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Tumor-selective Blood Flow Decrease Induced by an Angiotensin Converting Enzyme Inhibitor, Temocapril Hydrochloride

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To enhance chemotherapeutic efficacy against cancer, it is important to deliver anticancer drugs preferentially to cancer cells and to retain the drugs there for a prolonged time. The *in vivo* prolongation of the exposure time of anticancer drugs in tumors can be accomplished by decreasing tumor tissue blood flow (tBF) after anticancer drug administration. The present study demonstrated that temocapril hydrochloride, an angiotensin converting enzyme inhibitor, decreases tumor tBF markedly in LY80 tumor, a subline of Yoshida sarcoma in the rat, without affecting the blood flow in liver, kidney, bone marrow, and brain. In tumor areas with flow of above 20 ml/min/100 g, the tBF decreased by approximately 50% due to temocapril. In tumor areas with tBF of about 20 ml/min/100 g, it became less than 3 ml/min/100 g with temocapril and did not recover during the 2 h experiment. These findings were obtained not only in large tumors, but also in microfoci growing within a transparent chamber. Furthermore, even when temocapril was administered under the condition of increased tumor tBF by administering angiotensin II, tumor tBF decreased immediately. Using this technique, it should be possible to trap anticancer drugs selectively in tumor tissue for an extended period of time.

Key words: Tumor blood flow — Drug delivey system — Temocapril hydrochloride — Angiotensin II — ACE inhibitor

Even now, there are very few drugs which exhibit tumor-selective toxicity. However, even if an anticancer drug with great potential were discovered and administered, it would have little practical value unless it could be delivered to the tumor tissue at an effective concentration.[1–3] Furthermore, even if the anticancer drug were delivered to the tumor at a suitable level, the therapeutic efficacy would still be inadequate for some drugs unless the time of contact between the drug and the cancer cells were suitably long. Thus, one approach for enhancing the therapeutic effect of cancer chemotherapy is that a sufficient exposure time of tumors to anticancer drugs is attained after the delivery of anticancer drugs to tumor tissues was increased.[4,5] In this context we are now able to enhance delivery of anticancer drug to tumor tissue selectively by means of increasing tumor tissue blood flow (tBF) with angiotensin II (AII)[6,7]; this method has been approved by the Ministry of Health and Welfare in Japan as "AII-induced hypertension chemotherapy."[8] Drug delivery can be enhanced 1.5–2 fold, although the drugs are still readily washed out by tBF. To suppress the washout of anticancer drugs, it is important to reduce tumor tBF immediately after the drug has been delivered to tumor tissues.

Since the study of Algire *et al.*,[9] it has been found that serotonin,[10,11] hydralazine,[12] sodium nitroprusside,[4,13] flavone acetic acid (FAA),[14–16] tumor necrosis factor (TNF)-α,[17] vinca alkaloids,[18,19] 5,6-dimethylxanthenone-4-acetic acid (DMXAA),[20] nitric oxide synthase (NOS) inhibitor,[21] and combretastatin A-4 compounds[22–24] markedly decrease tumor tBF. However, the decrease in tumor tBF induced by some of these substances is not selective to tumors. In addition, with the exception of vinca alkaloids, NOS inhibitor and combretastatin A-4 compounds, it was found in animal experiments that the decrease in tumor tBF induced by these substances is associated with a marked reduction of the mean arterial blood pressure (MABP). Drugs that induce a continuous and severe reduction of MABP seem to be unsuitable for clinical use.

We have searched for drugs or mechanisms by which tumor tBF can be decreased selectively without greatly affecting the systemic arterial blood pressure. Recently, we have found that one of the angiotensin converting enzyme (ACE) inhibitors, temocapril hydrochloride has this action. The purpose of this paper is to investigate the effects of temocapril on normal and tumor tBF, and to demonstrate that temocapril selectively decreases tumor tBF.

