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Modulation of Tumor-selective Vascular Blood Flow and Extravasation by the Stable Prostaglandin I_2 Analogue Beraprost Sodium

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Improved delivery of macromolecular drugs to solid tumor is known as the enhanced permeability and retention (EPR) effect of macromolecular drugs and lipids. We report here that a prostaglandin I_2 (PGI_2) analogue induces enhancement of tumor-selective drug delivery, while it decreases tumor blood flow, in a rat tumor model (AH136B). Beraprost sodium (BPS) is an analogue of PGI_2 that is more stable than parental PGI_2 *in vivo* ($t_{1/2}$ for BPS is >1 h vs. a few seconds for PGI_2). Thus, BPS was administered to tumor-bearing rats to examine its effect on tumor vascular permeability as well as tumor blood flow. The amount of extravasation of the Evans blue–albumin complex in tumor tissue increased from two to three times, whereas tumor blood flow decreased almost 70%, in the group treated with BPS at 7 μg/kg compared with controls. Tissue blood flow of normal organs such as the kidney and the liver did not change to a significant extent. These findings establish a new role for BPS, not only in enhancing macromolecular drug delivery, but also in reducing the blood supply to tumor tissues.

Keywords: Enhanced permeability; Modulation of EPR effect; Prostaglandin I_2 analogue; Tumor delivery

Abbreviations: EPR, enhanced permeability and retention; PGI_2, prostaglandin I_2; BPS, beraprost sodium; sc, subcutaneously; ia, intra-arterial; iv, intravenous

INTRODUCTION

Extensive accumulation of polymer therapeutics, that is highly tumor selective is usually observed when these agents are administered to experimental animals with solid tumors and also in clinical cases (e.g. Matsumura and Maeda, 1986; Seymour *et al.*, 1995; Noguchi *et al.*, 1998; Maeda, 2001; Maeda *et al.*, 2001). This phenomenon of enhanced permeability and retention (EPR) effect of macromolecules in the vasculature of solid tumors is one of the most unique character not seen in normal tissues/organs and important concepts in the development of anticancer drugs with selective targeting characteristics. The pathophysiological mechanism of the EPR effect has been demonstrated by a number of vascular mediators such as bradykinin (BK), nitric oxide (NO), prostaglandins (PGs) and vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) (Matsumura and Maeda, 1986; Dvorak *et al.*, 1988; Wu *et al.*, 1998; Maeda *et al.*, 1999; Hori *et al.*, 2000; Maeda, 2001; Maeda *et al.*, 2001), as well as NO/peroxynitrate-activated matrix metalloproteinase (MMP) (Maeda *et al.*, 1994; Wu *et al.*, 1998; 2001). We previously found that both injection of angiotensin I-converting enzyme (ACE) inhibitors such as enalapril, which elevates the local concentration of BK (Matsumura *et al.*, 1988; 1991; Wu *et al.*, 1998; 2002; Hori *et al.*, 2000), and angiotensin II-induced hypertension resulted in a pronounced increase in the intratumor accumulation of macromolecular drugs, an albumin–dye complex, and SMANCS (Suzuki *et al.*, 1981; Hori *et al.*, 1985; Li *et al.*, 1993). In the latter condition, macromolecular drugs were "pushed" into the tumor. Low molecular weight mitomycin C did not manifest

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this effect (Li *et al.*, 1993) perhaps by back diffusion into the general circulation.

Prostaglandin I_2 (PGI_2, prostacyclin), originally described by Moncada *et al.* (1976), is a naturally occurring substance in many tissues including the vascular endothelium (Moncada *et al.*, 1977). PGI_2 has been shown to have a potent inhibitory effect on platelet aggregation (Whittle *et al.*, 1978; Inada *et al.*, 1998); it is also a potent vasodilator (Bunting *et al.*, 1976). Its effects are thus very similar to those of NO and the derivatives of NO (Wu *et al.*, 1998; 2001; Maeda, 2001; Maeda *et al.*, 2001). PGI_2 is chemically quite unstable, so it is difficult to use it exogenously as a vascular modulator. Consequently, more stable various PGI_2 derivatives have been developed by altering the chemical structure.

