Increase in Tumor Oxygenation and Potentiation of Radiation Effects Using Pentoxifylline, Vinpocetine and Ticlopidine Hydrochloride

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Radiosensitize/Hypoxic Cell/Oxygen tension.

The purpose of the present study was to investigate the effects of Pentoxifylline (PTX), Vinpocetine (VPT) and Ticlopidine Hydrochloride (TCD), used commonly for vascular disorders in humans, on the \( pO_2 \) in SCCVII tumors of C3H/HeJ mice and on the radioresponse of SCCVII tumors. The \( pO_2 \) in the SCCVII tumors, which were measured 30 min after intraperitoneal (i.p.) injection of PTX (5 mg/kg), VPT (5 mg/kg), or TCD (10 mg/kg) using polarography, was compared to that in saline-treated control tumors. All the three drugs, PTX, VPT and TCD, yielded significant increase of the \( pO_2 \) in the SCCVII tumors from 25.6 to 26.9 mmHg, from 18.6 to 22.9 mmHg, and from 22.6 to 25.9 mmHg, respectively. Frequency histogram of the \( pO_2 \) distribution in the saline-treated SCCVII tumors did not show hypoxic fraction of less than 10 mmHg. The radioresponses of the drugs were investigated by tumor growth delay assay. In the drug-treated groups, the SCCVII tumors were irradiated with a single dose of 15 Gy 30 min after injection of the drugs at the same doses as those used in the experiments for intratumoral \( pO_2 \) measurement. Compared with the irradiation alone group, significant tumor growth delays were observed in all the drug-treated groups. The time required to reach a four-fold increase in the initial tumor volume were 4 days in the saline-treated control group, 22 days in the irradiation (IR) alone group, 28 days in the PTX + IR group, 29 days in the VPT + IR group, and 32 days in TCD + IR group. In conclusion, VPT and TCD are potentially promising drugs for increasing the intratumoral \( pO_2 \) although the mechanism for radiopotentiation observed in the present study is unknown due to small hypoxic fraction in the SCCVII tumors. Further studies on other mechanisms for radiopotentiation of PTX, VPT or TCD, besides of increasing the \( pO_2 \) in the tumor, are needed.

INTRODUCTION

The presence of hypoxic cells in tumors is due, at least in part, to progressive deterioration of blood perfusion upon tumor growth. Thus, improvement of blood perfusion in the tumor may ameliorate tumor oxygenation. Blood perfusion in the tumor may increase when flow resistance is reduced, either by dilation of blood vessels or by reducing blood viscosity. Since the hastily formed vasculatures in tumors have quite different characters from those in normal tissues, various agents that dilate blood vessels in normal tissues are generally ineffective in tumor vessels.1–4 On the other hand, increase of blood fluidity can improve blood perfusion through the tortuous, twisted and focally constricted vessels in the tumor, which results in elevation of the intratumoral \( pO_2 \).

Pentoxifylline (PTX), a methylxantine derivative, has been used for the treatment of regional microcirculation disorders such as intermittent claudication, cerebrovascular disorders and reported to increase blood fluidity by multiple effects on the blood such as increasing the deformability of red blood cells and leukocytes, preventing the aggregation of platelets, and decreasing plasma viscosity.5–11 Song et al.12 and Lee et al.13,14 investigated the effects of PTX on the change of oxygenation in the tumor and on the radioresponses of the tumor, and concluded that PTX increased the radio-

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response of the tumors by improving tumor oxygenation. We had interest in two drugs, vinpocetine (VPT), a vasoactive vinca alkaloid, and Ticlopidine Hydrochloride (TCD), a thienopyridine derived antiplatelet drug. Both drugs have been reported to increase blood fluidity by the same mechanism as PTX.\(^{15-21}\) The purpose of the present study was to investigate the effects of PTX, VPT and TCD on the \(pO_2\) in the SCCVII murine tumors of the C3H/HeJ mice and on the radioresponses of the tumors.

**MATERIALS AND METHODS**

**Animals**

All experiments were performed on 7-8-week-old male C3H/HeJ mice. The mice were allowed food and water ad libitum, and rooms were maintained at 22 ± 2°C with a 12-h light / dark cycle. All the experiments were approved by Medical Science Research Animal Resources ethical committee of Suzuka University.

**Tumor**

SCCVII tumors were kindly provided by Dr. Chiyoko Murayama at the Tokai University. SCCVII tumor cells, derived from spontaneous squamous cell carcinoma (origin of murine cutaneous carcinoma) in C3H/He mice, were grown in vitro using RPMI 1640 medium supplemented with 10% calf serum. The cells in the exponential growth phase were harvested by treatment with a 0.25% trypsin solution. Approximately 1 × 10\(^6\) viable cells were injected subcutaneously (s.c.) into the right legs of male C3H/HeJ mice. The tumors were used when they grew 6–8 mm in diameter.