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MATERIALS AND METHODS

Rats and tumor Male Donryu rats (Crj-Donryu; Nippon Charles River, Yokohama) at 8–10 weeks of age and with a weight of 250–300 g were used for measurements of tBF. The same strain of rats, weighing 200–220 g each, was used for vital microscopic observation of tumor microcirculation using a transparent chamber in a skinfold.[25] The rats were bred and maintained in a ventilated, temperature-controlled (24±1°C), and a specific pathogen-free environment on a bed of wood shavings, with food and water freely available and a 12-h light-dark cycle. They were housed two or three per cage. Every rat that was fitted with a transparent chamber was caged individually.

The tumor cells used were LY80, a variant of the Yoshida sarcoma, which has been maintained in our laboratory by successive i.p. transplantation. For measurement of tBF in solid tumors, cells (2×10^6) in 0.1 ml were injected s.c. into the back of each rat. All rats developed tumors, and tumor volumes became approximately 3–6 cm^3 8–10 days after tumor cell implantation. No spontaneous regression occurred. For vital microscopic observation, a small fragment (about 0.1 mm^3) of LY80 prepared from the s.c. tumors of carrier rats was transplanted onto the tissue within the chamber when the transparent chamber was installed. All experiments were performed under anesthetic conditions in a controlled-temperature box (25°C) fitted with a suction duct. All animal experiments were conducted under the guidelines approved by the Animal Experiment Committee of our institute.

Drugs Temocapril hydrochloride powder was kindly provided by Sankyo Pharmaceutical Co., Tokyo. The powder was dissolved in a vehicle composed of ethanol, dimethyl sulfoxide and 0.9% NaCl solution (1:1:8), resulting in a final concentration of 2.25 mg/ml, just before use and injected i.v. at a volume of 2.0 ml/kg (4.5 mg/kg) using an infusion pump (Compact Syringe Pump; Harvard Apparatus Co., Inc., Millis, MA). The infusion rate was 0.18 ml/min. AII of human type was purchased from Sigma Chemical Co., Tokyo. AII was dissolved in 0.9% NaCl solution to a concentration of 5.0 μg/ml and infused i.v. continuously at the rate of 0.018 ml/min to maintain an MABP of approximately 160 mmHg for 4 min. Pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL) and enflurane (Ethrane; Abbott Laboratories) were used for anesthesia.

Anesthesia Rats were anesthetized with pentobarbital sodium administered intramuscularly with an initial dose of 25 mg/kg and enflurane (1.0% in the inhaled carrier gas at 1 liter/min). Supplemental subsequent doses (12.5 mg/kg) of pentobarbital were given at 90-min intervals. The concentration of enflurane was precisely maintained by means of an anesthetic apparatus for small laboratory animals.[26] Rats were laid on a heated stage at 34°C throughout the experiments and body temperature was monitored at the rectum, at a depth of 30 mm, using a temperature probe for small animals (PTC-201; Unique Medical Co., Tokyo) and maintained at 33.5–35.5°C. Under this condition, rats were kept at a stable light anesthetic level during 2 h of experimentation, and no emergencies were encountered.

Blood pressure measurement MABP was monitored in all of the rats used. MABP was measured via a catheter (PE-50; Clay Adams Co., Persippany, NJ) inserted into the right femoral artery. The pressure in the catheter was recorded continuously with a pressure transducer (TNF-R; Spectramed Medical Products, Singapore), whose output was fed into an amplifier (6M82; NEC-Sanei Co., Tokyo) adapted to the measurement of MABP. MABP was measured at multiple time points (i.e., 30 min, 1 h, and every subsequent 1-h interval until 4 h) after the temocapril administration.

Measurement of tBF tBF in normal tissues and tumor was measured with the hydrogen clearance method,[26, 27] the principles of which were described in detail by Aukland *et al.*[28] In brief, after saturation of the tissue with hydrogen following the inhalation of 7–9% hydrogen gas in air (at 1 liter/min), the tBF value (in ml/min/100 g tissue) was calculated from the half-life of the clearance curve obtained by a tBF meter with 2 separate amplifiers (PHG-201; Unique Medical Co.). Two hydrogen electrodes with 80 μm diameter and 25 cm length (UHE-201C; Unique Medical Co.) and two rod-type Ag/AgCl reference electrodes (TT-98012; Unique Medical Co.) were used per rat. A small incision was made in the dorsal skin and the reference electrode was inserted between the skin and the musculature.

