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Increased Intratumor Concentration of Fluorescein-isothiocyanate-labeled Neocarzinostatin in Rats under Angiotensin-induced Hypertension

Ikuo ABE, Katsuyoshi HORI, Sachiko SAITO, Shigeru TANDA, Yulin LI* and Maroh SUZUKI
Department of Experimental Oncology, Research Institute for Tuberculosis and Cancer, Tohoku University, 4-1 Seiryo-machi, Sendai 980

On the basis of the observation that the tumor tissue blood flow selectively increases under angiotensin (AT)-induced hypertension, the change of the drug concentration in the tumor and normal tissues was examined in male Donryu rats. The intratumor concentration of fluorescein isothiocyanate-labeled neocarzinostatin was about 2-fold higher in the AT-induced hypertension group than in the control up to 20 min after the drug injection. In the normal organs or the uninvolved organs of the tumor-bearing rats, however, no clear increase was seen in the experimental group compared with the control, as anticipated from the observation of the tissue blood flow. The present study supports the hypothesis that the enhanced anticancer effect in chemotherapy under AT-induced hypertension formerly reported is due to the tumor-selective enhancement of the drug delivery.

Key words: Drug concentration — FITC-NCS — Angiotensin-II — Tumor blood flow — Rat ascites hepatoma AH109A

Drug delivery to the tumors is one of the major factors affecting the response to the drug.[1-3] In fact, the drug delivery system is considered to be defective in tumors since the tumor blood flow is known, in general, to be very poor.[4-7] Recently, Suzuki *et al.*[6-8] found that the tissue blood flow increased several-fold selectively in the tumor under angiotensin (AT)-induced hypertension, without increase in any of the normal tissues examined. With the increase in the tumor blood flow, an increase in the tumor vascular area has been seen using a transparent chamber.[9] By utilizing this phenomenon, the effect of anticancer drugs was markedly enhanced in an experimental animal model.[6,7] The chemotherapeutic advantage of utilization of this finding was confirmed in clinical cancer therapy as well.[10-13] In this connection, it is essential to know the relation between the anticancer effect and the drug delivery to the tumor. Suzuki *et al.*[14] showed that the distribution of such fluorescence-emitting substances as daunomycin-HCl and fluorescein isothiocyanate (FITC)-dextran was enhanced under AT-induced hypertension in micro-tumors spreading in a transparent chamber on the back subcutis of rats. In the present paper, the effect of AT-induced hypertension on the delivery of FITC-labeled neocarzinostatin (FITC-NCS) to a solid tumor is reported.

MATERIALS AND METHODS

Drug FITC-NCS was kindly supplied by Kayaku Co., Tokyo. FITC-NCS (MW; 10,700) contained 1.7 mol of FITC per mole of NCS, and 1 mg of FITC-NCS was equivalent to 0.45 mg of NCS in terms of bactericidal activity (Dr. A. Ito, personal communication). A small amount of impurity (less than 5%) detected electrophoretically was considered to be negligible in the drug distribution experiment but was removed before electrophoresis, where indicated, as follows. FITC-NCS (0.3 mg eq./ml) dissolved in Ca^{2+}·Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS, pH 7.4) was passed through a Sep-Pak C18 cartridge minicolumn (Waters Assoc., MA) and recovered with 2.5 ml of 60% ethanol in PBS (found to be the best condition for recovery in the preliminary experiment) after washing the column with 4 ml of PBS. The eluate was made 90% in terms of ethanol concentration, stored at −80° for 2 hr and centrifuged at 10,000*g*, 4° for 20 min to precipitate FITC-NCS. FITC-NCS was stable after treatment with 90% ethanol when its stability was checked

* Present address: Pathology Department, Norman Bethune University of Medical Sciences, Changchun, China.

fluorometrically. The precipitate was dissolved again in a small amount of PBS. Purified FITC-NCS thus obtained formed a single band in electrophoresis and the recovery of fluorescence was about 90%.

