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Fluctuations in Tumor Blood Flow under Normotension and the Effect of Angiotensin II-induced Hypertension

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To elucidate the significance of angiotensin II (AII)-induced hypertension chemotherapy, changes of tissue blood flow both in normal subcutis and in tumors (AH109A, LY80) were measured with the hydrogen gas clearance method. A newly-developed anesthetic machine was used to keep the animals' condition constant. Tissue blood flow in normal subcutis and tumors always fluctuated with time under normotension. The nature and the rate of fluctuation in tumor blood flow were almost identical in two different types of tumors. However, the fluctuation of blood flow in tumor and that in normal subcutis were almost always inversely related when blood flows in these different tissues were measured simultaneously, i.e., when tissue blood flow in normal subcutis decreased, tumor blood flow increased, and *vice versa*. The findings supported the idea that the connection mode between the tumor vascular bed and normal vascular bed is a parallel circuit. Vascular resistance in the normal vascular bed under AII-induced hypertension seemed to be greater than that under normotension, because the AII-increased tumor blood flow always exceeded the maximum tumor blood flow under normotension. Due to the fluctuations of tumor blood flow, no-flow or low-flow areas, resistant to delivery of anticancer drugs, moved sporadically within the tumor under the normotensive condition. However, good conditions for drug delivery to tumor tissue were induced by AII-induced hypertension.

Key words: Tumor blood flow — Angiotensin II — Anesthetic machine — Hydrogen clearance method — No-flow area

Angiotensin II (AII)-induced hypertension chemotherapy,[1] which is a new therapeutic modality based on the functional characteristics of tumor microcirculation,[2,3] has been in clinical use since 1978 and has been verified to be superior to conventional cancer chemotherapy by clinical[4,5] and histological[6] criteria. By elucidating the reason why an adequate amount of anticancer drug is not delivered to all viable tumor cells under conditions of normotension, it may be possible to evaluate the clinical significance of AII-induced hypertension chemotherapy. For this purpose, we must measure time-dependent fluctuations of tumor blood flow under conditions of normotension, and compare them with the flow values observed under AII-induced hypertension. However, to data there has been no effective means to measure tumor blood flow quantitatively in a given region at different time intervals. In the present experiment we were able to perform such measurement by means of a novel technique employing a newly developed anesthetic machine.

The aim of this study was to examine the following two problems concerning tumor microcirculation. 1) How does tumor blood flow fluctuate under conditions of normotension? 2) Can maximum tumor blood flow under normotension exceed the flow value increased by AII-induced hypertension?

MATERIALS AND METHODS

Animal and tumors Male Donryu rats (Nippon Rat Co., Urawa), weighing 250–300 g, were used in this experiment. The tumors were AH109A, a Yoshida rat ascites hepatoma, and LY80 (established by Dr. H. Satoh), a subline of the Yoshida sarcoma, which have been maintained in our laboratory by successive i.p. transplantation. Tumors were used in experiments when they reached approximately 3 cm in diameter at 14–18 days after s.c. transplantation of 2×10^6 ascites tumor cells.

Blood pressure measurement and elevation Mean arterial blood pressure was measured by a catheter (PE-50; Clay Adams Co., Parsippany, NJ) inserted into the right femoral artery. The pressure in the catheter was recorded with a pressure transducer (TNF-R; Spectramed Medical Products (S) Pte. Ltd., Singapore) whose output was fed into a strain amplifier (6M82; NEC-Sanei Co., Tokyo). Blood pressure was elevated by continuous infusion into the tail vein of AII (angiotensin II human; Toa Eiyo Ltd., Tokyo) dissolved in physiological saline at a concentration of 2.0 μg/ml. AII (0.02–0.06 μg/min) was infused by means of an infusion pump (compact syringe pump; Harvard Apparatus Co., Inc., Millis, MA).

