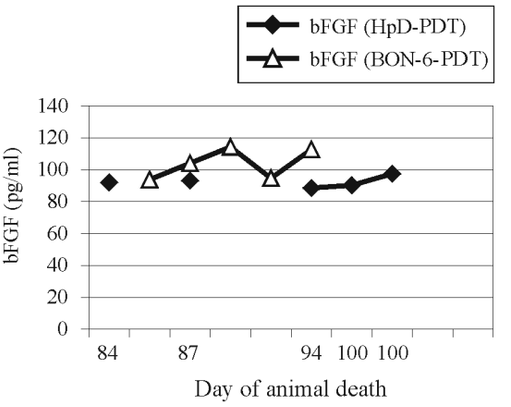

Fig. 2. Correlation between bFGF levels (pg/ml) and survival time (days) after HpD- and BON-6-PDT in tumor bearing mice. bFGF levels at 24 hours after PDT.

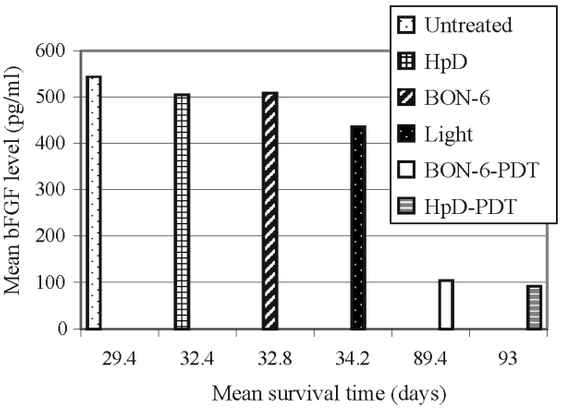

Fig. 3. Correlation between mean bFGF levels (pg/ml) and mean survival time (days) after HpD-, BON-6-PDT and in control groups in tumor-bearing mice. bFGF levels at 24 hours after PDT.

alkaline with 0.05M NaOH to achieve complete solubilization. Peritoneal injections were made with a dose of 2.5mg/kg of body weight.

Light source: a 250W halogen lamp (Penta Lamps, Teclas, CH) was used at the following wavelengths: 630±40nm to sensitize HpD, and 680±40nm to sensitize BON-6. In the case of BON-6, the area over the tumor was covered with a glass filter (Schott no. RG-645) to cut out wavelengths below 650nm. The total light dose applied in all cases was 64J/cm^2.

Animals: inbred BALB/c mice weighing 19 - 21g, 4 months old, were used. The mice were kept in plastic cages and fed according to regulations of Wrocław Medical University. Five animals were used in each group studied, including control groups treated with light or sensitizers only, and healthy untreated mice. All treatments, except injections, were made under *ketaminium hydrochloricum* anaesthesia. After blood collection, the mice were left to survive, and the time of survival in days was recorded.

Tumor: BFS1 fibrosarcoma was purchased from the Institute of Immunology and Experimental Therapy, Wrocław. Tumor samples (1mm^3), were inoculated subcutaneously in the left abdominal region. When the tumor reached a mean diameter of 5mm (about 6 days after inoculation), the photosensitizer was injected, and 24 hours later irradiated with the stated light dose. The tumors were then measured twice a week in the two available diameters.

Determination of bFGF: bFGF levels were measured at 24 and 96 hours after the exposure to light. Blood (200µl) was collected by heart puncture and left for 2 hours to clot. Eppendorf tubes containing the blood were centrifuged for 10 minutes at 500rpm. The sera so obtained were processed for bFGF concentration using monoclonal antibody to bFGF. Next, the second, polyclonal antibody (rabbit anti-mouse) and ABC kit were used to make visible the antigens. Recombinant mouse basic fibroblast growth factor was applied as a standard. The assay was run on 96-well microplates. Optical density was determined using a microplate reader set to 450 and 630nm. Basic FGF concentration was read using a standard curve and was given in pg/ml. The bFGF values were calculated as the mean of 5 measurements in a group and standard deviation (SD) was also given.

Results

On a weight basis, HpD was found to be slightly more effective in PDT than the hydroxygallium(III) phthalocyanine tetrasulfonate (BON-6). This feature was accompanied by lower levels of bFGF after HpD-PDT as measured with the ELISA kit.

