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Circadian Variation of Tumor Blood Flow in Rat Subcutaneous Tumors and Its Alteration by Angiotensin II-induced Hypertension[1]

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ABSTRACT

Circadian fluctuation in tumor blood flow of the rat subcutaneous tumor was investigated. Tumor tissue blood flows in the daytime zone (10 a.m. to 4 p.m.) and in the nighttime zone (10 p.m. to 4 a.m.) in both the first phase (doubling time of tumor volume = 1.7 days) and the second phase (doubling time of tumor volume = 5.7 days) of growth of the LY80 tumor in rats were measured using the hydrogen gas clearance technique. In the first phase of tumor growth, the tumor blood flow was 20.3 ± 12.2 ml/min/100 g in the daytime zone (n = 22) and 46.6 ± 19.3 ml/min/100 g in the nighttime zone (n = 22). In the second phase, tumor blood flow was 9.6 ± 5.7 ml/min/100 g in the daytime zone (n = 45) and 19.4 ± 8.2 ml/min/100 g in the nighttime zone (n = 38). Tumor blood flow in the nighttime zone was significantly higher than that in the daytime zone (first phase, $P < 0.001$; second phase, $P < 0.001$). However, there were no significant differences in the mean arterial blood pressure, tumor size, and body weight of rats between the daytime zone and the nighttime zone. There was also a marked difference in the effect of angiotensin II-induced hypertension on tumor blood flow between the daytime zone and the nighttime zone. These results suggest that circadian fluctuations in tumor blood flow should be carefully considered when developing strategies to maximize the effectiveness of cancer therapy in relation to the flow rate of circulating blood.

INTRODUCTION

Tumor blood flow plays an important role in various types of cancer treatment. In chemotherapy, tumor blood flow is a major factor determining drug delivery to tumor tissue (1, 2). In radiotherapy, the efficacy of treatment depends upon tumor tissue oxygenation which is governed in part by tumor blood flow (3, 4). In hyperthermia, damage to tumor can be enhanced by decreasing tumor blood flow (5, 6). Recently, the authors measured tumor blood flow in identical regions within a tumor time dependently and reported that tumor blood flow fluctuates with time under conditions of normotension (7). During these experiments, it was noticed that tumor blood flow tended to increase at night. There have been, however, no reports concerning circadian fluctuations in tumor blood flow. It is important to clarify the time zones in which tumor blood flow increases to the greatest value and those in which it decreases to the lowest value. By elucidating circadian fluctuations in tumor blood flow, it should be possible to establish the most effective timing for various types of cancer treatment with regard to the influence of tumor blood flow. This is particularly important in A II[3]-induced hypertension chemotherapy (8–11).

The purpose of this study is to report the new finding that there is a marked difference of the tumor blood flow between daytime and nighttime, and that the elevation of tumor blood flow by A II is also markedly different over the two time periods.

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[3] The abbreviation used is: A II, angiotensin II.

MATERIALS AND METHODS

Rats and Tumor. All experiments were performed on 8- to 9-wk-old male Donryu rats (Rij-Donryu; Nippon Charles-River Co., Ltd., Shizuoka, Japan). Animals were housed in plastic cages in an air-conditioned room. Food and water were available *ad libitum*, and a 12-h light/dark schedule (light onset at 7 a.m.) was maintained. To avoid the effect of diet on tumor blood flow, however, rats were forced to fast for 12 h before measurement. The tumor used was LY80 (established in 1966 by Dr. H. Satoh), a subline of Yoshida sarcoma, which has been maintained in our laboratory by successive i.p. transplantation. Cells (2×10^6) in 0.1 ml were injected s.c. into the back of each rat.

Growth of LY80. Tumor size was measured daily by calipers, and the volume (V) was calculated by the following formula: $V = (\pi/6) \times d_1 \times d_2 \times d_3$, where d_1, d_2, and d_3 were the long axis, short axis, and height of the tumor nodule, respectively. A growth curve of tumor following s.c. transplantation is shown in Fig. 1. The rate of growth decreased from approximately the 14th day following tumor implantation. The tumors were used for measurement of tumor blood flow when they were approximately 5 cm^3 and 20 cm^3 in volume, about the 9th to 13th days (I, first phase of tumor growth) and the 15th to 29th days (II, second phase of tumor growth) after s.c. transplantation, respectively.

