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Expression of vascular endothelial growth factor in mouse tumours subjected to photodynamic therapy

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Abstract

The aim of this study was to define the appropriate fractionation interval between photodynamic therapy (PDT) for the enhancement of its anti-tumour effects. Tumour reoxygenation and the kinetics of tumour vascular cells following PDT were evaluated in mice by means of immunohistochemical staining for the vascular endothelial growth factor (VEGF) and the proliferating cell nuclear antigen (PCNA), respectively. The VEGF labelling indices (LIs) of the tumour cells and the PCNA LIs of the tumour vascular cells were assessed at various time intervals after PDT. The tumour cell VEGF LIs of the experimental groups at time points from 0 to 6 h after PDT were significantly higher than those of the control groups, but subsequently returned to control levels at 24 h after PDT. The vascular cell PCNA LI of the experimental group at 24 h after PDT was significantly lower than that of the control group, but returned to the control level at 48 h. These results indicated that the tumour subjected to PDT might be reoxygenated, and that the maximum damage to the tumour vasculature emerged at 24 h after PDT. We propose here that the fractionation interval between PDTs should be 24 h.

Keywords: Photodynamic therapy; Vascular endothelial growth factor; Proliferating cell nuclear antigen; Fractionation interval; Tumour vasculature

1. Introduction

Photodynamic therapy (PDT) [1] as a cancer treatment consists of the systemic administration of a photosensitiser and laser irradiation. PDT is an effective treatment to control local cancer lesions [2,3]. However, recurrence of the tumour after PDT remains problematic in spite of a good initial response [4–6]. In addition, we have demonstrated experimentally that tumour cells adjacent to the surrounding normal tissue are unaffected by PDT [7].

The photochemical reaction depends on the presence of oxygen in the tumour [8], and Henderson and Fingar [9] have pointed out that the inhibitory mechanism affecting the photochemical effect results from the rapid induction of hypoxia by the PDT itself. Accordingly, fractionation of PDT light treatment is recommended to reoxygenate the hypoxic tumour tissue [10–14]. However, it is not clear how long an interval between laser irradiations is appropriate for the enhancement of PDT antitumour effects.

Vascular endothelial growth factor (VEGF) is a specific endothelial cell growth factor [15–17], which potentiates tumour growth and angiogenesis [18,19]. Moreover, hypoxic or anoxic conditions induce VEGF secretion in tissues [20,21].

In this study, VEGF levels in tumour tissues and proliferating cell nuclear antigen (PCNA) levels in vascular cells were examined immunohistochemically in mouse tumours that had been subjected to PDT to help define the appropriate fractionation interval between laser irradiations for a PDT course of treatment.

2. Materials and methods

2.1. Animals and tumours

Five to six-week-old, 22–24 g, male C3H/HeNCrj mice ($n = 56$, Charles River, Osaka, Japan) were used.

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They were housed in a room with subdued lighting and fed a standard pellet diet (Oriental Yeast, Osaka, Japan) and water.

NR-S1 mouse squamous cell carcinoma [22] (National Institute of Radiological Sciences, Chiba, Japan) was transplanted into the dorsum of each mouse. The tumours that grew to approximately 7×7 mm in size by about 12 days after transplantation were used in this experiment.

2.2. Photodynamic therapy (PDT)

PDT was carried out using haematoporphyrin oligomers [23] (Hp oligomers) (Seikagaku Kogyo, Tokyo, Japan) as the photosensitiser, and red light emitted from a pulsed Nd:YAG dye laser (Quanta-Ray® DCR-3 and PDL-2, Spectra Physics, Mountain View, CA, USA) as the light source. The laser was tuned to a wavelength of 630 nm, which was verified with a spectrometric multichannel analyser (SMA Systems, Tokyo Instruments, Tokyo, Japan), at a frequency of 10 Hz. The diameter of the irradiating laser beam entirely covered the tumour. A power meter (30 A-P Ophir Optics, Jerusalem, Israel) was used to measure the light intensity.

2.3. Protocols

In the experimental group ($n=28$), Hp oligomers were injected intraperitoneally (i.p.) (20 mg/kg body weight) 48 h before the laser irradiation, which was carried out at a power of 15 mJ/cm^2/pulse for 30 min (estimated energy dose: 270 J/cm^2). Groups of four mice were sacrificed at intervals of 0, 0.5, 2.5, 6, 24, 48 and 72 h after PDT. Hp oligomers and laser irradiation were not administered in the control animals. In the control groups ($n=28$), sub-groups of four mice were sacrificed at the same time points as for the experimental groups. The time points for the sacrifice of control mice were determined at the intervals when the size of the tumours reached 7×7 mm.