After 24 hours, the concentration of bFGF in the control group of healthy, untreated mice (Table 1, row 7) did not exceed 239.0 pg/ml (SD 8.0). The highest concentrations of bFGF were found in the group of tumor-bearing, untreated mice where the level was over 543.5pg/ml (SD 11.74; Table 1, row 6). In control tumor-bearing animals treated with HpD only or BON-6 only (Table 1, rows 3 and 4) the levels of

TABLE 1

Serum bFGF levels (pg/ml) following PDT and in control groups at 24 hours after treatment in tumor-bearing and healthy mice. SD - standard deviation, 1 - 5 mouse number.

	1	2	3	4	5	Mean	SD
HpD-PDT	97.4	90.2	88.5	93.1	92.0	92.2	3.37
BON-6-PDT	94.6	93.8	104.0	112.7	114.3	103.9	9.67
HpD	523.4	511.9	505.3	490.3	494.1	505.0	13.43
BON-6	528.2	497.6	498.0	507.7	511.2	508.5	12.5
Light	444.8	437.6	439.3	427.9	430.0	435.9	6.94
Untreated	556.1	552.4	538.0	544.3	526.7	543.5	11.74
Healthy	236.0	242.7	229.9	235.6	250.8	239.0	8.0

TABLE 2

Survival time (in days) of tumor-bearing mice treated with PDT and in control groups. SD - standard deviation, 1 - 5 mouse number

	1	2	3	4	5	Mean	SD
HpD-PDT	>100 - cured	>100 - cured	94	87	84	93.0	7.35
BON-6-PDT	91	85	87	94	90	89.4	3.51
HpD	34	33	36	30	29	32.4	2.88
BON-6	31	34	27	38	34	32.8	4.1
Light	37	39	29	33	33	34.2	3.9
Untreated	30	36	28	28	25	29.4	4.1

TABLE 3

Serum bFGF levels (pg/ml) following PDT and in control groups at 96 hours after treatment in tumor-bearing mice. SD - standard deviation, 1 - 5 mouse number

	1	2	3	4	5	Mean	SD
HpD-PDT	210.7	221.0	235.4	204.6	209.8	216.3	12.22
BON-6-PDT	226.0	234.8	235.5	219.3	224.9	228.1	6.92
HpD	563.3	547.6	572.0	551.7	534.6	553.8	14.43
BON-6	531.5	542.6	538.3	555.4	569.4	547.4	15.05
Light	480.7	497.4	477.8	498.9	506.1	492.2	12.3
Untreated	574.1	588.9	560.7	594.2	570.2	577.6	13.75

growth factor did not vary significantly from those observed in tumor-bearing and untreated mice (HpD: 505.0pg/ml, SD 13.43, BON-6: 508.5pg/ml, SD 12.5). Interestingly, tumor-bearing animals treated only with light showed a slight decrease in basic FGF (435.9pg/ml, SD 6.94). As stated, the largest decrease in bFGF was observed in the HpD-PDT animals: in that group it was below 100pg/ml (92.2pg/ml, SD 3.37), whereas in the BON-6-PDT group some animals exceeded 100pg/ml (103.9pg/ml, SD 9.67; Table 1, rows 1 and 2). These 24-hour results are summarised in Table 1. Figure 2 shows an informative comparison between the bFGF values and survival times in PDT treated groups of mice.

After 96 hours (Table 3) the mean bFGF levels increased in all control and PDT-treated groups. In BON-6-PDT treated animals the level was over 220pg/ml (228.1pg/ml, SD 6.92), whereas in HpD-PDT group it was 216.3pg/ml (SD 12.22). In untreated, tumor-bearing mice the level of bFGF also increased from 543.5 to 577.6pg/ml.

In both groups of mice treated with PDT the survival time was significantly longer than in control groups. The mean survival time in control groups did not exceed 34.2 days (Table 2, row 5). Mice which were given HpD-PDT survived 93.0 days (SD 7.35), while mice given BON-6-PDT survived 89.4 days (SD 3.51). The correlation between mean bFGF levels in pg/ml at 24 hours after PDT and mean

survival time is shown in figure 3. Measurement of tumor size showed total regression in 2 cases after HpD-PDT and no one after BON-6-PDT. Mice which survived for 100 days were considered as cured (no palpable tumour was found also later at 150 days). In all cases other than the healthy, untreated animals, the mice died because of the spread of the disease to other organs (e.g. lungs, liver); however, the tumor at the site of inoculation generally tended to decrease in size. Survival time in days in all the experimental groups is shown in Table 2: in comparison with control animals, the mean survival in PDT treated groups was almost three times longer (93.0 and 89.4 vs. 29.4 - 34.2 days).

Discussion

There are many forms of FGF, but the most important role in angiogenesis plays basic FGF. A number of various oncogenes code proteins that display up to 50% homology to FGF. In lower concentrations bFGF stimulates proliferation of endothelial cells and in higher - activates collagenase degrading type IV collagen in basement membrane of blood vessels [13].