Blood Pressure Measurement and Elevation. Mean arterial blood pressure was measured by a catheter (PE-50; Clay Adams Co., Persippany, 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) the output of which was fed into an amplifier (6M82; NEC-Sanei Co., Tokyo, Japan) adapted for measurement of mean arterial blood pressure. Blood pressure was elevated to approximately 150 mm Hg after 1 min of continuous infusion into the tail vein of A II (human angiotensin II; Toa Eiyo, Ltd., Tokyo, Japan) dissolved in 0.9% NaCl solution at a concentration of 2.0 μg/ml. A II (0.02 to 0.06 μg/min) was infused by means of an infusion pump (compact syringe pump; Harvard Apparatus Co., Inc., Millis, MA).

Maintenance of Anesthesia. To maintain anesthesia during measurement of tumor blood flow we used a newly developed anesthetic machine described previously (12). The concentration of anesthetic, enflurane (Ethrane; Abbott Laboratories, North Chicago, IL), was kept constant at 1.5% in air (1 liter/min) as carrier gas. Body temperature was maintained during anesthesia by placing the animal on a heated stage at 34°C. The experiments were performed in a controlled temperature chamber (24.5 ± 0.3°C) fitted with a suction duct.

Measurement of Tissue Blood Flow in Tumor. Tissue blood flow in tumor was measured by the hydrogen gas clearance technique (13). In the experiments, 2 hydrogen electrodes (UHE-201C; Unique Medical Co., Tokyo, Japan) 2 to 4 cm apart and 2 reference electrodes (UHE-001; Unique Medical Co.) were used per rat. The measuring range was within a volume of approximately 1 mm^3 of tissue. In all measurements the depth of the inserted electrode was 5 mm from the surface of the tumor nodule. The site in LY80 tumor was nonnecrotic histologically even in the large tumor. Assessment of tumor tissue blood flows in 2 different microareas was made simultaneously by clearance of the hydrogen gas which had saturated the tissue following the inhalation of 9% hydrogen gas in air (1 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 flow value (ml/min/100 g) was calculated from the half-life for hydrogen clearance from the exponential curve. Tumor blood flows in the daytime zone (10 a.m. to 4 p.m.) and in the nighttime zone (10 p.m. to 4 a.m.) in both the first phase of tumor growth and the second phase were

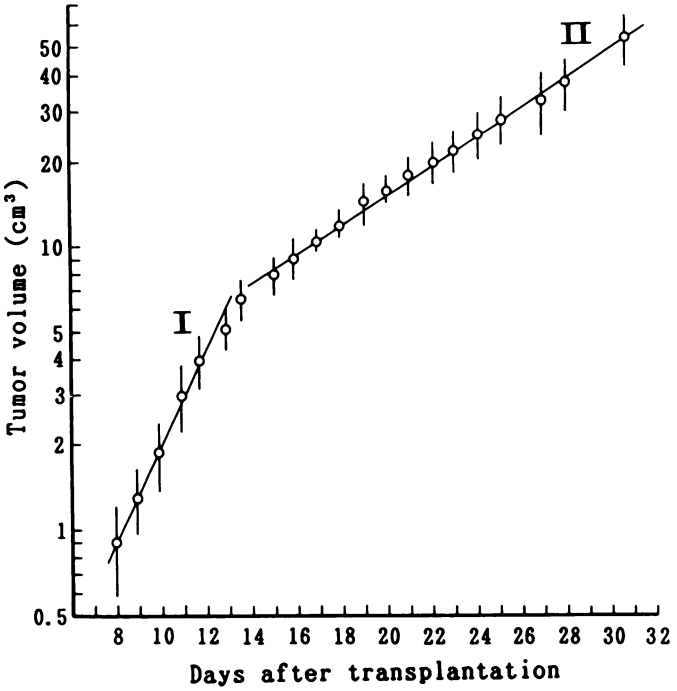

Fig. 1. Growth of tumor after s.c. transplantation of LY80 cells (n = 11). Tumor growth was divided into two stages, the first stage (I) ($Y = 0.1762X - 1.4938$, $r = 0.9975$, $P < 0.001$) and the second stage (II) ($Y = 0.0526X + 0.1160$, $r = 0.9956$, $P < 0.001$). *Bars*, SD.

measured by completely identical regimens. After each experiment, we cut the tumor with a safety razor carefully and checked that the hydrogen electrode was not in a necrotic region.