During laser irradiation in the experimental groups, animals were placed under general anaesthesia using pentobarbital sodium (40 mg/kg body weight) that was injected i.p. The control animals were similarly injected 30 min before sacrifice.

2.4. Haematoxylin and eosin (H&E) staining

The mice were sacrificed using inhalation of ether, and tumours were excised and fixed in 3.7% neutral buffered formalin for 24 h. This was followed by processing for routine paraffin embedding; three 4 μm sections were prepared for each specimen, mounted on poly L-lysine-coated glass slides, and dried overnight on a hot plate at 37°C to promote adhesion. In the first section from each of the specimens, routine haematoxylin and eosin (H&E) staining was carried out.

2.5. VEGF immunohistochemistry and labelling indices of VEGF

In the second sections, VEGF immunohistochemical staining (ABC kit, Vector Laboratories, Burlingame, CA, USA) was performed ($n=4$ for each group). Goat anti-VEGF polyclonal antibody (P-20, 1:100 diluted, Santa Cruz Biotechnology, sc-1836, CA, USA) was applied. The sections incubated with normal goat serum instead of VEGF served as negative controls.

Both the labelled and unlabelled tumour cells were counted with the aid of a squared eyepiece graticule (Nikon, Tokyo, Japan) in the square fields (0.0625 mm^2) at a magnification of 400×. The VEGF labelling index (LI) of tumour cells was defined as the percentage of strongly or intermediately VEGF-positive cells in 1000 tumour cells counted from four randomly selected fields.

2.6. PCNA immunohistochemistry and labelling indices of vascular cells

In the third sections, PCNA immunohistochemical staining (ABC Kit, Vector Laboratories, Burlingame, CA, USA) was carried out ($n=4$ for each group). Mouse anti-PCNA monoclonal antibody (PC10, 1:100 diluted, DAKO M0879, Denmark) was applied. The sections incubated with normal mouse serum instead of PCNA served as negative controls.

PCNA positivity was assessed by counting the entire number of vascular cells in each specimen. Only the vascular cells were counted in the square fields of a squared eyepiece graticule (Nikon, Tokyo, Japan) at a magnification of 400×. The eyepiece graticule was shifted over the entire area of the tumour in each specimen.

2.7. Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test. Differences between the control and experimental groups were considered statistically significant when $P<0.05$.

3. Results

3.1. Findings of H&E staining

In the control specimens, slides showed multiple mitotic tumour cells, and only a few necrotic areas in the tumours. In the experimental groups, there was no instance in which the tumour cells were completely destroyed, and histological findings at 0 and 0.5 h after

PDT were similar to those of the control groups. Tumour cells showing condensation of nuclei emerged at 2.5 h after PDT. The tumour necrosis gradually extended with time after PDT, although many surviving tumour cells remained, mainly in the peripheral areas in each specimen.

3.2. Findings of VEGF immunohistocheinistry and VEGF LIs

VEGF-positive tumour cells were distinguished by the brown-stained cells; the cytoplasm was stained in the positive tumour cells. In the control specimens, tumour cells adjacent to the blood vessels and to the normal tissues were strongly stained. However, the tumour cells in other regions were either unstained or weakly positive (Fig. 1). In the experimental group at 0 h following PDT, strongly positive tumour cells were observed throughout the tumour (Fig. 2).

The average LI was 18.1% in the control group at 0 h and 30.2% in the experimental group immediately after PDT. The value increased over time in the experimental group until 6 h after PDT (60.5%), subsequently decreasing to 44.1, 21.9 and 23.3% at 24, 48 and 72 h after PDT, respectively (Fig. 3). There were significant differences between the control and experimental groups at 0, 0.5, 2.5 and 6 h after PDT ($P=0.0433$ at 0 h after PDT; $P=0.0209$ at 0.5, 2.5 and 6 h after PDT). There were no significant differences between the control and the experimental groups at 24, 48 or 72 h after PDT ($P=0.1489$ at 24 h after PDT; $P=0.7728$ at 48 h after PDT; $P=0.2482$ at 72 h after PDT).