Drug Distribution Rat ascites hepatoma, AH109A (10^6 cells) was inoculated in the back subcutis of male Donryu rats (Nihon Rat Co., Urawa, Saitama). On day 9 after inoculation, the rats were divided into two groups; control and AT-induced hypertension groups. Both groups of rats were treated with pentobarbital sodium (Nembutal, 30 mg/kg) subcutaneously about 20 min before FITC-NCS injection. FITC-NCS (1 mg eq./2 ml/kg) was injected slowly over 40 sec at time 0 through the tail vein. Two rats of the hypertension group were treated at the same time; one rat was monitored for mean blood pressure through the femoral artery,[7] while the other was not. When the mean pressure reached around 145 mmHg in the monitored rat during continuous intravenous infusion of AT, the drug was administered to both rats. Hypertension was maintained for 10 min from time 0. The animals were sacrificed at the time points designated. Immediately after decapitation, the running blood was collected for plasma samples. The tumor and normal organs were excised, weighed, and homogenized in PBS. The homogenates and plasma were mixed with 1.5 volumes of cold ethanol (60% final), stored at $-20°$ for about 30 min and centrifuged at 3,000 rpm for 10 min. FITC-NCS in the supernatant was quantified fluorometrically (excitation, 490 nm; emission, 520 nm). Recovery of FITC-NCS from the tissue homogenates was more than 95% by the above extraction procedure. It was confirmed in advance that the drug distribution was not different in the rats monitored for mean blood pressure and their counterparts. PBS could not be replaced with either saline or phosphate buffer alone for full strength of fluorescence emission.

Electrophoresis on Cellulose Acetate Membrane and Gel Chromatography Electrophoresis on cellulose acetate membrane was performed for detection of degradation or metabolism of the drug by the standard method.[15] Sixty percent ethanol extract was dialyzed against 40% polyethylene glycol 6,000 overnight at 0° to concentrate the extract and to remove ethanol, where indicated. The concentrated extract was recovered with PBS and made 90% in terms of ethanol concentration, stored at $-80°$ for 2 hr, and centrifuged. The precipitate was dissolved again as described above and subjected to electrophoresis after sedimentation of insoluble materials. Samples (2 μl unless otherwise stated) were applied on cellulose acetate membrane and electrophoresed in 0.06*M* barbital buffer, pH 8.6. Applied current was 0.8 mA/cm width of the cellulose membrane. Dialysis was omitted in the case of the plasma and kidney since the FITC-NCS concentration was very high in these tissues. Gel chromatography was performed according to Maeda *et al.*[16] as described in the legend to Fig. 2 to detect the possible production of low-molecular-weight metabolites, which may not be precipitated with cold ethanol.

Results and Discussion

An electrophoregram (Fig. 1) of the extracts of the tissues sampled 20 min post-injection showed a single major band in each case, coincident with purified FITC-NCS. In the unextracted plasma sample, a weakly fluo-

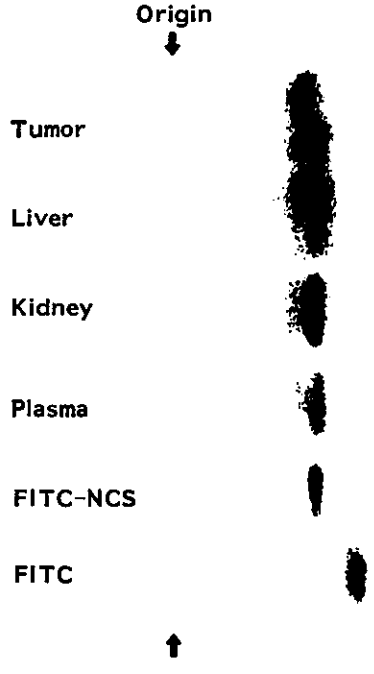

Fig. 1. Electrophoresis of the extracts of the plasma, tumor, liver, and kidney of the animals injected with FITC-NCS. The ethanol extracts (60% final) were prepared from the tissues of the rats 20 min after FITC-NCS injection, dialyzed against 40% polyethylene glycol and precipitated with ethanol as described in the text. Two microliters of extracts were applied to the cellulose acetate membrane. Electrophoresis on a cellulose acetate membrane was performed at pH 8.6 for 50 min at 0.8 mA/cm.[15] Anode, left side; cathode, right side. FITC-NCS levels in the tumor and liver were so low that 10-μl samples were applied. The lower two lanes show authentic FITC-NCS and FITC.