For measurements of tumor tBF, a pin hole was made into the skin overlying the tumor with a syringe needle (23G; Termo Co., Tokyo), and an electrode was inserted from the hole into the tumor tissue. The depth of the inserted electrode was less than 5 mm from the surface of the tumor nodule. To measure the tBF of the brain cortex, a small hole was drilled at the right parietal bone with a dental drill and an electrode was introduced to the depth of 1.5–2 mm below the brain surface. To measure the tBF of the liver, the rats underwent laparotomy at the mid-line and electrodes were inserted into the median lobe. For measurements of tBF in the kidney, the right kidney was exposed after cutting in the lower back region and electrodes were inserted into the cortex to a depth of approximately 1.5–2 mm from the ventral surface. The tBF in the bone marrow was measured by one electrode inserted at the junction of the upper and middle thirds of the shaft of the left femur through a small hole bored in the intercondyloid fossa.

Change in tBF due to temocapril Before the administra-

tion of temocapril, tBF was measured 2 or 3 times at 30-min intervals. When the blood flow had stabilized, temocapril was administered via the lateral tail vein by an infusion pump. tBF was measured at multiple time points (i.e., 30 min, 1 h and 2 h) after the administration.

Changes in tumor tBF due to AII and subsequent temocapril administration To investigate changes in tumor tBF due to AII and subsequent temocapril administation, two syringe needles (27G; Termo Co.) were introduced into the left and right lateral tail veins of each rat. By administration of AII via the tail vein with a syringe, MABP was elevated to approximately 160 mmHg. Once a hypertensive state had developed, it was maintained for 4 min, and then AII administration was stopped. Tumor tBF was measured before, during, and 10 min, 30 min, and 1 h after the AII administration. After tumor tBF at 1 h had been measured, MABP was again elevated by AII and the hypertensive state was maintained for 4 min. Then AII administration was stopped, and at the same time the infusion of temocapril was initiated via another syringe needle placed on the opposite side. Tumor tBF was measured before, as well as during AII-induced hypertension, and at 10 min, 30 min, and 1 h after the start of the temocapril administration.

Vital microscopic observation of tumor tBF due to AII and subsequent temocapril administration Rat transparent chambers were implanted in the dorsal skin flaps under aseptic conditions for vital microscopic observation. Each chamber consisted of two identical titanium frames containing a circular quartz glass window. Tumor neovascularization usually began to develop within the chamber 4–5 days following tumor implantation. Observations were made both on comparatively early tumors (6 days after tumor implantation) and advanced tumors (12 days after tumor implantation) growing in the chamber. Each rat with a chamber was placed in the right lateral position on a heated (34°C) stage (MATS-SFA; Tokai HIT Co., Ltd., Tokyo), which was attached to the mechanical stage of the microscope. AII and temocapril were administered as described earlier. Tumor tBF changes brought about by the drugs were observed directly through a light microscope (Fluophoto; Nippon Kogaku K.K., Tokyo) with a ×10 ocular and a ×4–20 objective. The image of the microcirculatory bed was viewed with a CCD video camera (CS-900; Olympus Kogaku K.K., Tokyo), displayed on a TV monitor (PVM-14M4J; Sony Corp., Tokyo) and recorded on a videocassette recorder (SVO-2100; Sony Corp.). Segments of the video tape that contained the desired images were transferred to computer hard disk (Power Machintosh 8600/200; Apple Japan, Inc., Tokyo). Final images were produced by a digital printer (Pictrography 4000; Fuji Photo Film Co., Ltd., Tokyo).

Statistics All results were expressed as mean±SD. Since MABP, and tBF of normal tissues and tumors did not change significantly in response to the vehicle solution during the experimental period (data not shown), the statistical significance of each time point after the temocapril administration was compared to the initial time points and evaluated with paired *t* tests. *P* values of 0.05 or below were considered significant, and those of 0.001 or below were considered highly significant.

RESULTS

Effect of temocapril on MABP The effect of temocapril on MABP is shown in Fig. 1. MABP decreased after the drug administration, reaching 87% of the control value after 2 h and thereafter the value remained relatively constant. Although the decrease in MABP induced by temocapril was small it was highly significant throughout the experiments.