**Drug Treatment**

PTX \{3,7-dimethyl-1(5-oxohexyl) xanthine\}, VPT \{(13aS, 13bS)-13a-ethyl-2,3,4,6,13a,13b-hexahydro-1H-indololo[3,2-1-de]pyrido[3,2,1-ij][1,5]naphthyridine-12-carboxylate\} and TCD \{5-(2-chlorobenzyl)-4,5,6,7-tetrahydrothieno[3,2-c]pyridine monohydrochloride\} were purchased from Sigma Chemical Co. (Yokkaichi, Mie, Japan). Solutions of PTX, VTP and TCD in a sterile saline solution (0.9% NaCl) were freshly prepared prior to each experiment. The tumor-bearers were i.p. injected with PTX (5 mg / kg), VPT (5 mg / kg) or TCD (10 mg / kg) in a volume of 0.01 ml / g body weight. In the present study, we used the dose of each drug at which pharmacological effects on microcirculation were demonstrated in the studies using experimental animals.\(^{13,16,20}\)

**Measurement of intra tumor \(pO_2\) using \(O_2\) microelectrodes**

The tumor \(pO_2\) was measured polarographically (POG-203: RIKA DENKI, Tokyo, Japan) using recessed tip microelectrodes with diameters of 100 µm (\(pO_2\) Clark needle: UOE-50, Unique Medical, Tokyo, Japan). The 100-µm-wire-type electrode has been reported to be effective for detection of the intratumoral hypoxic fraction of less than 5 mmHg.\(^{12}\) The electrodes were calibrated by immersion in a series of 0.9% saline solutions at 34.0°C saturated with 100% \(N_2\), 5% \(O_2\) plus 95% \(N_2\), and 10% \(O_2\) plus 90% \(N_2\). The mice were anaesthetized with an i.p. injection of 20 mg / kg of a mixture of ketamine and xylazine (87:13 in 0.1 ml of saline). The anaesthetized mice were placed on an electrically insulated isothermal pad to keep the body temperature at approx. 37.5°C. The peritoneum was cannulated with a 26-gauge needle connected to a 1-ml syringe containing a particular concentration of freshly made PTX, VPT or TCD. The skin over the tumors was carefully removed using a pair of fine scissors and a scalpel, and the exposed tumor surface was immediately covered with gauze moistened with 0.9% saline. The oxygen electrode was carefully inserted into the tumor with a micromanipulator, and a reference electrode was also inserted into the periphery of the tumor. The \(pO_2\) was measured at five points along two or three tracks in each tumor by advancing the microelectrode along the tracks in steps of 600 µm and moving backward 100 µm within a tumor, to up to 5 mm in depth. In each treatment group, measurements of the tumor \(pO_2\) at total 100 points were performed on 8–10 tumors, and the data were grouped into intervals of 2 mmHg for frequent histogram analysis of the \(pO_2\) distribution in the tumor.

The tumor \(pO_2\) measurements were performed 30 min after an i.p. injection of the drugs. The changes in the tumor \(pO_2\) were also studied 30 min after an i.p. injection of saline solution alone for control. The interval time, 30 min, was determined on the basis of the study in which the tumor oxygenation was reported to increase to the peak during 20–60 min after injection of 5 mg/kg PTX.\(^{12}\) Since VPT and TCD have the same effects on blood fluidity as PTX does,\(^{15-21}\) we measured the tumor \(pO_2\) 30 min after an i.p. injection of the drugs. Preliminary to the present study, we carried out continuous measurements of the \(pO_2\) at a single position in one tumor in each treatment group, and observed elevation of the tumor \(pO_2\) during 20–60 min after injection of the drugs (data not shown).

**X- Irradiation**

Mice legs with the tumors were locally exposed to x-rays at a single dose of 15 Gy 30 min after injection of each drug using a 200 kVp orthovoltage machine (Philips Medical Systems, Tokyo, Japan). The radiation parameters were 200 kVp, and 9 mA, with an added filtration of 0.2 mm Cu at a dose rate of 0.419 Gy/min. During irradiation, the mice were anaesthetized with an i.p. injection of 20 mg/kg of a mixture of ketamine and xylazine that was the same dose as that used in the tumor \(pO_2\) measurements.

**Tumor growth of SCCVII tumors**

In the growth delay assay, tumors were checked 3–4 times...
Fig. 1. Frequency distribution of measured intra-tumour $pO_2$ in saline-treated (dark bars) and PTX treated (light bars) SCC tumors, constructed as a function of oxygen tension, with grouping in 2-mmHg intervals (Fig. 1-A). The frequency distributions of intra-tumor $pO_2$ in VPT treated (Fig. 1-B) and TCD-treated (Fig. 1-C) tumors are also shown.
a week for 35 days. The tumor volume was calculated using the formula: \( V = 0.5 \times a \times b^2 \), where \( a \) and \( b \) were the longer and shorter diameters of the tumors, respectively. The diameters, \( a \) and \( b \) were measured with caliper.

Data analysis
Statistical analyses were performed using Mann-Whitney U test. A \( p \)-value less than 0.05 was considered statistically significant.

