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Lymphotropic Accumulation of an Antitumor Antibiotic Protein, Neocarzinostatin*

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Abstract—In vivo *distribution of the antitumor antibiotic neocarzinostatin (NCS) at about the clinical dose level, based on bioactivity, was studied in rats, in the presence of proteolytic inhibitors preventing degradation of NCS during the bioassay. The activity of NCS was found to accumulate to a much larger extent in the lymph nodes, especially popliteal, axillary and lumbar lymph nodes, than in other organs except the bladder, after intravenous (i.v.) injection. In addition, accumulation in regional lymph nodes was found to be very high after subcutaneous (s.c.) injection. Bioactivity of NCS was detected also in other organs and tissues such as the kidney, bone marrow, blood, lung, small intestine, liver and spleen, but it was lower than expected. In the heart, stomach, large intestine, muscle and prostate, activity of NCS was not detected. Excretion of NCS in urine* 10 min *after an i.v. injection was about* 80 *times more than that after an s.c. injection. Urinary recovery of NCS was almost completed in* 90 min, *which yielded* 44 *and* 12% *of the total i.v. and s.c. dosage, respectively. The present findings of the highly lymphotropic nature of NCS recommends its application for control of the lymphatic metastasis in man.*

INTRODUCTION

NEOCARZINOSTATIN (NCS) is the first proteinaceous antitumor antibiotic (mol. wt about 10,700) used clinically [1–5], and its primary structure is determined [6]. The action of NCS at the molecular level has been elucidated; it causes strand scission of DNA in bacterial and animal cells [7–9]. Later NCS was shown to cause a sequence specific cleavage at thymidine residues, leaving both of the cleaved ends (3′ and 5′) phosphorylated [10–12].

Previously, [^{14}C] succinyl-NCS was found to accumulate predominantly in the bladder when administered i.v. [13], and this result led to the systemic administration of NCS for successful treatment of human bladder cancer [14, 15]. The distribution of biological activity of NCS after i.v. administration was reported by Fujita *et al.* [16], but the dose of NCS was extremely high (100 times of LD_{50}), exceeding the level of clinical practice [14, 15]. In addition, no attention was paid to the proteolytic breakdown of the drug during the experiments, and indeed this was found to be one of the major inactivation pathways [17, 18].

In the present experiments, the designated dose of NCS was close to clinical level. Furthermore protease inhibitors were added to the samples to prevent the proteolytic breakdown of NCS during the preparation of assay samples.

In cancer therapy, the metastasis of tumor cells is responsible for many therapeutic failures. Metastasis is also known to progress very frequently via the lymphatic system [19]. The accumulation of NCS with bioactivity in lymph nodes after i.v. and s.c. administrations, described in this report, will provide a very important and promising clue in the therapy of malignant tumors which metastasize via the lymphatic system.

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MATERIALS AND METHODS

Materials

NCS was obtained from Kayaku Antibiotic

Research Co. Ltd., Tokyo, and it was purified as reported previously [20]. Two mg of the drug were dissolved in 1.0 ml of 0.01 M phosphate buffered saline (0.15 M NaCl) (PBS, pH 7.0). Three protease inhibitors, leupeptin, pepstatin and antipain (gifts of Professors Matsushima and Umezawa, Tokyo), were dissolved together in PBS at a concentration of 0.3% for each and this is hereafter referred to as inhibitor solution. Both sensitivity-test-agar, used at 1.0% in agar concentration, and Tryptosoy broth for bacterial growth were purchased from Eiken Chemical Co. Ltd., Tokyo.