Anesthetic machine for small laboratory animals A schematic diagram of the new anesthetic machine for small laboratory animals used in this experiment is shown

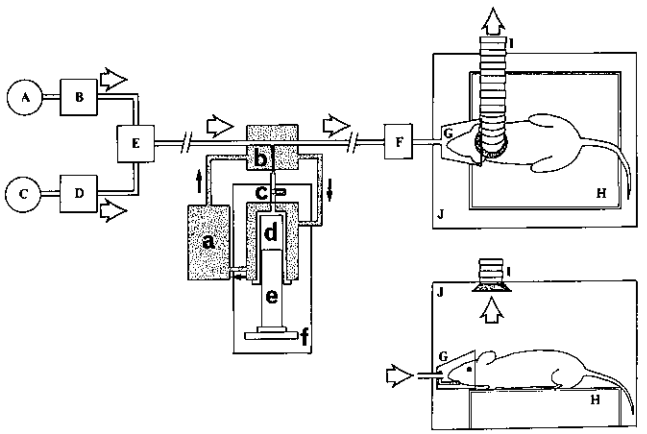


Fig. 1. Inhalation anesthetic system for small laboratory animals. A, air pump; B, flow meter for air; C, hydrogen gas cylinder; D, flow meter for hydrogen gas; E, gas mixer; F, humidifier; G, face mask; H, heated stage; I, suction duct; J, container; a, circulator; b, vaporizer; c, three-way valve; d, volatile anesthetic; e, glass syringe; f, microinfuser. ⇨, carrier gas flow; ➡, circulating water.

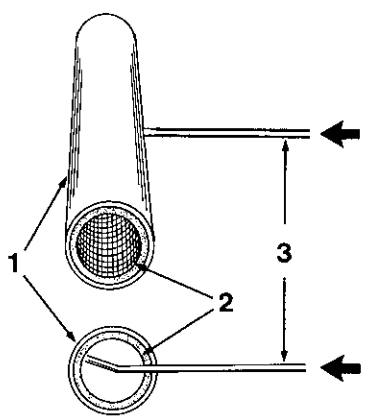


Fig. 2. Structure of vaporizer. 1, stainless tube; 2, metal mesh; 3, syringe needle; ⬅, flow direction of volatile anesthetic.

in Fig. 1. Enflurane (Ethrane; Abbott Laboratories, North Chicago, IL), a volatile liquid anesthetic, was gradually introduced onto a metal mesh inside the vaporizer (Fig. 2) by using a microinfusion pump (compact syringe pump; Harvard Apparatus Co.). The anesthetic evaporated immediately within the vaporizer and was transported by carrier gas to the face mask. The concentration of the anesthetic could be selected easily, by adjusting the ratio between the flow of anesthetic and that of carrier gas. Enflurane was used at a concentration of 1.5% in air (60 liter/min) in all experiments described below. This concentration seemed to have no direct effect on tissue blood flow. However, a concentration of more than 3% enflurane clearly decreased microvasculature reaction to AII. To prevent a decrease in temperature within the vaporizer as a result of the evaporation of anesthetic, the vaporizer was maintained at constant temperature (18 ± 0.5°C) with circulating water. Decrease in body temperature following anesthesia was prevented by placing the animal on a heated stage at 34°C. The experiments were performed in a constant-temperature (24.5 ± 0.3°C) container fitted with a suction duct. Using this device, rats were kept under moderately deep anesthesia for 5–12 h.

Pupil response, analgesia and blood pressure were observed to determine depth of anesthesia. The depth of anesthesia during experiments seemed to be comparable to the first plane of the third stage in human ether anesthesia.[7]

Measurement of local tissue blood flow Tissue blood flow was measured with the hydrogen clearance method developed by Aukland *et al.*,[8] as previously reported in detail.[3] A tissue blood flow meter with 2 separate amplifiers (PHG-201; Unique Medical Co., Tokyo) was used. In the present experiment, 2 hydrogen electrodes (UHE-201C; Unique Medical Co.) 2–4 cm apart and the same number of reference electrodes (UHE-001; Unique Medical Co.) were employed per rat. For measurement of tumor blood flow, a minor incision of skin just sufficient to allow an electrode to be inserted was made using a syringe needle and a hydrogen electrode was inserted into the site. The depth of the inserted electrode did not exceed 10 mm from the tumor surface. For measurement in the subcutis, electrodes were placed in the low back portion by means of a 23-gauge syringe needle. Reference electrodes were placed on a subcutaneous pouch in the caudal region.