RESULTS

Tumor \( pO_2 \)
To reduce the effects of the condition such as temperature or atmospheric pressure, the measurements of the tumor \( pO_2 \) in the drug-treated group and in the control group were performed consecutively on the same day. Figure 1 shows frequency histograms of the \( pO_2 \) distribution in the SCCVII tumors. In the PTX-treated group, the mean \( pO_2 \) in the tumor increased significantly from 25.6 \( \pm \) 7.4 to 26.9 \( \pm \) 5.9 mmHg \( (p = 0.0079) \). In the VPT-treated group, the mean \( pO_2 \) in the tumor increased significantly from 18.6 \( \pm \) 4.7 to 22.9 \( \pm \) 5.6 mmHg \( (p < 0.0001) \). In the TCD-treated group, the mean \( pO_2 \) in the tumor increased significantly from 22.6 \( \pm \) 5.4 to 25.9 \( \pm \) 5.0 mmHg \( (p < 0.0001) \).

Tumor growth
In the drug-treated groups, the tumors were irradiated with a single dose of 15 Gy 30 min after an i.p injection of the drugs. Figure 2 shows the mean relative tumor volume as a function of time after various treatments. Each point represents the mean of 7–10 tumors. The volume of the SCCVII tumors increased four-fold in 4 days in the control group, and in 22 days in the irradiation alone group. The time for the tumor volume to increase four-fold were 28, 29 and 32 days in the PTX-treated group, in the VPT-treated group, and in the TCD-treated group, respectively. The growth rate in each drug-treated group significantly decreased compared with that in the irradiation alone group \( (p < 0.01) \).

DISCUSSION

In the present study, we revealed that PTX, VPT and TCD, which have been widely used for various vascular disorders, have radiopotentiation effects on the tumor growth using a murine tumor model. Although the radiosensitization effects of PTX have been reported to be caused by the increase of the tumor \( pO_2 \),\(^{12-14} \) no report is available on the changes in the tumor \( pO_2 \) by VPT or TCD and on the effects of the combined treatments of these drugs and radiation. Both VPT and TCD have been reported to be effective for improvement of regional microcirculation and to be used for cerebral ischemic diseases or peripheral arterial occlusive disease such as intermittent claudication.\(^{15-17} \) In addition, using an experimental animal model, VPT and TCD were reported to be effective for improvement of blood fluidity by reducing plasma viscosity and increase of red blood cell deformability,\(^{15-23} \) which are the same rationale for elevation of the tumor \( pO_2 \) as that by PTX treatment. Therefore, we designed the present study to reveal whether VPT and TCD can yield the radiopotentiation effects by increasing tumor \( pO_2 \) as reported in the studies using PTX and radiation.\(^{12-14} \)
As would be expected, all the three drugs, PTX, VPT and TCD yielded significant increase of the $pO_2$ in the SCCVII tumors from 25.6 to 26.9 mmHg, from 18.6 to 22.9 mmHg and from 22.6 to 25.9 mmHg, respectively. We observed significant tumor growth delay by the combined treatment of all the three drugs, PTX, VLD and TCD. However, an increase in $pO_2$ above 15 mmHg would not affect the radioreponse of the tumors significantly. Therefore, the radiopotentiation effects of PTX, VCT or TCD, which were observed regardless of the small hypoxic fraction in the SCCVII tumors in the present study, evokes the following question; whether other mechanisms, besides of elevation of tumor $pO_2$, might cause the radiosensitizing effect of these drugs. Bohm et al. reported that inhibition of DNA repair by PTX in homologous recombination (HR) is one of the mechanisms for the radiopotentiation effect. According to the report by Wang et al, VPT was inhibitor of the protein, calmodulin-dependent protein kinases, which was implicated in the process of double strand brake (DSB) rejoining, which may suggest that VPT has some effects on radiosensitization. Chen et al. reported that antplatelet drugs including PTX and TCD induced apoptosis in cultured cancer cells. Since radiation also activates apoptotic pathway, combination of antplatelet drugs and radiation may activate same or different apoptotic pathway leading to enhancement of cell killing.

In the present study, the contribution of the elevation of the $pO_2$ by VPT and TCD to the significant tumor growth delay is unclear due to small hypoxic fraction in the SCCVII tumors. However, two important results were obtained in the present study. First, increase of the tumor $pO_2$ was demonstrated after an injection of VPT and TCD which have been widely used for the treatment of vascular disorder such as cerebral ischemic diseases or intermittent claudication and has been confirmed the safety in clinical practice. Therefore, further studies to confirm the relationship between the increase of the tumor $pO_2$ and radiosensitizing of the tumors by VPT or TCD are needed using the tumors with large hypoxic fraction. Second, regardless of small hypoxic fraction in the SCCVII tumor, PTX, VPT and TCD demonstrated significant tumor growth delay in combination with radiation. Therefore, further studies for revealing other mechanisms for radiosensitizing of PTX, VPT and TCD, such as inhibition of DNA repair or activation of apoptotic pathway, should be carried out for seeking appropriate sequence of the combined therapy.

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