Changes in tBF due to temocapril The effects of temocapril on the tBF of normal tissues (liver, kidney, bone marrow, brain) and tumors are shown in Fig. 2. In all normal tissues measured, mean tBF did not change significantly in response to temocapril (Fig. 2, A–D). By contrast, tumor tBF decreased markedly. In the tumor areas with a flow of more than 20 ml/min/100 g (n=10), the tBF decreased by approximately 50% in response to temocapril. In the tumor areas with tBF of less than 20 ml/min/100 g (n=10), it fell below 3 ml/min/100 g and did not recover during the 2 h experiment. The difference of tumor tBF before and after temocapril administration was highly significant at every time point (Fig. 2, E and F).

Changes in MABP and tumor tBF due to AII and subsequent temocapril administration The changes in MABP and tumor tBF induced by AII and subsequent

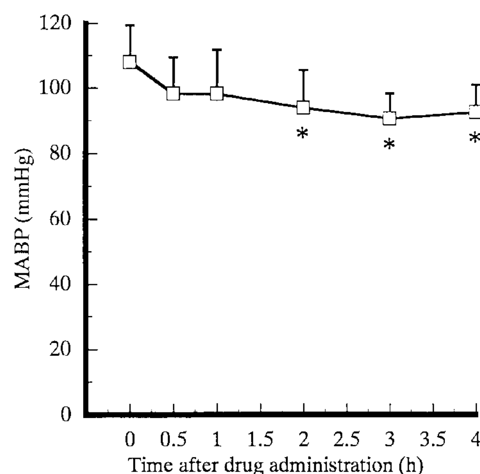

Fig. 1. The effect of temocapril on MABP. Each time point shows the mean±SD (n=10). Temocapril was injected i.v. at 0 min. * P<0.001.

temocapril administration are shown in Fig. 3. When MABP was elevated from 93.3±6.4 to 144.9±10.8 mmHg with AII, tumor tBF increased from 39.2±14.3 to 66.0±28.2 ml/min/100 g (n=14). The values of tumor tBF 10 min, 30 min, and 1 h after the end of AII administration were 40.6±13.0, 35.1±14.5, 35.3±12.8 ml/min/