For example, beraprost sodium (BPS) (sodium DL-4-[1*R*, 2*R*, 3a*S*, 8b*S*)-1, 2, 3a, 8b-tetrahydro-2-hydroxyl-1-[(3*S*, 4*RS*)-3-hydroxy-4-methyl-oct-6-yne-(E)-1-enyl]-5-cyclopenta-[*b*] benzofuranyl] butyrate; Fig. 1) was designed to improve the chemical stability by introducing a benzofuranyl backbone into the structure of PGI_2. BPS thus obtained is indeed more stable than PGI_2. It can be administered orally and has been demonstrated to possess essentially the same pharmacological profile as PGI_2, but its half-life in humans is 1.1 h, which is in great contrast to the half-life (seconds) of native PGI_2 (Sim *et al.*, 1985; Umetsu *et al.*, 1987).

Previous studies reported that PGI_2 decreased the vascular permeability in skeletal muscle and in ischemic and inflammatory diseases (Muller *et al.*, 1987; Moller and Grande, 1999). It is thus being approved for oral treatment of impaired peripheral blood circulation such as in colonic ulcer as well as of pulmonary hypertension. However, the effects of PGI_2 on vascular permeability and blood flow in solid tumors are not known. To investigate these effects, we performed an *in vivo* study with intravenous (iv) and intra-arterial (ia) injections of the PGI_2 derivative BPS into tumor-bearing animals. The results showed that BPS may have clinical value because of its enhancement of the EPR effect.

MATERIALS AND METHODS

Animals and Implantation of AH136B Tumor

Donryu rats, weighing 160–180 g, were obtained from a commercial supplier (SLC, Inc., Shizuoka, Japan). They were handled according to the guidelines of the Experimental Animal Center of Kumamoto University. AH136B tumor (hepatoma) cells were maintained by serial passage in ascitic fluid in Donryu rats as described previously (Doi *et al.*, 1996). The cells were implanted subcutaneously (sc) at a dorsal site on the foot of the rats (inoculum size, 1×10^7 cells per injection site), and tumors were usually allowed to grow for 14 days to reach a diameter of 10–15 mm. The rats were then used for the experiments.

Measurement of Evans Blue Accumulation in the Tumor Tissues

BPS, with $t_{1/2}$ of 5.3 and 1.1 h in rats and humans, respectively, was obtained from Toray Research Laboratories, Kamakura, Japan. Evans blue dye, which forms a complex with albumin in blood, was injected at 7.5 mg/kg into tumor-bearing rats before or after BPS injection. BPS was injected either iv or ia in the dose range of 0.07–70 μg/kg. For a few minutes after Evans blue injection, rats were reperfused with 10 ml of heparinized physiological saline, and then the tumors were excised while the rats were under deep anesthesia induced by ether. Tumors were minced, and dye was extracted with formamide after incubation at 60°C for 48 h. The amount of dye was quantified by measurement of absorbance at 620 nm, which was used as the parameter of extravasation of albumin (Matsumura and Maeda, 1986).

Measurement of Tissue Blood Flow and Systemic Blood Pressure

Blood flow in tumor tissue and normal organs was measured by using a laser-Doppler flow meter

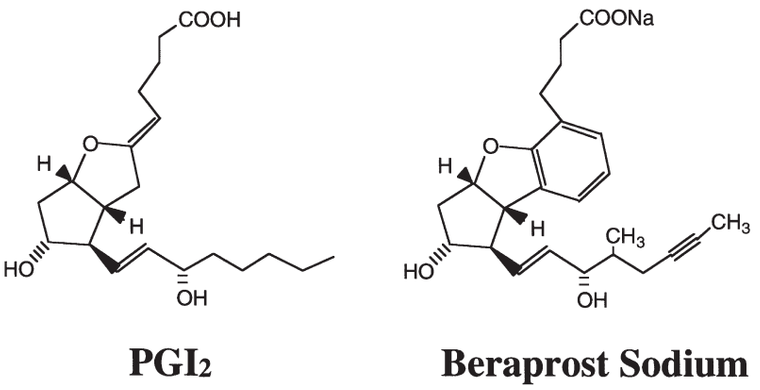

FIGURE 1 Chemical structures of PGI_2 and its stable analogue beraprost sodium (BPS).