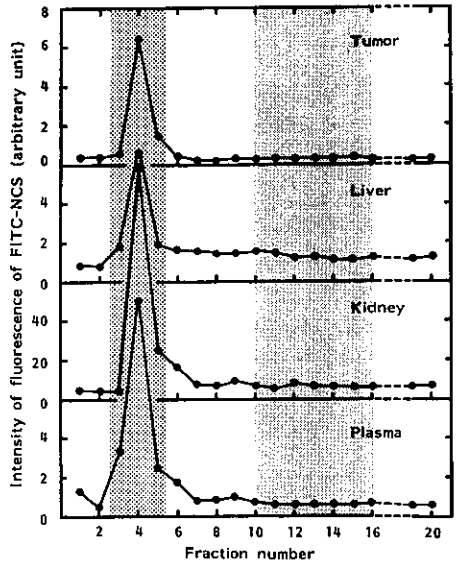

Fig. 2. Gel chromatography of the extracts of the tissues from the rats given FITC-NCS. Sixty percent ethanol extracts prepared as described in the text were placed on evaporating dishes at −20° overnight and/or diluted with PBS to lower the ethanol concentration to less than 15%, applied to Sephadex-G25 columns and eluted with PBS according to Maeda *et al.*[16] Almost 100% of FITC-NCS-derived fluorescence was recovered in the whole eluate. ▨, FITC-NCS; ▩, mitomycin C (MW; 334) in place of FITC (MW; 389), which could not be used because of its adsorption on Sephadex G-25.

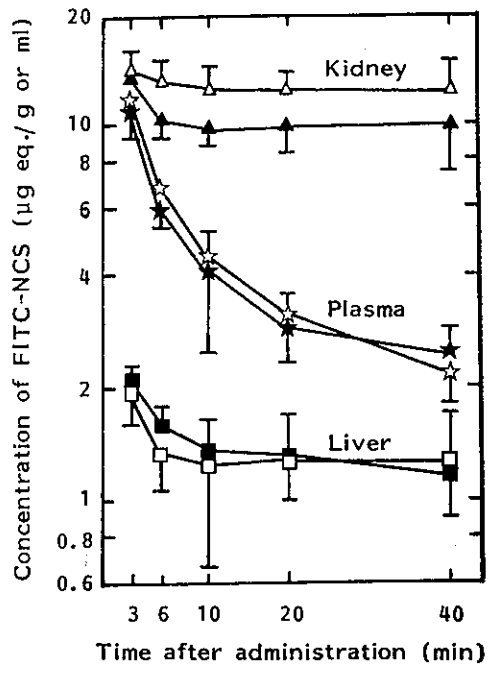

Fig. 3. Effect of AT-induced hypertension on the concentration of FITC-NCS in the tissues of the normal rats. After injection of FITC-NCS (1 mg eq./kg), the rats were sacrificed and the tissues were sampled as described in the text. Closed symbols show the control and the open symbols the AT-induced hypertension groups. Each point and bar show the mean and standard deviation of values from 4 animals.

rescent band was also detected in the location of albumin (not shown), suggesting that at least a portion of the drug was albumin-bound. Minor bands, though not discernible in this electrophoregram, were detected before and after the main bands in the dialyzed extracts of all the tissues tested; they were estimated to show less than 10% of the fluorescence intensity of the major band. These minor bands were not observed in the undialyzed extracts of the plasma or kidney or purified FITC-NCS (not shown). Thus, the degradation of FITC-NCS might have occurred in the dialysis step as an artifact. Degradation of the drug was not detected by gel chromatography with fluorescence monitoring (Fig. 2) in contrast to the finding with [^{14}C]succinyl NCS.[16] Thus, the fate of FITC-NCS might not necessarily be the same as that of NCS, the mother compound. The same seems to be true for immunological identification of NCS.[17] FITC-NCS, however, may still be useful, within limits, for estimation of the role of the tumor-selective increased blood flow under AT-induced hypertension in the delivery to and the effect on the tumor of anticancer drugs.

Decrease in the plasma concentration was rapid, in accordance with the earlier reports.[16, 18] The drug concentration was at similar levels in the control and AT-induced hypertension groups in the normal organs (Fig. 3) or in the uninvolved organs of the tumor-bearing rats (Table I) other than the kidney, which tended to give slightly higher values, though statistically insignificant, in the experimental group. In the case of daunomycin-HCl, the concentration rather tended to de-

Table I. Effect of AT-induced Hypertension on the Concentration of FITC-NCS in the Tissues of the Tumor-bearing Rats 20 min after Injection[a]

Tissues	Control	AT-induced hypertension
	[μg/ml or g (mean±SD of 4 animals)]	
Plasma[b]	3.20±0.92	3.61±0.94
Heart	0.46±0.15	0.54±0.11
Lung	0.79±0.20	0.99±0.17
Liver	1.29±0.28	1.26±0.60
Kidney[b]	9.86±1.25	12.79±4.58
Spleen	0.75±0.25	0.69±0.05
Tumor	0.31±0.14	0.53±0.08[c]

a) Dose: 1 mg eq./2 ml/kg, intravenously.
b) Twelve animals. *c*) $P<0.01$.