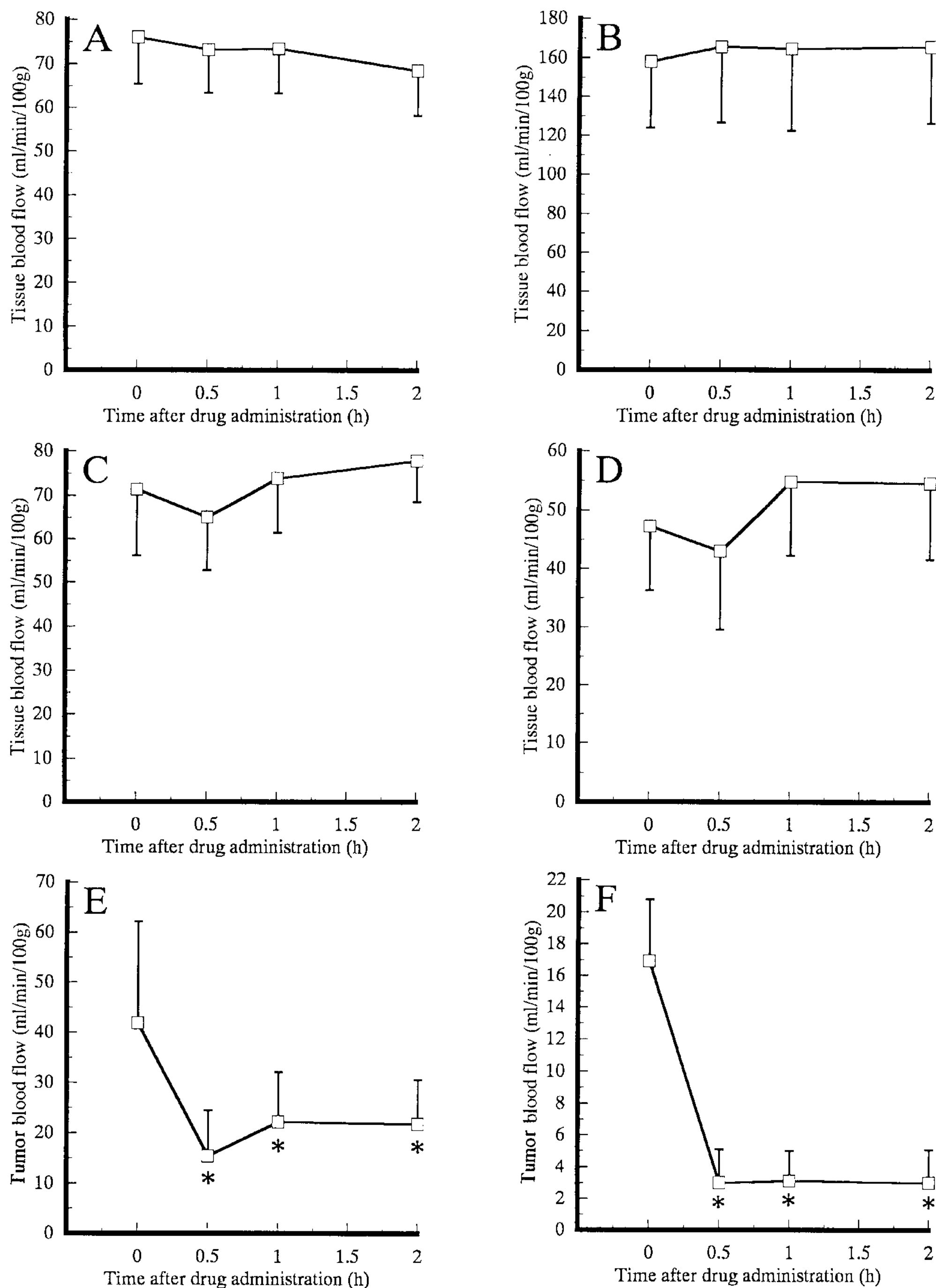

Fig. 2. Changes in tBF of normal tissues and LY80 tumors due to temocapril. A, liver (n=17); B, kidney (n=14); C, bone marrow (n=10); D, brain (n=10); E, tumor (regions with flow of more than 20 ml/min/100 g) (n=10); F, tumor (regions with flow of less than 20 ml/min/100 g) (n=10). Temocapril was injected i.v. at 0 min. In all normal tissues, the mean tBF did not change significantly in response to temocapril. By contrast, tumor tBF decreased highly significantly at all time points. * P<0.001.

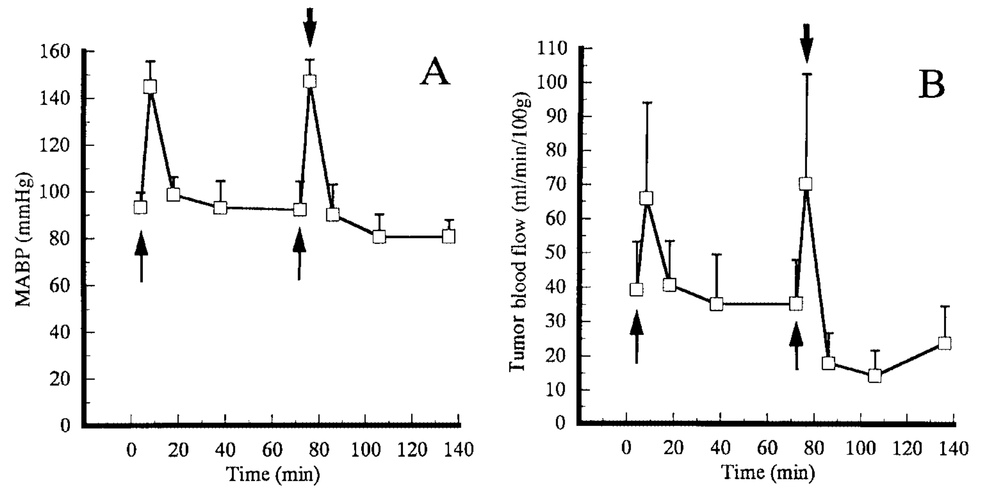

Fig. 3. Changes in tumor tBF due to AII-induced hypertension and subsequent administration of temocapril. A, MABP change; B, tumor tBF change; ↑, AII injection; ↓, temocapril injection. Temocapril decreased tumor tBF rapidly when administered under AII-induced hypertension.