Measurement of Change in Blood Flow under A II-induced Hypertension. Changes in tumor blood flow accompanying blood pressure changes were analyzed according to the method reported by Suzuki *et al.* (14). Briefly, in the case of a flow level below approximately 30 ml/min/100 g, following the recording of hydrogen clearance under conditions of normotension, blood pressure was elevated to over 140 mm Hg, and the inflectional curve was also recorded for 4 min. The half-life could be computed from each slope of the clearance curve at the times of normotension and hypertension. In the case of a flow level above 30 ml/min/100 g, after the clearance curve under normal pressure was obtained, the rat inhaled hydrogen gas again, and when saturation of hydrogen was achieved, A II was infused continuously. When the blood pressure rose to over 140 mm Hg the inhalation of hydrogen was halted, and the tumor blood flow was measured.

Statistical Analysis. All data were presented as the mean ± SD. Student's t test was used for comparison of body weight, tumor size, mean arterial pressure, and tumor blood flow in 2 different time zones. $P < 0.05$ was considered significant.

RESULTS

Differences of Tumor Blood Flow in 2 Different Time Zones. The mean doubling time of tumor volume in the first phase of tumor growth was 1.7 days. Tumor blood flows from individual tumors under conditions of normotension in the daytime zone and the nighttime zone in the first phase are compared in Fig. 2. Tumor blood flow was 20.3 ± 12.2 ml/min/100 g in the daytime zone (I d) (blood pressure, 97.8 ± 7.7 mm Hg; tumor size, 4.9 ± 1.9 cm³; body weight, 319.4 ± 18.3 g; animal number, 11; sample number, 22) and 46.6 ± 19.3 ml/min/100 g in the nighttime zone (I n) (blood pressure, 102.0 ± 6.7 mm Hg; tumor size, 4.8 ± 1.4 cm³; body weight, 320.7 ± 14.9 g; animal number, 11; sample number, 22). There were no significant differences in blood pressure, tumor size, and body weight among rats measured in the daytime zone and those measured in the nighttime zone. However, tumor blood flow was significantly higher in the nighttime zone than in the daytime zone ($P < 0.001$).

The doubling time of tumor volume in the second phase of tumor growth was 5.7 days. Tumor blood flow under conditions of normotension in the daytime zone and the nighttime zone in the second phase is shown in Fig. 3. Tumor blood flow was 9.6 ± 5.7 ml/min/100 g in the daytime zone (II d) (blood pressure, 100.8 ± 10.4 mm Hg; tumor size, 21.3 ± 7.9 cm³; body weight, 360.2 ± 53.8 g; animal number, 23; sample number, 45) and 19.4 ± 8.2 ml/min/100 g in the nighttime zone (II n) (blood pressure, 100.7 ± 7.5 mm Hg; tumor size, 18.7 ± 4.1 cm³; body weight, 353.3 ± 37.1 g; animal number, 20; sample number, 38). There were also no significant differences in blood pressure, tumor size, and body weight among rats in the daytime zone and those measured in the nighttime zone. However, tumor blood flow was also significantly higher in the nighttime zone than in the daytime zone ($P < 0.001$). Furthermore, tumor blood flow in the first phase of tumor