Assessment of the tissue blood flow of tumor and subcutis was made by following the clearance of the inert hydrogen gas which had saturated the tissue following the inhalation of 9% hydrogen in air (60 liter/min); after the inhalation was halted, the washout of hydrogen was monitored at intervals of 1 min for 4 min on a recorder (Unicorder UR-3P; Unique Medical Co.). The counts were analyzed by an NEC personal computer program to derive the half-life for hydrogen clearance from the exponential curve. The flow value (in ml/min/100 g tissue) was calculated from the half-life. Tumor areas with blood flow below 10 ml/min/100 g were defined as "low-flow areas." Measurements of tissue blood flow in the present experiments were performed during 12 h between 8 a.m. and 8 p.m.

Change in tissue blood flow under AII-induced hypertension After the clearance curve had been recorded for 4 min under conditions of normotension, AII was infused

intravenously. When the mean blood pressure had increased over 140 mmHg and stabilized, the inflectional curve was again recorded for 4 min. The half-life was calculated and in turn tissue blood flow was calculated.

Fluctuation of tissue blood flow in a given region under normotension Tissue blood flow in a given region under normotension was measured every 1 h for 4 h. The correlation between maximum flow and minimum flow during the experimental period of 4 h was estimated. Furthermore, we examined whether the fluctuations of tissue blood flow in two different microareas were similar or not. When blood flows in two microareas increased or decreased concomitantly, the fluctuation was defined as "parallel." When blood flows in two microareas changed inversely with respect to each other, the fluctuation was defined as "opposite." "Parallel rate" ("opposite rate") was defined as the percentage of parallel (opposite) changes among all samples.

Observation of tumor blood flow in a given region Observation of the tumor blood flow in a given region was performed according to the method described previously.[9-11] The anesthetic machine and heated stage were used under the same conditions as in the case of blood flow measurement. The temperature in the container for observation was held at 24.5±0.3°C during the experimental period. Microscopical findings of tumor vessels were photographed on instant color film (FP-100; Fuji Photo Film Co., Tokyo) or recorded using a closed-circuit video system consisting of a color video camera (OV100; Olympus Kogaku K.K., Tokyo), TV monitor (Trinitron color monitor KX-13HG1; Sony Corporation, Tokyo), and a video cassette recorder (U-matic VO-5800; Sony Corporation). Mean arterial blood pressure was monitored throughout the experiments.

Statistical analysis The significance of differences was analyzed by using Student's *t* test. The criterion of statistical significance was taken as $P<0.05$.

RESULTS

Fluctuation of tissue blood flow An example of fluctuation of tissue blood flow in normal subcutis with time is shown in Fig. 3. Though the experimental environment was held constant and the mean arterial blood pressure was almost constant throughout the experiments, tissue blood flow in the subcutis fluctuated considerably with time. Tissue blood flows of two different microareas 2–4 cm apart fluctuated in parallel in many cases (parallel rate 74.4%, opposite rate 15.7%, n=43).

An example of fluctuation of tumor blood flow in AH109A over 8 h is shown in Fig. 4. Tumor blood flow in two microareas 2–4 cm apart fluctuated considerably even though mean arterial blood pressure was essentially constant. The fluctuations of tumor blood flow were also

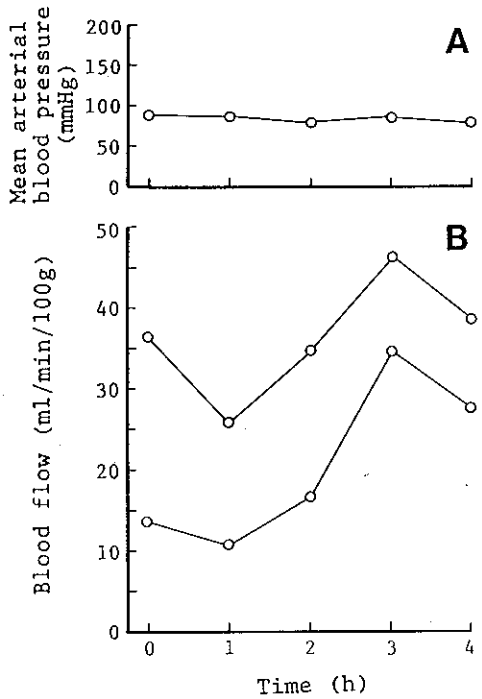


Fig. 3. Fluctuations of tissue blood flow in normal subcutis under conditions of normotension. A, mean arterial blood pressure; B, tissue blood flow. Tissue blood flows in two different microareas were measured simultaneously.

parallel in many cases (parallel rate 64.0%, opposite rate 29.0%, n=100), which was analogous to the case of fluctuation in normal subcutis.