Fig. 4. The effects of AII and temocapril on the blood flow of comparatively early tumors (6 days after tumor implantation) growing in the rat transparent chamber. Views in A through D are the identical site of the same animal. A, before drug administration (MABP, 100 mmHg); B, AII-induced hypertension (MABP, 155 mmHg); C, 5 min after temocapril administration (MABP, 115 mmHg); D, 1 h after temocapril administration (MABP, 90 mmHg).

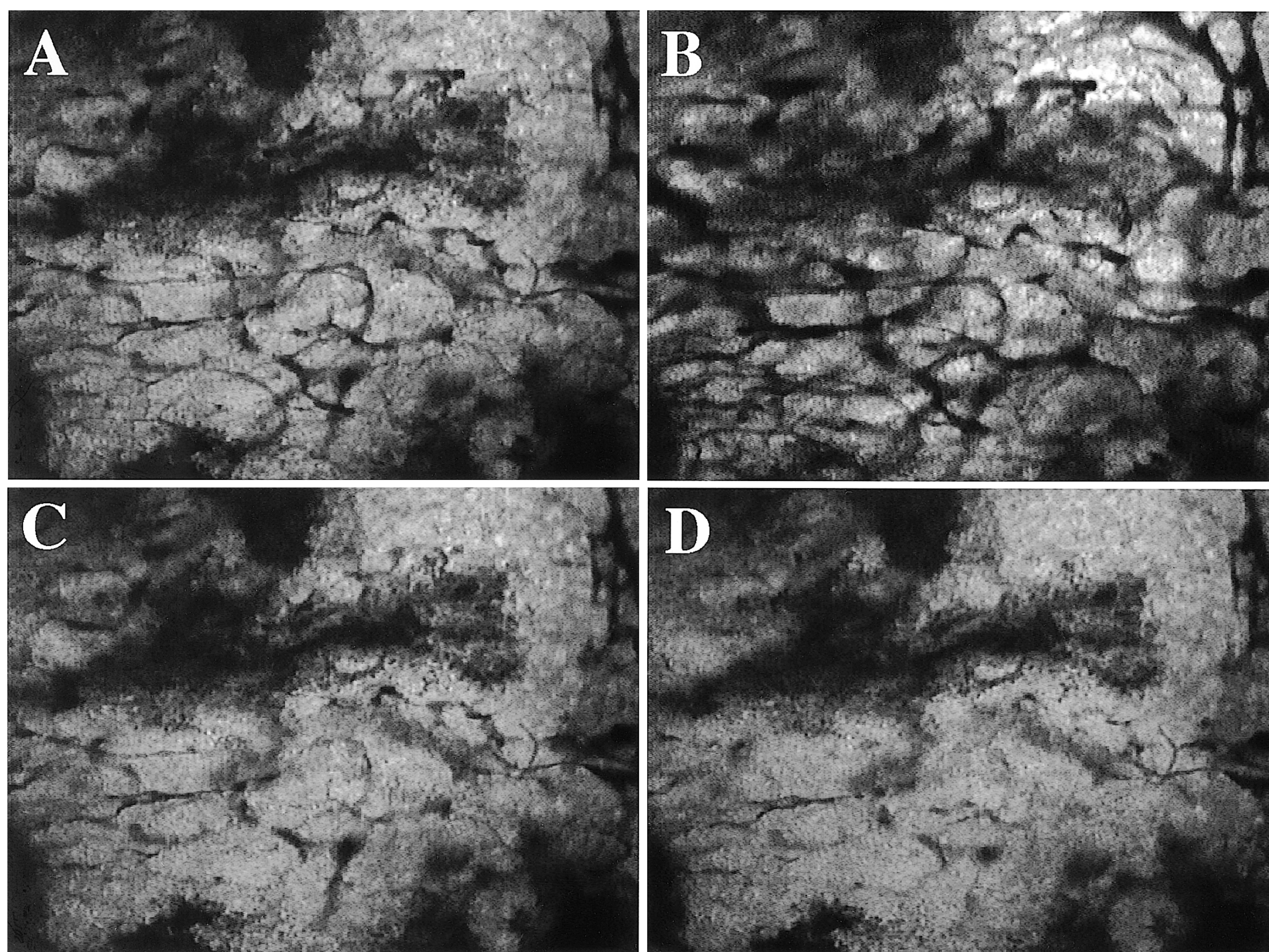

Fig. 5. The effects of AII and temocapril on the blood flow of comparatively advanced tumors (12 days after tumor implantation) growing in the rat transparent chamber. Views in A through D are the identical site of the same animal. A, before drug administration (MABP, 95 mmHg); B, AII-induced hypertension (MABP, 160 mmHg); C, 5 min after temocapril administration (MABP, 110 mmHg); D, 1 h after temocapril administration (MABP, 88 mmHg).

100 g, respectively. At 1 h after the first administration of AII, AII was again administered. When MABP was elevated from 92.1±12.4 to 147.0±9.5 mmHg, tumor tBF increased from 35.3±12.8 to 70.4±32.3 ml/min/100 g. Next, temocapril solution was infused into each rat with hypertension induced by AII infusion, and a decrease of MABP from 147.0±9.5 to 90.0±13.0 mmHg was observed. Tumor tBF decreased to 18.0±8.8 ml/min/100 g. At 30 min and 1 h after the start of the temocapril administration (the end of AII infusion) the flow rates became 14.4±7.4 and 23.9±10.9 ml/min/100 g, respectively.