Animals, treatments and preparations of specimens

Male rats of HO strain weighing 200–300 g were used for the experiments. The drug was injected i.v. through the tail vein or s.c. bilaterally at midpoint between the ankle and the knee of hind legs, while the rats were under ether anesthesia. The dose of injection was 2 mg/kg of the rat's body weight. The time course of the distribution of the drug at 10, 30, 90 and 180 min was studied, because in preliminary experiments the biological activity of NCS in popliteal lymph nodes reached a maximum level 30 min after s.c. injection in foot pads. At 10, 30, 90 and 180 min after injections, each rat was anesthesized again, and sacrificed by bleeding from bilateral inguinal arteries. An aliquot of blood (0.5 ml) was mixed with an equal volume each of the chilled inhibitor solution and PBS, yielding the final concentration of the inhibitors of 0.1% respectively, and stored in an ice box till assay. Urine samples were collected on a polypropylene sheet in a cage and also obtained from the vessical cavity with a syringe. They were stored with inhibitors similar to the blood. Bone marrow specimens were obtained by flushing with 0.2 ml of the chilled inhibitor solution at first, and then rinsed with 0.2 ml of chilled PBS, by applying hydrostatic pressure to one side of the femurus using a 23 gauge needle attached to a syringe. Bone marrow specimens were stored similarly. Each organ and tissue was removed immediately, and weighed by a torsion balance after removal of excess liquid with soft tissue paper, and then frozen in dry ice. The contents of the stomach, small intestine and large intestine were removed by gentle rinsing with PBS. All or a part of the organs or tissue was minced and homogenized with the inhibitor solution at cold temperature with a Sorvall omni-mixer, which was then rinsed with PBS. The homogenates and rinses were combined, each sample was centrifuged at 1500 rev/min at 4°C for 10 min, and the supernatants were used for the assay of bioactivity.

Measurement of biological activity

The biological activity of the drug in the specimens was assayed using a growth inhibition of a Gram-positive bacteria, *Sarcina lutea* PCI 1001 [1]. The assay system was as follows: 0.05 ml of fully grown culture of *S. lutea* in Tryptosoy broth was mixed with 100 ml of sensitivity test agar (1.0%) at about 40°C, and poured over a glass plate with four edgings (30×21 cm^2). The agar plate had a thickness of about 0.8 mm. Glass cylinders (8.0 mm i.d., 10 mm in length) were placed on the agar plate. A 50 μl aliquot of each assay sample or the standard NCS solution was placed in each glass cylinder at 4°C. The drug was allowed to be diffused for 5 hr at 4°C, and then the assay plate was incubated for 12 hr at 37°C in order to facilitate bacterial growth. During diffusion and incubation, the agar plate was kept in the dark, because NCS is known to be inactivated by light [21]. The minimum detectable concentration of the drug was approximately 0.01 μg/g of the specimen (Fig. 1). Antibacterial activity parallels well with antitumor activity [1], or effect on DNA [7–10].

Effect of protease inhibitors on the inactivation of NCS by tissue homogenates

A given amount of NCS was incubated with various tissue homogenates, as prepared above, in the presence or absence of the

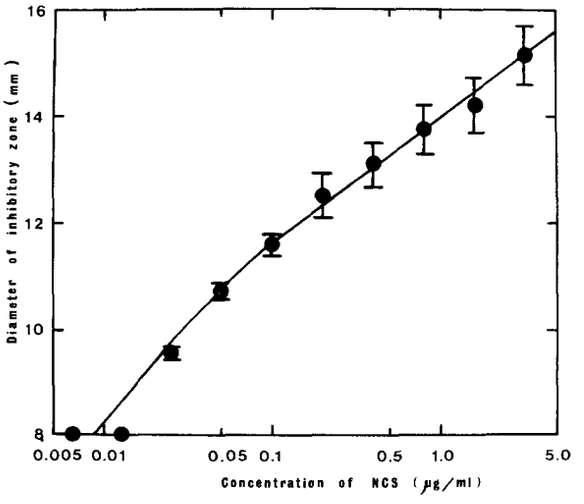

Fig. 1. Standard dose response assay of neocarzinostatin on agar plate with Sarcina lutea. *See detail in the text.* (n = 10).

inhibitors. The inhibitors were added to the homogenates to give a final concentration of 0.1% each. The concentrations of NCS were either 2 μg/ml for the homogenates of the lung, bladder, lymph nodes and the muscle, or 10 μg/ml for the liver and the kidney.

RESULTS

The results of standard assay of antibacterial activity is shown in Fig. 1, in which a linear relationship between log of drug concentrations and diameter of the growth inhibitory zone is seen. A reliable quantification is, thus, possible as low as about 0.008 μg/ml.