When one of the electrodes was placed in the tumor and the other was placed in the normal subcutis 3–4 cm away from the tumor, fluctuations of blood flow under normotension were opposite in most cases (parallel rate 13.3%, opposite rate 86.7%, n=20), i.e., when tissue blood flow in normal subcutis increased, tumor blood flow decreased and *vice versa* (Fig. 5).

The correlation between maximum flow and minimum flow in a given region during 4 h is shown in Fig. 6. A highly significant correlation was observed between maximum flow and minimum flow in both AH109A ($P<0.001$) and LY80 ($P<0.001$). The slopes of the lines in the two kinds of tumor were almost identical. It was calculated from the regression lines that tumor blood flow fluctuated 1.6-fold in AH109A and 1.7-fold in LY80 under normotension. However, the fluctuation was not periodic as long as observation was performed for a maximum of 12 h. The mean value of maximum flow under normotension was 18.6±15.5 (n=25) in AH109A and 22.0±15.8 ml/min/100 g (n=48) in LY80.

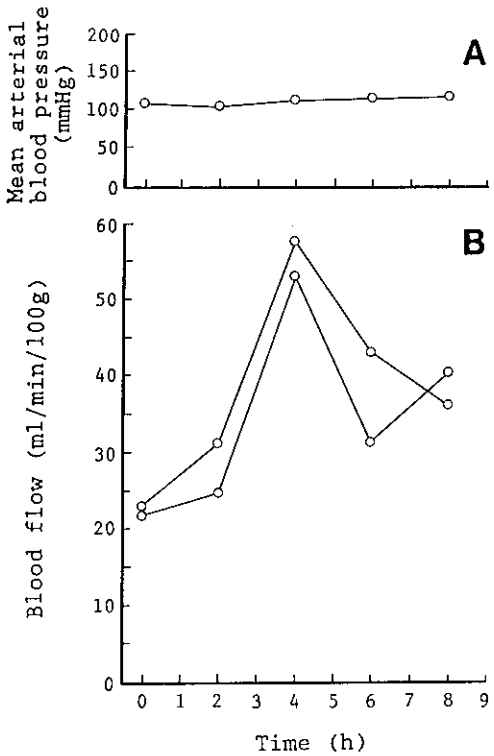


Fig. 4. Fluctuations of tumor blood flow in AH109A under conditions of normotension. A, mean arterial blood pressure; B, tumor blood flow. Tumor blood flows in two different microareas were measured simultaneously.

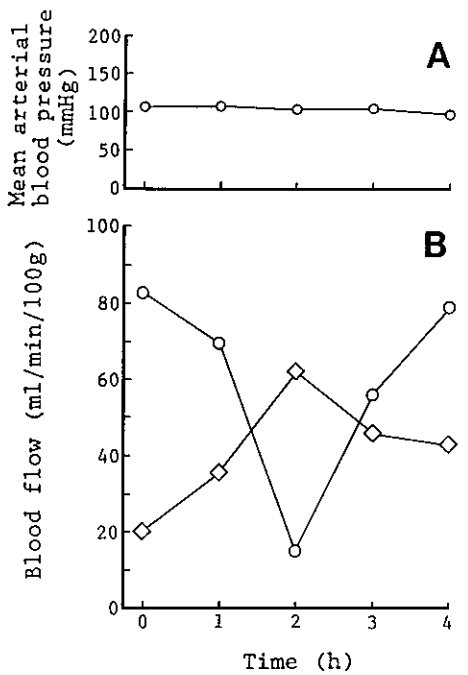


Fig. 5. Fluctuations of tissue blood flow in normal subcutis and tumor (LY80) under conditions of normotension. A, mean arterial blood pressure; B, tissue blood flow in normal subcutis (◇) and tumor (○). Tissue blood flows in two different microareas were measured simultaneously. Fluctuations of blood flow in the two tissues seem to be inversely related.