Vital microscopic observation of the change in tumor tBF due to AII and subsequent temocapril administration The effects of AII and temocapril on the blood flow of the comparatively early tumors growing in the rat transparent chamber are shown in Fig. 4. Fig. 4A shows the finding before drug administration. When MABP was elevated from 100 to 155 mmHg by AII, both tumor vessel diameter and blood velocity increased, resulting in an increase in tumor tBF (Fig. 4B). Under this condition, while tumor tBF was increased, temocapril solution was infused and the MABP decreased from 150 to 115 mmHg. The blood flow at this time appeared to be almost stopped and consequently functioning tumor vessels disappeared, as shown in Fig. 4C. The tBF in many tumor vessels did not recover even 1 h after the temocapril administration (Fig. 4D).

The effects of AII and subsequent temocapril administration on comparatively advanced tumors are shown in

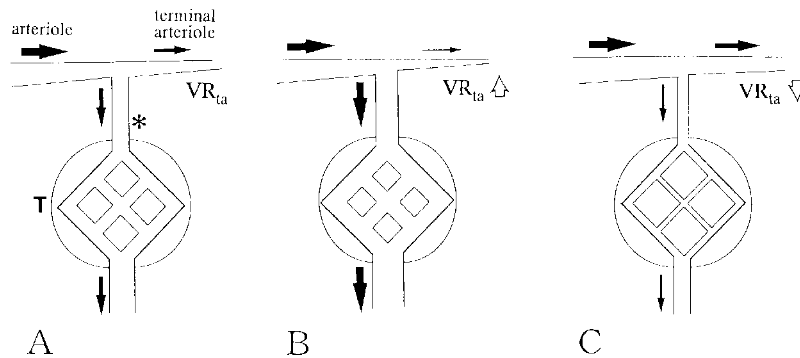

Fig. 6. The shunt-model of changes in tumor tBF induced by AII or ACE inhibitor. A, resting condition; B, AII administration; C, temocapril administration. Black arrows, vector of blood flow; VR_{ta}, vascular resistance of terminal arteriole; *, tumor vessel; T, tumor. The tumor vascular network characterized by a passive vascular bed and the preexisting vascular bed are parallel circuits (A). AII causes greater vascular resistance of the terminal arteriole, resulting in inflow of blood into tumor vessels (B). Temocapril inhibits the action of endogenous AII, leading to relaxation of the vascular tonus of the terminal arteriole, and resulting in a reduction of inflow of blood to the tumor vascular network (C).