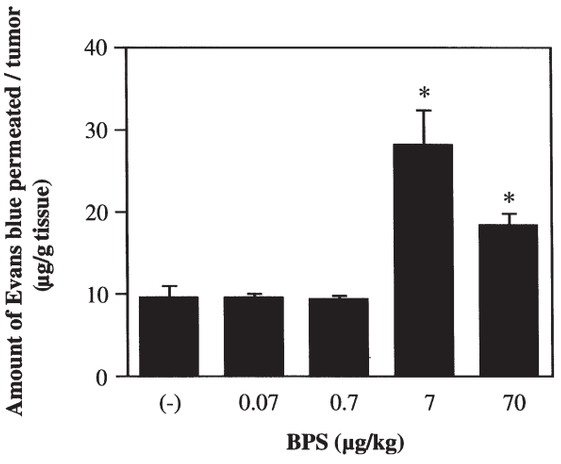

FIGURE 2 Effect of BPS on extravasation and accumulation of the Evans blue-albumin complex in tumor. Evans blue in saline was injected at 7.5 mg/kg at the tail/vein iv into AH136B tumor-bearing rats 5 min before iv BPS injection in the dose range of 0.07–70 μg/kg. Six hours after the Evans blue injection, rats were reperfused with 10 ml of heparinized physiological saline; the tumors were excised. Dye in minced tumor tissue was extracted with formamide after incubation at 60°C for 48 h. The amount of dye in the supernatant after centrifugation was quantified by measurement of absorbance at 620 nm. *, $P < 0.01$ vs. control. $n = 4$ for each group. Data shown are means ± SE in bar. See text for details and see reference by Matsumura and Maeda (1986).

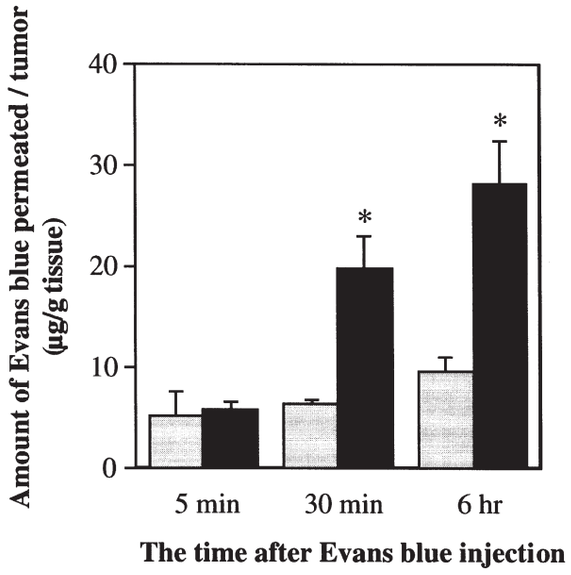

FIGURE 3 The time course change of Evans blue accumulation in the solid tumor after BPS injection. Evans blue was injected iv as described above to the tumor-bearing rats 5 min before injection of 7 μg/kg BPS (■) or vehicle (▩). The tumors were excised at 5, 30 min, and 6 hr after Evans blue injection, and the amount of dye was quantified as described for Fig. 2. *, $P < 0.01$ vs. control. $n = 3$ for each group. Data shown are means; bars, SE. See text for details.

(Advance Laser Flow Meter, ALF21, Tokyo, Japan). A probe-needle (Advance, model NS: 1.0 mm in diameter, 5 mm in length) of the flow meter was placed into the tissues and provided primarily relative measures of blood flow (ml/min/100 mg of tissue) at the capillary level. The drug BPS was injected either iv or ia in the dose range of 0.07–70 μg/kg as described in Fig. 2 legend or elsewhere, after which systemic blood pressure was measured at the common carotid artery by inserting a probe (Koden Engineering Model SEN-6102 hemomanometer Tokyo) into the artery.

RESULTS

Effect of BPS on Evans Blue Accumulation in Tumor Tissues after Intravenous (iv) Injection

Administration of BPS at doses of 7 and 70 μg/kg iv to tumor-bearing rats produced a significant increase in the accumulation of the dye 6 h after administration compared with the control group (no BPS, Fig. 2). Dose of 0.07 and 0.7 μg/kg iv, however, resulted in no significant accumulation of the dye (Fig. 2) perhaps due to very short duration of drug action at low doses, i.e. only less than 4 min (Fig. 5). To quantify the time-dependent increase in accumulation of the dye-albumin complex for the BPS dose of 7 μg/kg iv, tumors were excised 5 or 30 min after Evans blue injection, after which tissue was analyzed. At 5 min after injection of Evans blue, there was no significant difference in accumulation of the dye between the control and the BPS groups. However, at 30 min the BPS group showed a significantly higher accumulation compared with the control group (Fig. 3). Furthermore, the amount of Evans blue that had accumulated at 30 min was already about 70% of the amount found at 6 h (Fig. 2).