3.3. PCNA immunohistochemistry and PCNA LIs of vascular cells

PCNA-positive vascular cells were distinguished by the brown-stained nuclei in immunohistochemical staining (Fig. 4). Fig. 5 shows PCNA LIs of vascular cells in the control and experimental groups. The average LI was 15.2% in the control group at 0 h. In the experimental groups, the average LI decreased to 7.4% 24 h after PDT, subsequently increasing to the control level by 48 h after PDT (15.8%). There was a significant difference between the control and the experimental groups at 24 h after PDT ($P=0.0209$). However, there were no significant differences between the control and experimental groups at 0, 0.5, 2.5, 6, 48 and 72 h after PDT.

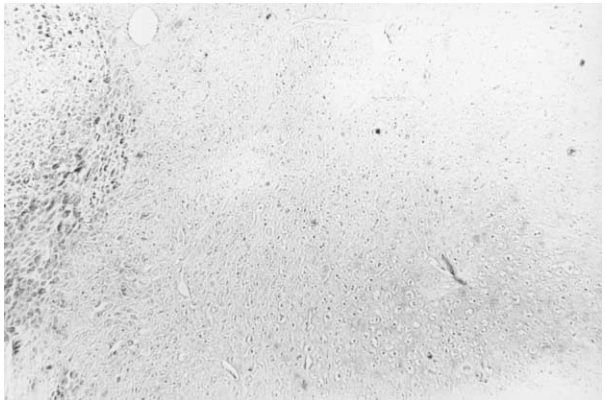

Fig. 1. Vascular endothelial growth factor (VEGF) immunohistological findings for a control specimen. VEGF-weak positive or negative tumour cells were found (original magnification: ×50).

4. Discussion

In the control specimens, the VEGF expression in the tumour was similar to those of a previous report [24].

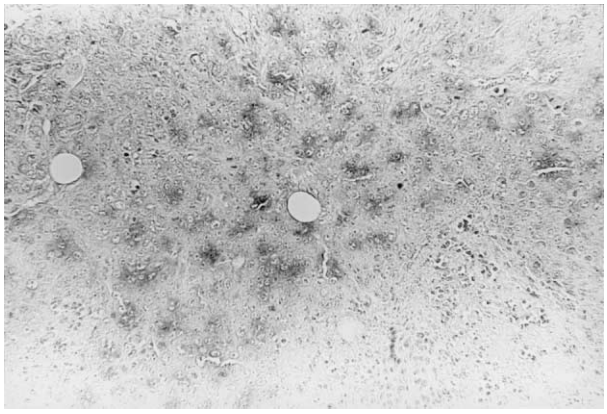

Fig. 2. VEGF-strongly positive tumour cells in the experimental group at immediately after photodynamic therapy (PDT) (original magnification: ×50). Labelled cells have dark brown stained cytoplasm.

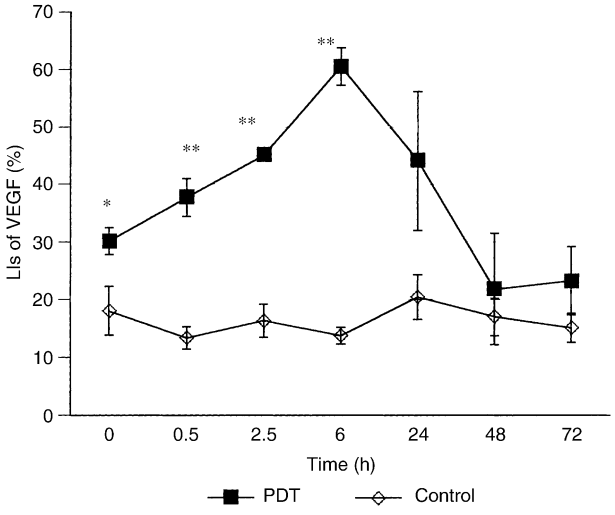

Fig. 3. Vascular endothelial growth factor (VEGF) labelling indices (LIs) of tumour cells. Each plot represents the mean; bars show standard error of the mean (SEM). Significant differences exist between the control group and the experimental groups at 0, 0.5, 2.5 and 6 h after photodynamic therapy (PDT) (*$P=0.0433$, **$P=0.0209$).