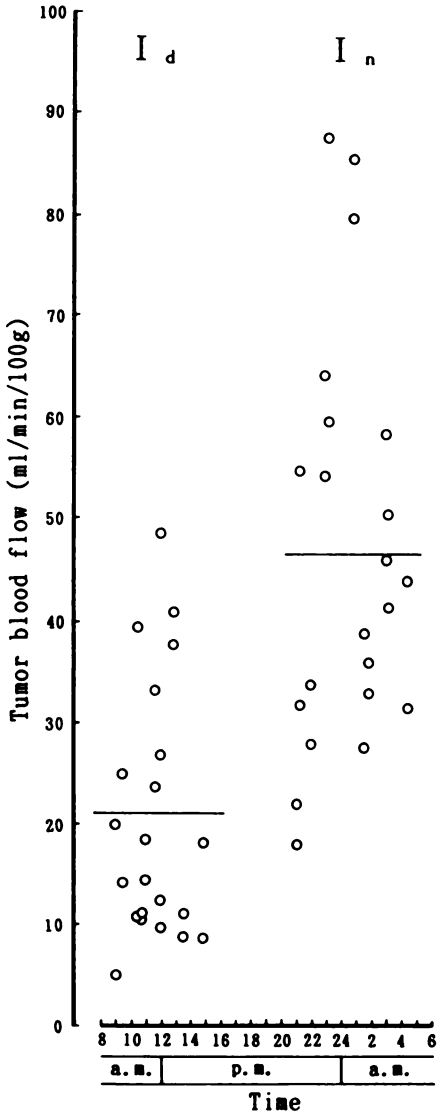

Fig. 2. Tumor blood flow in the daytime zone (I d) and in the nighttime zone (I n) of the first phase. Tumor blood flow was 20.3 ± 12.2 ml/min/100 g in the daytime and 46.6 ± 19.3 ml/min/100 g in the nighttime. Blood flow was significantly higher in the nighttime than in the daytime ($P < 0.001$).

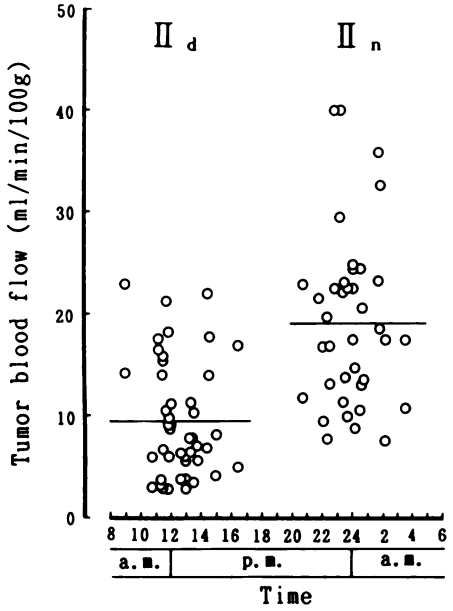

Fig. 3. Tumor blood flow in the daytime zone (II *d*) and in the nighttime zone (II *n*) of the second phase. Tumor blood flow was 9.6 ± 5.7 ml/min/100 g in the daytime and 19.4 ± 8.2 ml/min/100 g in the nighttime. Blood flow was significantly higher in the nighttime than in the daytime ($P < 0.001$).

growth was significantly higher than that in the second phase (I *d versus* II *d*, $P < 0.001$; I *n versus* II *n*, $P < 0.001$). Many low-flow areas (areas with blood flow of under 10 ml/min/100 g) (7) were measured in the daytime zone of the second phase of tumor growth. However, there were very few such areas in the nighttime zone.

Differences of Increased Values of Tumor Blood Flow by A II-induced Hypertension in 2 Different Time Zones. The increase in tumor blood flow caused by A II in 2 different time zones during the first phase of tumor growth is shown in Fig. 4. In the daytime zone, when mean arterial blood pressure was elevated from 97.8 ± 7.7 to 158.0 ± 5.7 mm Hg, the flow values increased significantly from 20.3 ± 12.1 to 62.3 ± 41.1 ml/min/100 g ($n = 22$, $P < 0.001$). In the nighttime zone, with the rise in blood pressure from 102.0 ± 6.7 to 160.0 ± 3.6 mm Hg, the flow increased significantly from 46.6 ± 19.3 to 103.4 ± 36.3 ml/min/100 g ($n = 22$, $P < 0.001$).

The increase in tumor blood flow by A II in 2 different time zones during the second phase of tumor growth is shown in Fig. 5. In the late second phase (end stage), especially in the daytime zone, it was not easy to elevate blood pressure to around 150 mm Hg. Samples when mean arterial blood pressure did not increase to 140 mm Hg were omitted from the analysis (daytime zone, 21 of 45 samples; nighttime zone, 11 of 38 samples). In the daytime zone, when mean arterial blood pressure was elevated from 104.3 ± 8.2 to 148.5 ± 8.0 mm Hg, the flow increased significantly from 10.9 ± 6.7 to 22.5 ± 11.6 ml/min/100 g ($n = 24$, $P < 0.001$). In the nighttime zone, with the rise in blood pressure from 103.3 ± 7.2 to 149.1 ± 7.6 mm Hg, the flow increased significantly from 21.6 ± 9.1 to 51.2 ± 28.0 ml/min/100 g ($n = 27$, $P < 0.001$). The increases in blood flow resulting from A II-induced hypertension were significantly greater in the nighttime zone than in the daytime zone (first phase, $P < 0.01$; second phase, $P < 0.001$).