Effect of BPS Administered Intravenously on Blood Flow and Blood Pressure in Tumor, Liver, and Kidney

Blood flow in kidney and liver in normal or tumor bearing rats was not affected by BPS to a significant extent at 0.07 ~ 7 μg/kg, i.e. there was less than a 10–20% decrease (Fig. 4). Tumor blood flow, however, was affected much greater extent by BPS at a dose of 7 μg/kg: there was a decrease of almost 70%, with less than 30% of tumor blood flow retained (Fig. 4). BPS did not affect tumor blood flow or the systemic blood pressure to a significant level, and it affected, only for a short period (~ 5 min) at a dose of 0.7 μg/kg or below. In contrast the systemic blood pressure rapidly decreased about 50% at BPS doses of 7 (Fig. 5B) and 70 μg/kg and lasted more than 30 min; while the tumor blood flow at higher dose of BPS (70 μg/kg) was greatly suppressed also for much longer period. This may result in less Evans blue accumulation at 70 μg/kg. In the kidney and liver, there was very little or only transitory suppression of blood flow at 0.7 μg/kg iv but clearly different from tumor (Figs. 4–6), and both tumor blood flow and blood pressure returned to the normal level within 5 min. The profile of blood flow was a mirror image of the profile of enhanced

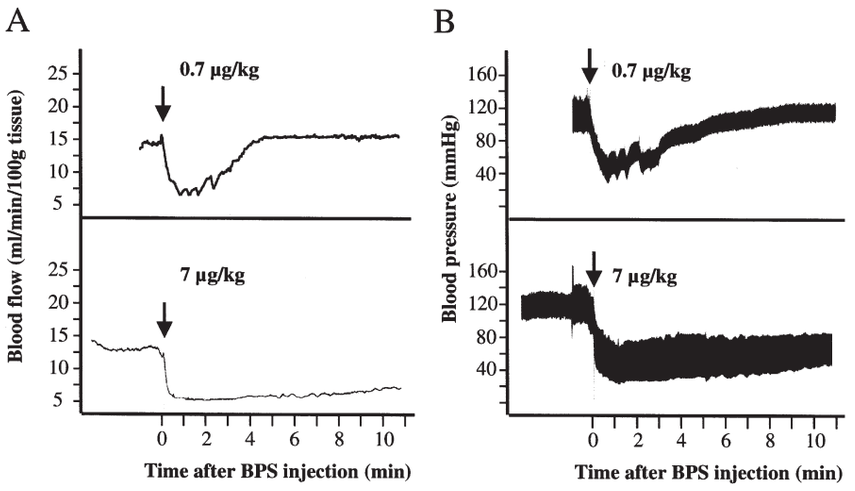

FIGURE 4 Change in tumor blood flow and systematic blood pressure after iv injection of BPS. (A) Blood flow in tumor tissue, measured by placing a probe needle of a laser-Doppler flow meter into the tumor tissue. (B) Systemic blood pressure at the common carotid artery was measured by using a hemomanometer. After injection of 0.7 μg/kg of BPS, both tumor blood flow and systemic blood pressure returned to normal level after 5–6 min. BPS was injected iv at a dose of 0.7 or 7 μg/kg. Data were analyzed by using a Macintosh computer with a MacLab data recording and analysis unit. $n = 3$. See text for details.

permeability with respect to the dose response for BPS (Fig. 2 vs. 4).

Effect of Intra-arterial (ia) Injection of BPS on Tumor Tissue

To evaluate the difference in the effect of BPS when administered by different routes (i.e. iv or ia), BPS was also administered ia to tumor-bearing rats via the feeding artery (common iliac artery) at a dose of 7 μg/kg. The effects of the ia BPS injection on Evans blue accumulation, tumor blood flow, and systemic blood pressure were similar to the effects of the iv injection (Fig. 6). At the BPS dose of 7 μg/kg, the accumulated amount of Evans blue was about three times greater in the tumor tissues than the amount in the control group (no BPS). In parallel, tumor blood flow was significantly decreased, whereas the blood flow in the liver and the kidney and the systemic blood pressure and were not significantly affected. These results suggest a reverse correlation between increased tumor-selective accumulation of the Evans blue and decreased blood flow, as was seen with iv administration of BPS.