Appearance of no-flow areas and recovery of tumor blood flow under normotension During the measurement of fluctuations of tumor blood flow, it was observed that one perfused area became a "low-flow area" within a few hours (Fig. 7) and in one "no-flow area"[11] blood flow gradually recovered with time and recirculation was recognized (Fig. 8). By using a transparent chamber technique,[9,10] we observed directly that tumor blood flow changed hourly. Some tumor vessels initially showed a high-flow and later a low-flow or a no-flow state at variable intervals, and *vice versa*. An example of serial observation for 12 h is shown in Fig. 9.

Change in tissue blood flow under AII-induced hypertension Changes in tissue blood flow of normal subcutis and tumors (AH109A, LY80) under AII-induced hypertension are shown in Fig. 10. The results obtained from 30 electrodes in the normal subcutis in 15 rats are summarized in Fig. 10A. When the mean arterial pressure was elevated from 98.8 ± 10.8 to 149.9 ± 6.8 mmHg, the flow values decreased from 16.4 ± 8.1 to 9.7 ± 3.7 ml/min/100 g ($P < 0.001$). A decrease was seen in all rats without exception. The results from 25 electrodes in AH109A in 13 rats are shown in Fig. 10B. When the pressure was elevated from 106.0 ± 6.9 to 158.0 ± 9.8 mmHg, the flow values increased significantly from 12.8 ± 9.4 to 34.0 ± 24.7 ml/min/100 g ($P < 0.001$). The results from 48 electrodes in LY80 in 24 rats are shown in Fig. 10C. With the rise in blood pressure from 96.1 ± 6.2 to 145.6 ± 11.0 mmHg, the flow values increased significantly from 12.4 ± 10.5 to 30.4 ± 16.8 ml/min/100 g ($P < 0.001$). Increase in tumor blood flow under AII-induced hypertension was always observed. The mean flow values increased by AII-induced hypertension were significantly higher than the mean value of maximum flow under normotension (AH109A: $P < 0.025$, LY80: $P < 0.025$). Though tumor blood flow changed hourly under conditions of normotension, an increase in tumor blood flow by AII-induced hypertension was brought about without exception at any stage of fluctuation. An example is shown in Fig. 11.

DISCUSSION

Characterization of tumor blood flow fluctuation It is well known that the microvasculature of various tissues

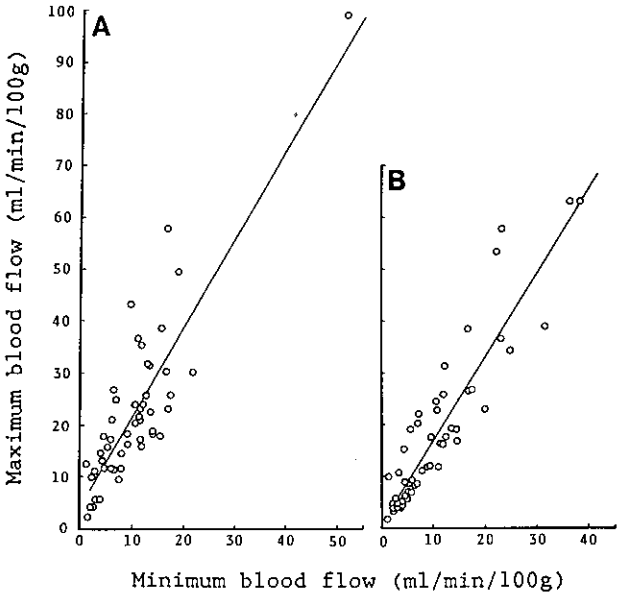


Fig. 6. Correlation between maximum blood flow and minimum blood flow in a given region within a tumor for 4 h. Highly significant correlations between the maximum blood flow and the minimum blood flow were observed in LY80 (A) ($y=1.7x+4.3$; $r=0.86$ ($P<0.001$); n=51) and AH109A (B) ($y=1.6x+1.6$; $r=0.91$ ($P<0.001$); n=51).