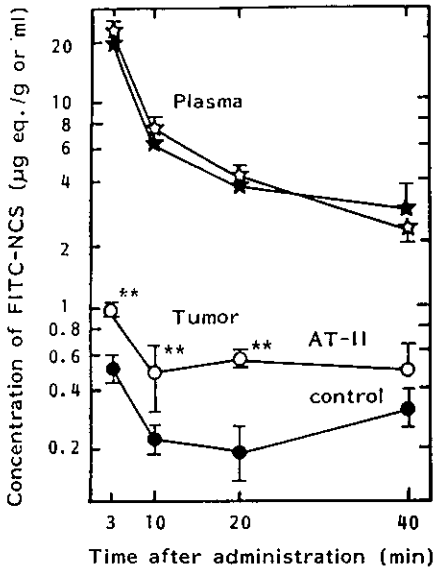

Fig. 4. Effect of AT-induced hypertension on the concentration of FITC-NCS in the plasma and tumor. Change of the concentration of FITC-NCS in the plasma (asterisks) and tumor tissue (circles) in the control (closed symbols) and AT-induced hypertension (open symbols) groups. The tumor concentrations at 3, 10, and 20 min after injection showed statistically significant differences at $P<0.01$ (**) between the two groups. Each value is the mean with standard deviation of 4 animals.

crease in the kidney.[19] That the renal tissue blood flow decreases to half of the control level under AT-induced hypertension[8] might be relevant to these findings in the kidney. Although the precise mechanism of the different effect on the kinetics of drug distribution between FITC-NCS and daunomycin-HCl in the kidney is uncertain, it might be related to whether the decreased renal blood flow brings about decreased excretion or reabsorption of the drugs in the kidney under AT-induced hypertension. In any case, hypertension-induced change in the drug concentration was considered to be negligible in the normal organs.

The effect of AT-induced hypertension on the drug distribution is obvious in the tumor (Fig. 4). FITC-NCS level in the experimental group was roughly twice that in the control up to 20 min.

Thus, the concentration of FITC-NCS increased only in the tumor under AT-induced hypertension, reflecting the fact that the tissue blood flow selectively increased in the tumor under the same conditions.[6-8] The concentration of daunomycin-HCl was also increased selectively in the tumor under AT-induced hypertension.[19] Very recently, Okamoto *et al.*[20] reported a tumor-selective increase in 5-fluorouracil and doxorubicin in sarcoma 180-bearing mice. Sato *et al.*[13] observed a tumor-selective increase in the accumulation of contrast medium in the dynamic computed tomography of metastatic liver cancer under AT-induced hypertension. Tumor-selective increase in the drug concentration should be fundamental to the improved anticancer effect of the drugs, since a non-selective increase should result in an adverse effect on the host. The present results support the hypothesis that AT-induced hypertension improved the chemotherapeutic effect on cancer observed experimentally[6,7] and clinically[10-13] through a tumor-selective increase in the tissue blood flow.

The FITC-NCS level in the tumor was increased about 2-fold in the experimental group, as in the case of daunomycin-HCl in large solid tumors[19] and in microtumors.[14] Wakui and Sato[12] reported 1.5 to 3-fold increased accumulation of [^{3}H]5-fluorouracil and *cis*-diaminedichloroplatinum in rat ascites hepatoma growing in the subcutis. Our preliminary observation revealed that enhancement of the distribution of mitomycin C in the tumor was about 2-fold or less compared to the control value under the present experimental conditions. Since the increase in

the tumor tissue blood flow has been reported to be 5-fold or so,[6] a greater increment of the drug concentration might have been expected. So far, the reason for this discrepancy is not clear. The fact that the tissue blood flow contributes to both influx and efflux of drugs to and from the tissues may be relevant. Further investigation is needed to clarify the relation between the tissue blood flow and the drug distribution, and its significance in cancer chemotherapy.

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