Effect of Timing of BPS Administration

The effect of the timing of BPS injection on vascular permeability (i.e. accumulation of macromolecules) and tumor blood flow was investigated. A group in which BPS was injected 5 min before the Evans blue injection was compared with a group given a BPS injection 5 min after Evans blue. BPS injected 5 min before the dye did not enhance the EPR effect (Fig. 7). This finding, therefore, suggested that a high plasma level of polymer drugs is

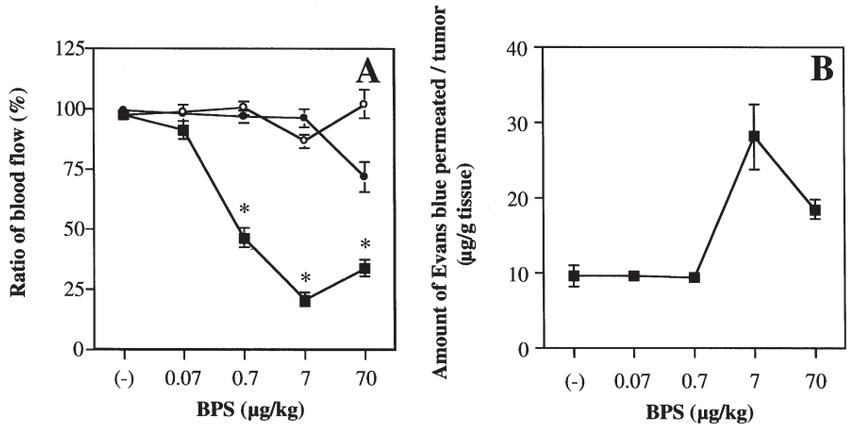

FIGURE 5 Effect of BPS on blood flow in tumor (■), the liver (○), and kidney (●). Blood flow was measured 2 min after BPS injection as described for Fig. 5 *, $P < 0.01$, tumor vs. liver or kidney. $n = 3$ for each group.

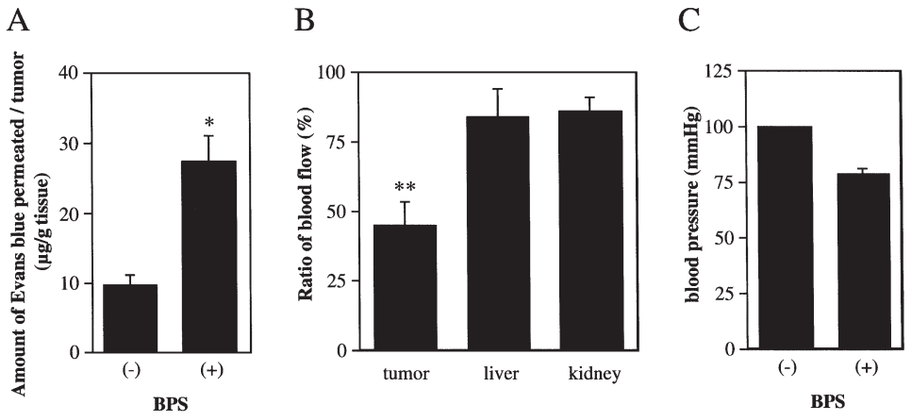

FIGURE 6 Effects of ia BPS on: accumulation of Evans blue (A), blood flow of tumor or normal organs (B), and systemic blood pressure (C). BPS was injected via the common iliac artery which is the tumor feeding artery at a dose of 7 μg/kg. All measurements were the same as those shown in Figs. 2 and 4. *, $P < 0.01$; **, $P < 0.05$. $n = 3$ for each group. Data shown are means ± SE in bar. See text for details.

necessary before the BPS injection. A decrease in tumor blood flow by prior injection of BPS did not enhance the accumulation of the macromolecular drug in the tumor (Figs. 5 and 7).

DISCUSSION

PGI_2 is known to improve blood flow and prevent platelet aggregation, thereby preventing occlusion. Its stable analogue BPS is in clinical use for the treatment of occlusive arterial diseases because of its potent vasodilatating effect, which improves peripheral blood circulation and thus prevents peripheral circulatory insufficiency and ischemia–reperfusion or hypoxia-induced necrosis (Bunting *et al.*, 1976; Whittle *et al.*, 1978). PGI_2 can also be cytoprotective with the inhibition of leukocyte activation, adhesion to the vascular wall, and of platelet aggregation being important. Therefore, production of PGI_2 by endothelial cells is physiologically logical and valuable (Granger and Kubes, 1994).