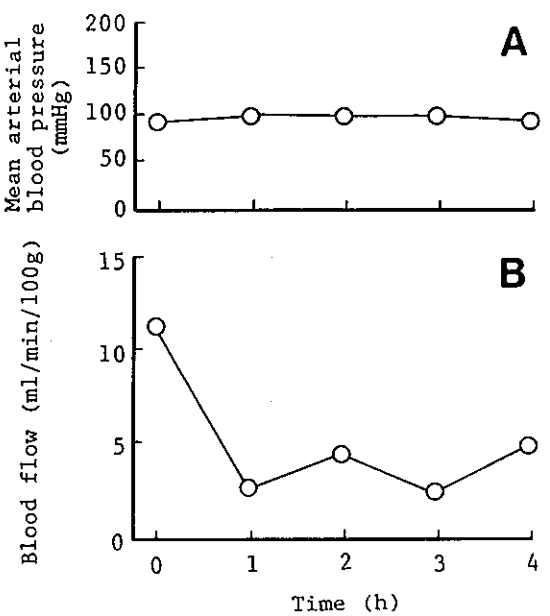


Fig. 7. Transition from the perfused state to low-flow state in LY80 tumor under normotension. A, mean arterial blood pressure; B, tumor blood flow.

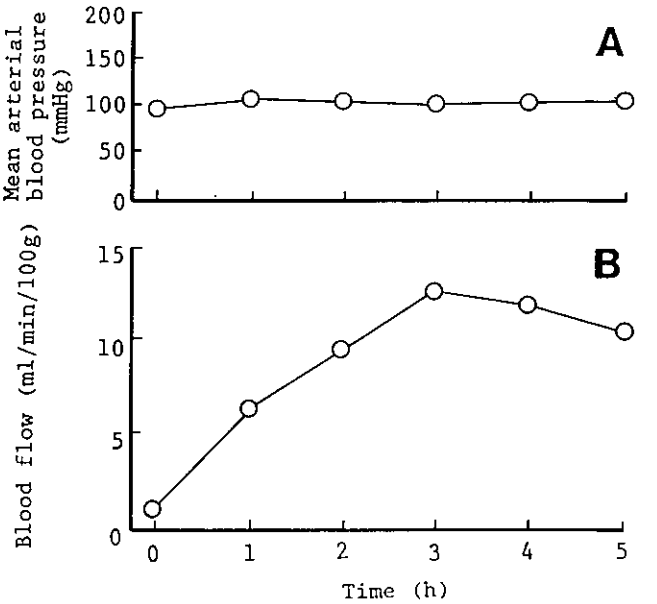
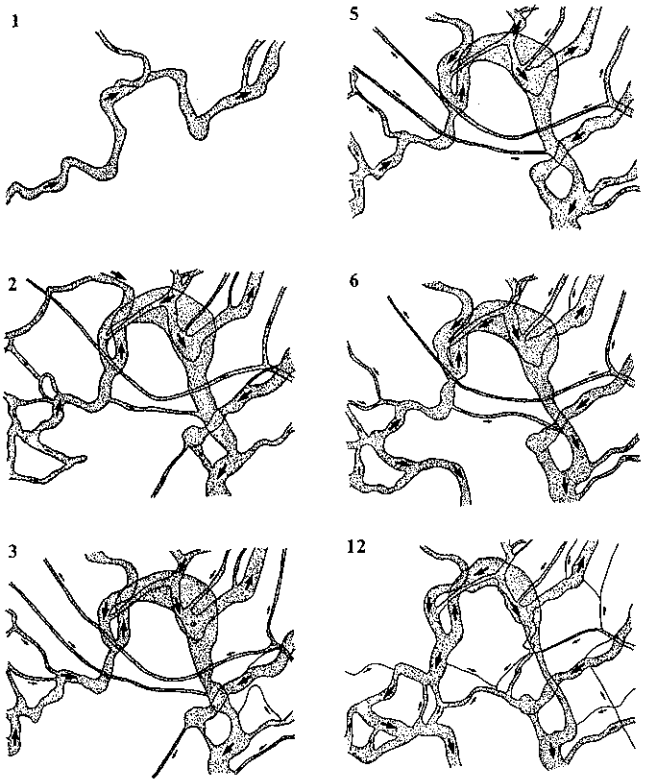


Fig. 8. Recovery of tumor blood flow in no-flow area of LY80 tumor under normotension. A, mean arterial blood pressure; B, tumor blood flow.