The VEGF LIs of the experimental groups at 0, 0.5, 2.5 and 6 h after PDT were significantly higher than those of the control groups (Fig. 3). *VEGF* mRNA is induced in cells treated with a variety of stresses, e.g. hypoxia [20], anoxia [21], X-ray irradiation [25], heavy metal ions [21], H_2O_2 and ultraviolet (UV) B light irradiation [26]. PDT induces severe hypoxia in the tumour tissue immediately after initiation [9]. This hypoxic condition would thus be responsible for the induction of VEGF in the tumour cells. VEGF secretion in hypoxic cells returns to the original level when the oxygen supply is resumed [20]. Accordingly, there are two possible explanations for the subsequent decrease of the VEGF LI: tumour cell death or re-oxygenation of tumour tissue. In our previous study, we observed that large numbers of tumour cells survived PDT exposure using the same dose as in the present study [7]. It is therefore logical to assume, based on the results of the present study, that the surviving tumour cells might have been reoxygenated to the control level during the 24 h following PDT. This tumour reoxygenation might be responsible for the repopulation of residual tumour cells at 48 h after PDT [27].

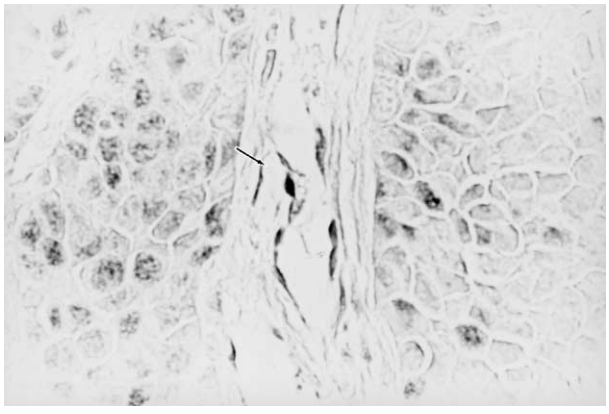

Fig. 4. Proliferating cell nuclear antigen (PCNA) immunohistological findings for a control specimen. The arrow shows the positive vascular cells. Labelled cells have dark brown stained nuclei (original magnification: ×200).

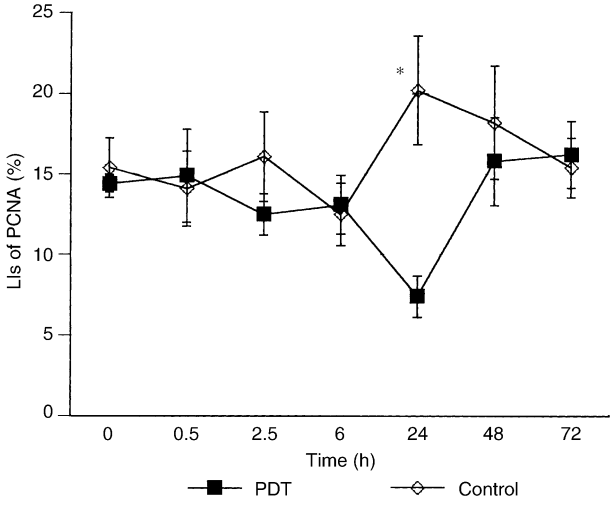

Fig. 5. Proliferating cell nuclear antigen (PCNA) labelling indices (LIs) of tumour vascular cells. Each plot represents the mean; bars show standard error of the mean (SEM). A significant difference exists between the control and experimental groups at 24 h after PDT (*$P = 0.0209$).

Vascular cells are an important target of PDT [28], and damage to tumour vascular cells was investigated using PCNA immunohistochemistry in the present study. At 24 h after PDT, the PCNA LI was significantly lower than that of the control group. At 48 h after PDT, the PCNA LIs of the tumour vascular cells had nearly returned to their original level. This upward trend in the proliferative potential of the tumour vasculature followed the peak of tumour cell VEGF LI. These findings indicate that VEGF secreted by the surviving tumour cells from immediately after the PDT (0 h) to 6 h induces proliferation of the tumour vascular cells.

In summary, the present study suggests that the maximum damage to the tumour vasculature emerged at 24 h after PDT, and that recovery of the damaged vasculature commenced at 48 h after PDT. Additionally, we showed that the residual tumour cells might be reoxygenated at 24 h after PDT. In conclusion, the fractionation interval of PDT laser irradiation should be 24 h.

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