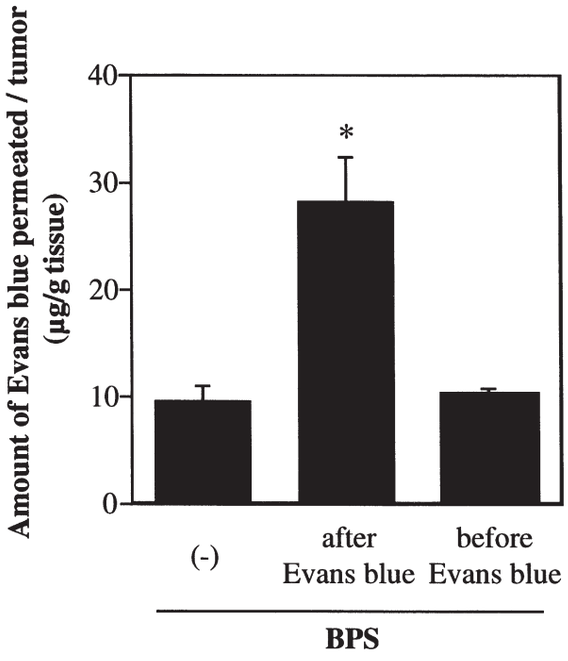

FIGURE 7 Effect of timing of iv BPS injection on the accumulation of Evans blue complex in tumor. BPS was injected iv at 7 μg/kg into the tumor-bearing rats 5 min before or 5 min after Evans blue injection. Six hours after the Evans blue injection, the tumor was excised and the amount of the dye was quantified as described in "Materials and Methods" section. *, $P < 0.01$ vs. control. $n = 4$ for each group. Data shown are means ± SE in bar. See text for details.

Additional analogues and related agents also have similar effects on vascular pressure, leukocytes adheresion, and platelets aggregation. Iloprost, for example, another stable analogue of PGI_2, inhibited exacerbation of liver injury in an ischemia–reperfusion-induced hepatic injury model (Harada *et al.*, 1999) via enhancing hepatic blood flow and inhibiting leukocyte activation and platelet aggregation. Similarly, in normal tissue in the hamster cheek pouch, iloprost at a nonhypotensive dose (0.05 μg/kg/min, iv) inhibited platelet aggregation and significantly attenuated the vascular permeability induced by ischemia-reperfusion (Muller *et al.*, 1987; Jiang *et al.*, 1998).

These effects are very similar to those of NO such as vasodilatation, vascular permeability enhancement, inhibition of platelet aggregation, and increased blood flow (Moncada *et al.*, 1991; Maeda *et al.*, 1994; Doi *et al.*, 1996). Vascular permeability in microvascular endothelial cells has been reported to be mediated PGI_2 production, which was triggered by VEGF/VPF (Murohara *et al.*, 1998). This permeability enhancing effect by PGI_2 had not discussed previously in cancer tissue. Furthermore, NO production is known to be upregulated by VEGF (Leung *et al.*, 1989; Papapetropoulos *et al.*, 1997; Wu *et al.*, 1998). BK, another vascular mediator, may be involved in cross-talk to upregulate NO production via a mechanism different from that used by VEGF (Bernier *et al.*, 2000) and may induce PG production (Chand and Eyre, 1977;

Cahill *et al.*, 1988; Maeda *et al.*, 1999), and, thus BK-PG-VEGF-NO cross-talk may also occur in cancer tissue. In addition, deleterious vasodilatory effects and enhanced vascular permeability effects of PGI_2 given at 0.4 μg/kg/min during development of pulmonary edema have been reported in animal studies; these effects may be related to enhanced extravasation into the alveolar space induced by PGI_2 (Krausz *et al.*, 1982; Ogletree, 1982; Yoshimura *et al.*, 1989).