is composed of a large number of microvascular modules.[12-14] The structural unit of the vascular bed is likewise its functional unit.[12] Generally, but not always, the modules respond in the same manner to stimuli. Tissue blood flows within two different microareas 2–4 cm apart either in the tumor or in the normal subcutis fluctuated in parallel under normotension in many cases. However, fluctuations of blood flow in tumor and that in normal subcutis were almost always opposite, i.e., when tumor blood flow increased from a lower to a higher level under normotension, tissue blood flow in normal subcutis decreased to a lower level, and *vice versa*. Furthermore, tissue blood flow of the two areas always decreased in normal subcutis and always increased in tumor under AII-induced hypertension. The findings support the idea that the connection mode between the preexisting vascular bed and the tumor vascular bed is a parallel circuit.[15-18] The fact that the nature of fluctuation was almost identical in two different types of tumors suggests that the fluctuation of tumor blood flow is not caused by the tumor vascular bed but rather by the normal vascular bed. We believe that the fluctuation in blood flow through the tumor vascular network is largely determined by the fluctuation of vascular resistance in the normal vascular bed under normotension.

Menke and Vaupel[19] reported that in animals anesthetized with methoxyflurane, tumor blood flow in-



creased slightly with time. Zanelli and Fowler[20] investigated the effects of inhalation anesthetics on the response of the tumor vascular bed to several vasoactive agents and reported that these anesthetics alter the responses in a variety of ways. In our experience, tumor blood flow often changes in various ways when inhalation anesthetics are administered to rats with a simple mask. However, it was found that the changes were caused by instability of the concentration of anesthetics. Accordingly, for measuring long-term fluctuations of tumor blood flow it was essential to use our anesthetic machine, which permits the concentration of anesthetic to be adjusted precisely. Since the anesthetic evaporated gradually at a constant rate during the experiment, there seemed to be little possibility of an acute variation in microcirculation being due to the anesthetic itself. Since the enflurane used in this experiment has no prominent side effect even after long-term inhalation, it was suitable for inhalation anes-

Fig. 9. Microscopic observation of tissue blood flow fluctuation in AH109A tumor under normotension. Functioning vessels were traced each hour. The number to the upper left of each trace indicates the time in hours after the initiation of observation. Arrows: direction of blood flow. There was no tumor blood flow at the first observation (0 h). However, blood flow was seen at 1 h and fluctuated hourly thereafter.

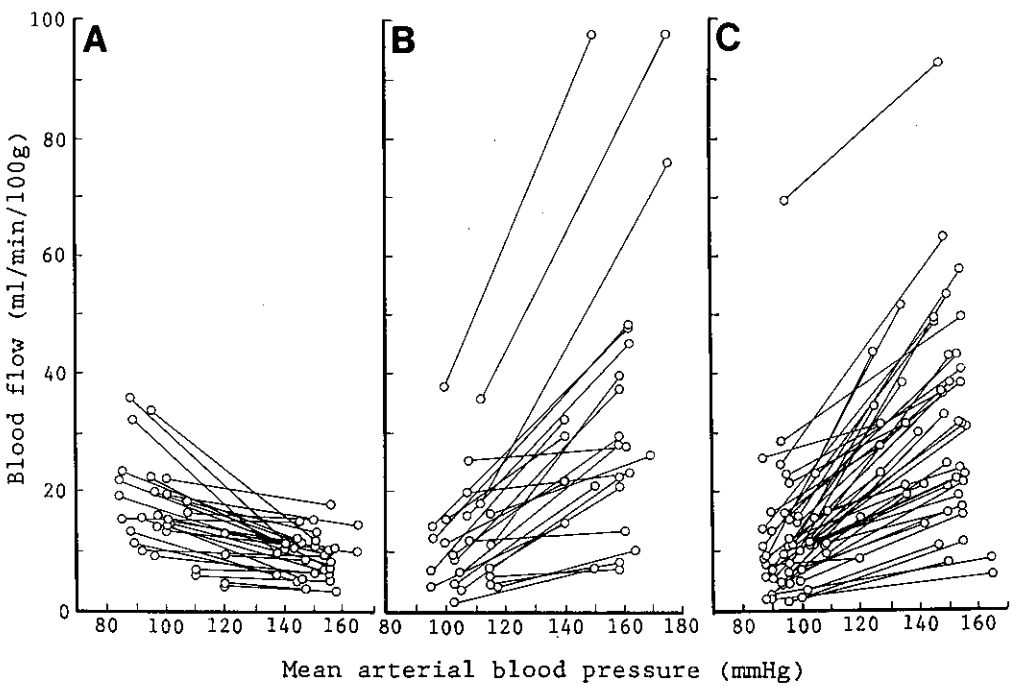


Fig. 10. Change in tissue blood flow by AII-induced hypertension. A, normal subcutis; B, AH109A; C, LY80.