Fig. 5. Fig. 5A is the finding before drug administration. When MABP was elevated from 95 to 160 mmHg due to AII, tumor tBF increased markedly and consequently many vessels were enlarged and became visible (Fig. 5B). When MABP decreased from 160 to 110 in response to temocapril, blood flow in most tumor vessels in the observed area stopped completely within 5 min, and many blood vessels shrank and were no longer visible under the microscope (Fig. 5C). Tumor tBF remained suppressed even 1 h after drug administration (Fig. 5D).

DISCUSSION

The present study clearly demonstrated that temocapril induces a marked decrease in tumor tBF without a severe reduction in MABP and this decrease of tBF is tumor-specific. In areas with comparatively high flow, the mean tBF decreased by approximately 50%. In areas with comparatively low flow, tumor tBF decreased to almost zero following temocapril administration and did not recover during the experimental period. On the other hand, tBFs in normal tissues did not change in response to the drug. Recently we have found that a similar selective decrease in tumor tBF is also induced by captopril, another ACE inhibitor (Hori *et al.*, unpublished data), and by losartan, an angiotensin type I receptor (AT1 receptor) antagonist (Saito *et al.*, unpublished data). Although the reason is not yet clear, a selective decrease in tumor tBF seems to be caused by inhibiting the action of endogenous AII.

In a previous study, we demonstrated that the increase in tumor tBF by AII is the result of a secondary response to the vessel reaction of preexisting arterioles.[29] That is, the tumor vascular bed and preexisting vascular bed are parallel circuits (Fig. 6), and AII has greater effects on the preexisting terminal arterioles, causing greater vascular resistance and thus, simultaneously, greater perfusion pressure of upper arterioles, resulting in inflow of blood to the passive vascular network of the tumor (Fig. 6B). The tumor tBF reduction induced by inhibiting the action of endogenous AII is also considered to be a secondary reaction that is mediated by the interaction between AII and AT1 receptors, which exist abundantly on the preexisting arterioles and mediate the contractile response in vascular smooth muscle cells. Inhibition of this interaction might lead to relaxation of vascular tonus in some regions of the preexisting terminal arteriole and decrease in arteriolar pressure, resulting in reduction of inflow of blood to the tumor vascular network (Fig. 6C). In recent years, it has been demonstrated that there are fewer AT1 receptors in poorly differentiated cancers.[30–34] Our hypothesis regarding the microvascular mechanism of the decrease in tumor tBF by temocapril is further supported by the fact that there are fewer AT1 receptors in tumors.

The fact that tumor tBF decreases rapidly in response to temocapril administered under AII-induced hypertension strongly suggests that by using the combination of AII and temocapril, we can enhance the delivery of anticancer drugs to tumor tissue, and prevent the washout of the drugs by tBF from the tumor tissue. Furthermore, the immediate decrease in tumor tBF induced following the increase in tumor tBF was observed not only in large tumors, but also in microfoci growing within the transparent chamber. This means that the prolongation of intratumoral exposure time of anticancer drugs occurs both in large tumors and in micrometastatic foci. Using our image analyzing system combined with the transparent chamber

technique, we are now analyzing quantitatively how long the exposure time of tumors to intratumoral drugs can be extended by temocapril.

In another series of experiment, Maeda *et al.*[35–37] demonstrated that a macromolecular anticancer drug SMANCS, a conjugate of neocarzinostatin and poly-(styrene-comaleic acid/anhydride), administered i.v. was retained in tumor tissues for a longer period than a lower-molecular-weight drug NCS, neocarzinostatin, and that a marked therapeutic efficacy was obtained using SMANCS. In addition, it has been shown that the antitumor activity is enhanced by a combination of anticancer drugs with agents that inhibit tumor blood flow.[38–40] Pruijn *et al.* reported that DMXAA-induced inhibition of tumor blood flow can be used to entrap melphalan in tumors, resulting in enhancement of the therapeutic effect.[5] All these results suggest that the chemotherapeutic efficacy might be enhanced by the prolongation of the exposure time of tumors to anticancer drugs and that the prolongation of the exposure time might be achieved by suppressing blood flow.

In conclusion, tumor tBF can be selectively decreased by use of temocapril without a severe reduction of MABP or tBF of normal tissues. Using this approach, it should be possible to trap anticancer drugs selectively in tumor tissue for an extended period of time.

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