In a different context, in normal tissues (not in tumor), BPS inhibited a thrombin-induced increase in permeability of cultured human umbilical vein endothelial cells grown on a type I collagen gel matrix layer (Imai-Sasaki *et al.*, 1995). In this study, fluorescein isothiocyanate conjugated albumin passed through the endothelial cells on the collagen layer into the lower chamber. A similar effect was noted for histamine, leukotriene B4, and phorbol myristate acetate (Imai-Sasaki *et al.*, 1995). This thrombin-induced permeability may occur via cAMP formation (Williams *et al.*, 1994; Uchida, 1996; Inada *et al.*, 1998) or activation of MMP by NO derivatives (e.g. peroxynitrite, $ONOO^-$) (Wu *et al.*, 2001), or it may involve activation of proteases: plasminogen activator and the prekallikrein to kinin cascade (Matsumura *et al.*, 1988; Maeda *et al.*, 1999; Maeda, 2001; Wu *et al.*, 2001).

In our present study, the amount of Evans blue extravasated in the tumor tissue in the group treated with BPS at 7 μg/kg via either ia or iv route increased two to three times compared with the non-treated control group; this increase did not occur at 0.7 μg/kg or less, perhaps due to short duration of drug action at this dose. This result suggests that tumor vascular permeability was increased by BPS at a relatively high dose but not at a very low dose. Thus, the effect of BPS, or PGI_2, on vascular permeability may vary with the local environment or pathophysiological state of the tissues, or it may vary in a biphasic manner (Williams *et al.*, 1994). Another PG, PGE_1, is known to have very similar vascular effects (Morita *et al.*, 1991; Ueno *et al.*, 1996), and interesting to see the result.

As mentioned above, PGI_2 is known to contribute to increased blood flow in healthy muscle and skin, as well as in brain tumor in several species as shown by experimental studies (Nomura *et al.*, 1996; Ueno *et al.*, 1996; Duffy *et al.*, 1998). In contrast to these results in normal tissues, however, our present experiments in tumor showed that 7 μg/kg BPS given either ia or iv decreased blood flow to a great extent (70–80%), which accompanied with a slight decrease in systemic blood pressure (about 25–50%). Similarly, and also in contrast to results in normal tissues, other PGs, PGE_1 and $PGF_2\alpha$, have been reported to reduce tumor blood flow selectively in VX2 liver carcinoma in rabbits (Morita *et al.*, 1991) and in malignant neoplasms of the small intestine and colon in humans (Kusano *et al.*, 1983).

Taken together, all these reports clearly support our present observation that vessels in tumors seem to respond to BPS differently from vessels in normal organs. Therefore, a new application in tumor biology may be possible: control of tumor blood flow and hence tumor growth, as well as anticancer drug delivery based on the EPR effect. In this context, we postulate that BPS, and possibly other PG analogues such as PGE_1, will be useful for enhancement of macromolecular drug delivery. The basic principle of the EPR effect has been discussed and verified for various macromolecular anticancer agents, such as SMANCS, and a polymer-doxorubicin complex (Matsumura and Maeda, 1986; Duncan *et al.*, 1992; Maeda *et al.*, 1999; Kataoka *et al.*, 2000; Maeda, 2001; Seymour *et al.*, 1995; Maeda *et al.*, 2002). It is intriguing that the considerable suppression of tumor blood flow may realize tumor suppressing effect, as was the case with ischemia-induced necrosis and apoptosis. In addition, a number of experiments demonstrated antimetastatic effects of PGI_2 and its analogues through the suppression of interactions between the tumor cells and the blood vessels of the host (Honn *et al.*, 1992; Schneider *et al.*, 1994; Yoshida *et al.*, 1999).

It should be noted that pronounced improvement in macromolecular drug delivery was observed with the ACE inhibitor temocapril given iv or ip, which also elevated the local BK concentration, and thus levels of NO and PGs. This increased EPR effect as measured by the extravasation of Evans blue was obtained without affecting the systemic blood pressure (Matsumura *et al.*, 1988; 1991; Wu *et al.*, 1998; Hori *et al.*, 1999; Maeda *et al.*, 1999; Hori *et al.*, 2000; Maeda, 2001).

In conclusion, we report here that BPS, the PGI_2 analogue, increased the delivery of macromolecules to tumor by about three fold at 2 μg/kg ia and two to three-fold also at 7 μg/kg iv. Furthermore, BPS selectively suppressed tumor capillary blood flow to a great extent without a severe reduction in the systemic blood pressure and in the blood flow of normal organs. A combination of macromolecular drugs followed by BPS administration may thus have useful therapeutic applications.

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