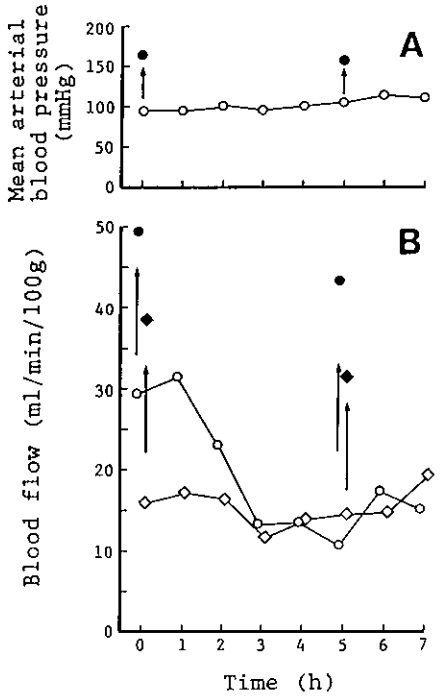


Fig. 11. Increase in tumor blood flow by AII-induced hypertension (LY80). A: mean arterial blood pressure. The blood pressure was elevated by venous infusion of angiotensin II at the first measurement of blood flow (0 h) and the 5th hour following the measurement. B: tumor blood flow. When mean arterial blood pressure was elevated from 94 to 163 mmHg at the first measurement, tumor blood flow in two microareas increased from 28.9 (○) and 16.1 (◇) to 49.5 (●) and 38.5 (◆) ml/min/100 g, respectively. With the rise in blood pressure from 105 to 157 mmHg at the 5th hour following the measurement, tumor blood flow increased from 10.7 (○) and 14.7 (◇) to 43.3 (●) and 31.5 (◆) ml/min/100 g, respectively.

thesia over several hours. When inhalation was stopped, the rats recovered from anesthesia in a short time.

A hydrogen gas clearance technique, based upon Kety's principle,[21] has great practical value because of its simplicity. The base line was maintained even after 8 h. An about 10 min inhalation of 9% hydrogen gas at 1 h intervals within an observation period of 4 h had no apparent effect on blood pressure or respiration. Accordingly, it seems that our machinery allows repeatable quantitative assessment of tissue blood flow in a given region within a tumor to be made at various time points.

Utility of AII-induced hypertension for enhancement of drug delivery In recent years we have reported that low-flow or no-flow areas appear inevitably with the progression of tumor growth.[11] Due to fluctuations of tumor blood flow, such low-flow or no-flow regions move sporadically.[22] Thus, tumor blood flow is very unstable under normotension. In the present study there was a tendency for the development of the no-flow state in low-flow areas. Well-perfused areas very rarely changed to low-flow or no-flow areas. The presence of areas within the tumor where blood flow had temporarily ceased, i.e., no-flow areas, was also reported by Brown[23] and Chaplin *et al.*[24] The existence of such areas, resistant to delivery of anticancer drugs, might be one reason for the poor results of conventional cancer chemotherapy performed under conditions of normotension.

The present results imply that effective drug delivery to all tumor tissue cannot reasonably be expected under conditions of normotension. It is noteworthy that tumor blood flow was increased by AII-induced hypertension at all points of fluctuation in flow, and the mean value of tumor blood flow in AII-induced hypertension was significantly higher than the mean maximum blood flow under normotension. The results suggest that the vascular resistance in the normal vascular bed under AII-induced hypertension might always be greater than that under conditions of normotension.

In conclusion, because of the fluctuation in tumor blood flow under normotension, low-flow areas and no-flow areas which are inaccessible to anticancer drugs appear sporadically. However, good conditions for drug delivery to tumor tissue were induced by AII-induced hypertension, regardless of the spatial and temporal inhomogeneity of tumor blood flow.

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