DISCUSSION

The present series of experiments demonstrated that there are circadian fluctuations in tumor blood flow of rat subcutaneous tumors and that the tumor blood flow reaches its greatest value at night.

To analyze circadian fluctuations in tumor blood flow it is necessary to minimize the influence of factors which might obscure accurate measurement. Since tumor blood flow is very susceptible to the presence of tumor necrosis (15, 16), efforts were made to eliminate the influence of necrosis. In LY80 tumors, necrotic areas were hardly ever seen at a depth of 5 mm from the surface of the tumor nodule, even in the end stage of tumor-bearing rats. This was the reason why we chose LY80 tumor from among the various available experimental tumor models. The hydrogen clearance method adopted measured tumor tissue blood flow. Since tissue blood flow is the blood flow of only a very limited area (16), the measured values are not greatly affected by the presence of central necroses. The conditions of measurements in the daytime were matched as much as possible with those at night. Tumor blood flow at

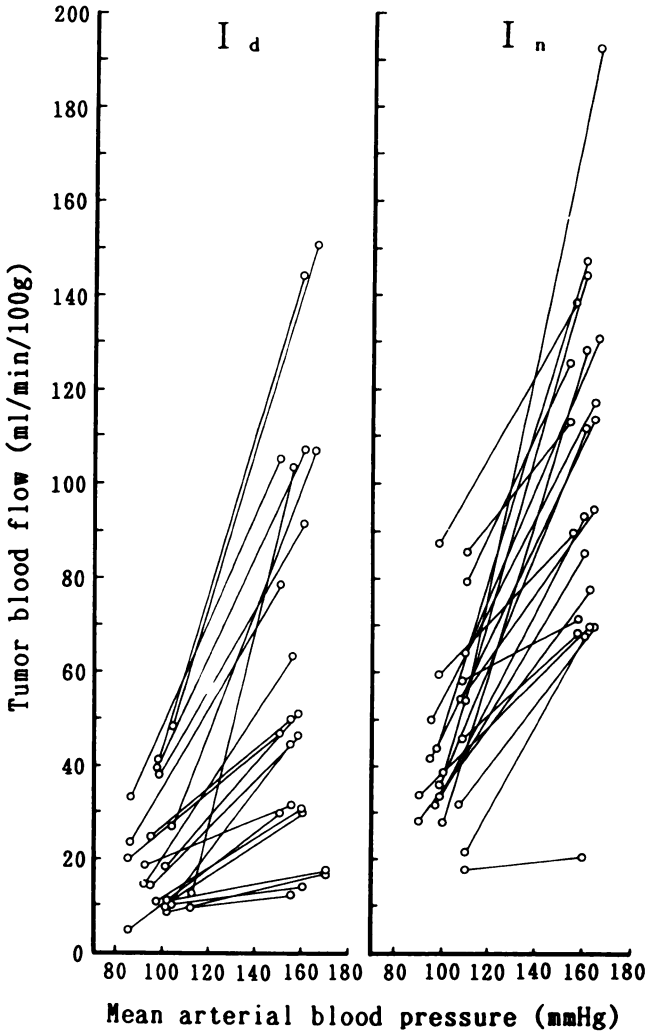

Fig. 4. Tumor blood flow increased by A II-induced hypertension in the daytime zone (I *d*) and in the nighttime zone (I *n*) of the first phase. In the daytime, when mean arterial blood pressure was elevated from 97.8 ± 7.7 to 158.0 ± 5.7 mm Hg, the blood flow increased from 20.3 ± 12.1 to 62.3 ± 41.1 ml/min/100 g ($n = 22$, $P < 0.001$). In the nighttime, with the rise in blood pressure from 102.0 ± 6.7 to 160.0 ± 3.6 mm Hg, the flow increased from 46.6 ± 19.3 to 103.4 ± 36.3 ml/min/100 g ($n = 22$, $P < 0.001$).