An inappreciable number of contributions dealing with PDT *in vivo* effects on basic fibroblast growth factor, persuaded us to carry out present experiment.

Serum analysis of basic fibroblast growth factor levels were studied in 53 patients with renal cell carcinoma, RCC [7]. It was found that no correlation occurs between bFGF and the presence or absence of metastases, nor it was any correlation between bFGF and survival time of patients [7]. Thus, the authors suggested that the importance of bFGF as a tumor marker should be limited [7]. These results are in contrast with our findings. In present study, we found that decreased levels of bFGF following PDT within 24 hours after treatment resulted in extended time of survival and, in single cases, complete tumor response. The direct comparison between these two studies is however very speculative since the RCC patients were not treated with photodynamic therapy.

In other study, Statius van Eps et al. [18] investigated whether PDT-generated free radicals could inactivate cell-associated bFGF normally released with cell injury. It is well known that procedurally related vascular injury results in smooth muscle cell proliferation which is partially initiated by smooth muscle cell release of mitogen, bFGF. This all may lead to intimal hyperplasia (IH) development. PDT produces free radicals resulting in localized smooth muscle cells eradication and may, in turn, inhibit IH [18]. After PDT with high light doses (100J/cm^2), cell-associated bFGF content was reduced by 88%, and together with other results, it has suggested that optimal doses of PDT can locally eradicate vascular smooth muscle cells without resulting in bFGF-induced initiation of cell proliferation [18]. In another study, the same authors investigated PDT effect on extracellular matrix-bFGF using chloroaluminum sulfonated phthalocyanine as photosensitizer [11]. In that case, PDT inactivation of matrix-resident bFGF provided a mechanism by which PDT suppresses smooth muscle cell proliferation in vessel wall, and contributes to excellent vascular healing when PDT is used for the inhibition of injury-induced IH [11].

Our results confirmed that local PDT may result in inhibition of bFGF production or release into serum of mice.

The presence of tumor secreted cytokines may enhance PDT-mediated toxicity of tumor associated endothelial cells; this effect was found with regard to acidic FGF, transforming growth factor-beta (TGF-β) and interleukin-1-alpha (IL-1-α) [5]. Platelet-derived growth factor (PDGF) and tumor necrosis factor-alpha (TNF-α) did not significantly modulate this toxicity [5]. Reduction in vascular perfusion associated with PDT-mediated microvascular injury produces tumor tissue hypoxia [8].

In this paper we present new information about the effect of PDT on bFGF levels and the correlation between post-PDT bFGF concentration and subsequent tumor growth rate. This is the first demonstration that decreased post-PDT bFGF levels may influence tumor growth rate and survival time.

Our studies have been carried out *in vivo* under conditions which differ significantly from those of earlier approaches carried out *in vitro*. We have employed a bFGF-specific antibody to detect the bFGF protein in the sera of mice undergoing PDT. We have used an antibody that measures natural bFGF, which stimulates endothelial cell mitogenic activity. We have found bFGF concentrations to be considerably decreased 24 and 96 hours after PDT. Similar effects were observed with the established photosensitizer HpD and the novel photosensitizer BON-6. Our results show that lower levels of bFGF after PDT are associated with slower tumor growth, with single cases of tumour total regression, and the significant prolongation of survival time of treated animals: the survival time of PDT-treated animals was almost three times that in any of the control groups. The early decrease in bFGF level (e.g. HpD-PDT:untreated mice = 1:5.9) correlated with longer survival. Tumor measurement in the PDT-treated groups showed almost constant decrease in tumor size. This effect was more obvious in cases where the decrease in bFGF level was more distinct.

This relationship could be attributed to poorer vascularization of tumor tissue and its vicinity after PDT. One of the major histological features of soft-tissue sarcomas (such as BSF 1 studied here) is the poor vascularisation of such tumors [14]. In cases where the tumor sizes diminished, but the animals succumbed in spite of applied phototoxic treatment, the death occurred following dissemination and meta-

stasizing to distant organs, as confirmed on autopsy and histological examination. This may suggest that in such cases our treatment did not prevent neovascularisation and subsequent metastasis formation.

The common effect of PDT *in vivo* is to lower the content of various proteins inside or outside the tumor tissue. Since bFGF is expressed by a wide variety of cell types, including tumor cells, we suggest that following PDT the ability of these cells to produce bFGF is reduced. We presume that photodynamic therapy may influence the capability of tumor tissue and its vicinity to form new vessels *via* a decrease in bFGF serum levels.

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