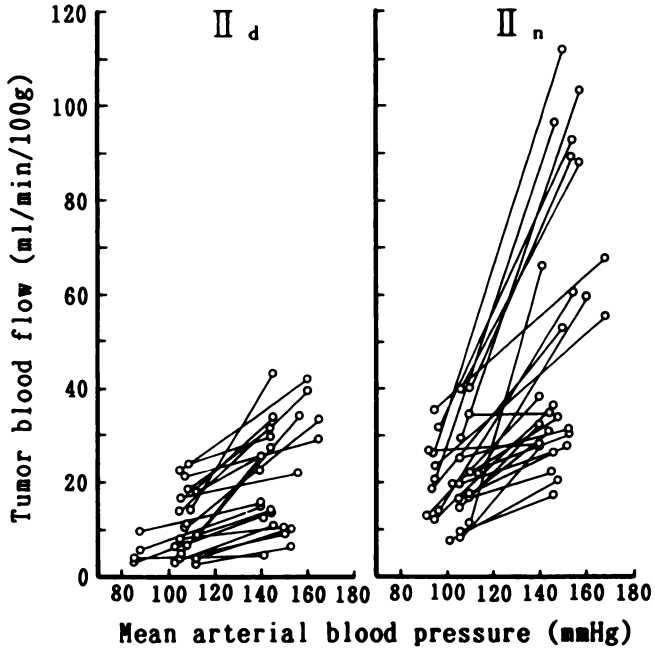

Fig. 5. Tumor blood flow increased by A II-induced hypertension in the daytime zone (II *d*) and in the nighttime zone (II *n*) of the second phase. In the daytime, when mean arterial blood pressure was elevated from 104.3 ± 8.2 to 148.5 ± 8.0 mm Hg, the blood flow increased from 10.9 ± 6.7 to 22.5 ± 11.6 ml/min/100 g ($n = 24$, $P < 0.001$). In the nighttime, with the rise in blood pressure from 103.3 ± 7.2 to 149.1 ± 7.6 mm Hg, the flow increased from 21.6 ± 9.1 to 51.2 ± 28.0 ml/min/100 g ($n = 27$, $P < 0.001$).

night was significantly higher than that in the daytime under the above measurement conditions.

Tumor blood flow is known to be quite heterogeneous at different locations even within a single tumor (1, 17–19). Furthermore, in recent years, time-dependent fluctuations of tumor blood flow have been reported (7, 20–22). We reported that tumor blood flow in LY80 shows variation of approximately 1.7 times during a 4-h period in the daytime (7). However, this does not mean that tumor blood flow in all areas increased by 1.7 times simultaneously. Menke and Vaupel (22) reported that the mean tumor blood flow in a rat subcutaneous tumor was almost constant during an observation period of 80 min, although in individual sites the flow rates can vary with time. This is probably due to changes of distribution of tissue blood flow within the tumor.

On the other hand, in the present study, the mean tumor blood flow in the nighttime was approximately twice as much as that in the daytime. This seemed to be due to an increase of inflow to the tumor because low-flow areas (7) which were prominent in the daytime of the second phase of tumor growth were seldom observed in the nighttime. Moreover, high-flow areas, such as 50 to 90 ml/min/100 g, observed during the nighttime of the first phase were not observed in the daytime. Hirst *et al.* (23) measured the relative perfusion of tumors at 4 time points throughout the day and suggested that relative perfusion might be changing at different times.

From these results, we concluded that there are circadian fluctuations in tumor blood flow. Furthermore, the tumor blood flow increase produced by A II-induced hypertension at night also exceeded that in the daytime. At least, in the present conditions, there was no doubt that A II-induced hypertension produced in the daytime could not make tumor blood flow maximum. However, it was not clear from only the present results why tumor tissue blood flow had circadian fluctuations and why the response of tumor blood flow to A II changed with the administration time of the day. Though the autonomic nervous system-mediated vascular reaction and/or circadian rhythms of enzyme activities might take part in these results, speculation about the mechanisms involved in these effects is not included in the present report.

In recent years it has been reported that the therapeutic index of anticancer drugs is affected by the time of day when treatment is given (24–26). The present results give rise to speculation that the time of administration would influence the efficacy of anticancer drugs through circadian fluctuations in tumor blood flow. From the size, clinically ordinary solid tumors seem to reach the second phase of growth described above. We would like to emphasize the importance of the analysis of circadian fluctuation in tumor blood flow at the clinical level. These analyses can pave the way for such cancer treatments depending on tumor blood flow as A II-induced hypertension chemotherapy (8–11), if human cancers have circadian fluctuation in blood flow.

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