The distribution of NCS based on the bioactivity in lymph nodes after an s.c. injection is shown in Fig. 2A. Data are expressed as the mean value of four rats in two experiments, and the vertical bars show the deviation of two experiments. Biological activity of NCS in popliteal nodes was 9.5 μg/g at 10 min and increased to the highest level (11.0 μg/g) at 30 min. From 30 to 90 min (8.5 μg/g), the activity decreased slowly, and rapidly to 180 min (0.57 μg/g). Figure 2B shows the recovery of activity (%) of the administered NCS in the popliteal nodes alone. The average weight of the popliteal lymph nodes of a rat was about 10 mg, which corresponds to 0.004% of total body weight, whereas recovery of NCS in the popliteal nodes was 0.021% at the highest level. Therefore, Fig. 2 indicates that NCS accumulated in popliteal lymph nodes effectively to a high level. The bioactivity of the lumbar and the axillary lymph nodes were 1.1 and 0.03 μg/g, respectively. That in other lymph nodes, mesenteric, mediastinal and cervical were at about 0.01 μg/g at 10 min, however, the drug became undetectable after 10 min except popliteal and lumbar lymph nodes.

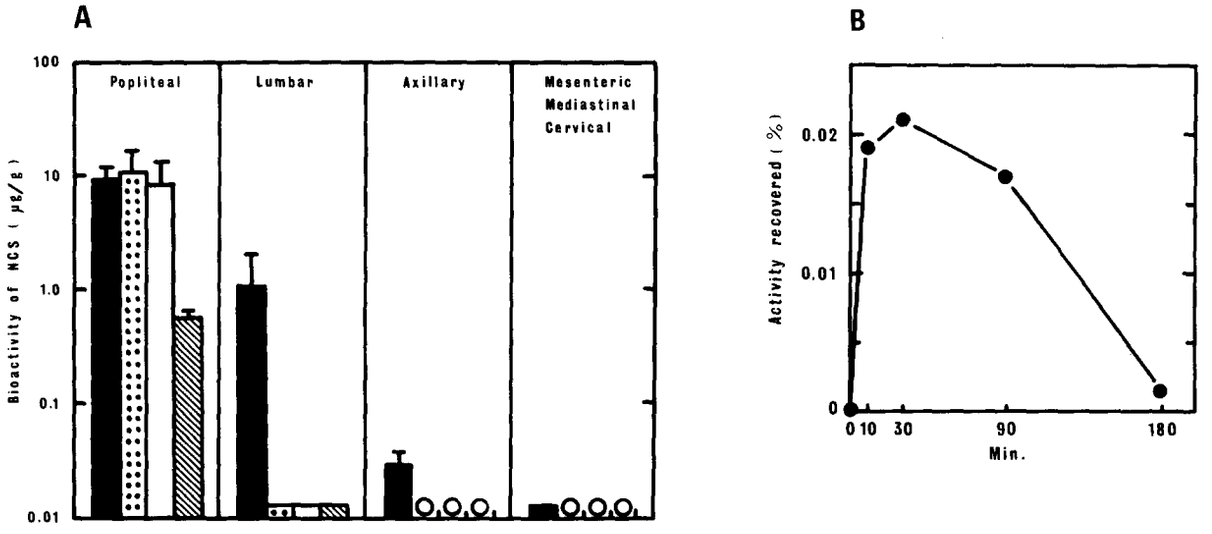

Fig. 2. (A) Distribution of bioactivity of neocarzinostatin (NCS) in various lymph nodes (LN). NCS was injected s.c. at the midpoint between knee and ankle of hind leg with a dose of 2 mg/kg. *Columns and vertical bars are the mean values and deviations obtained with four rats. Different columns with black, dotted, blank and streaks indicate the results obtained at* 10, 30, 90 *and* 180 min, *respectively. Blocks on the base line show that approximately minimum concentration* (0.01 μg/g) *was detected with bioassay using* Sarcina lutea. *Open circles on the base line show activity was undetectable. (B) Percentage of recovered activity of NCS in popliteal LN in Fig. 2A. Expressed in percentage of injected amount.*

Figure 3 shows the distributions of NCS in lymph nodes after an injection through the tail vein. In all lymph nodes assayed, NCS activity was higher than 0.01 μg/g at 10 min. It reached the highest level in 30 min in popliteal (0.27 μg/g), lumbar (2.5 μg/g) and axillary (0.78 μg/g) lymph nodes, however, that of mesenteric, mediastinal and cervical lymph nodes remained similar to 10 min. At 90 min it was still high in lumbar lymph

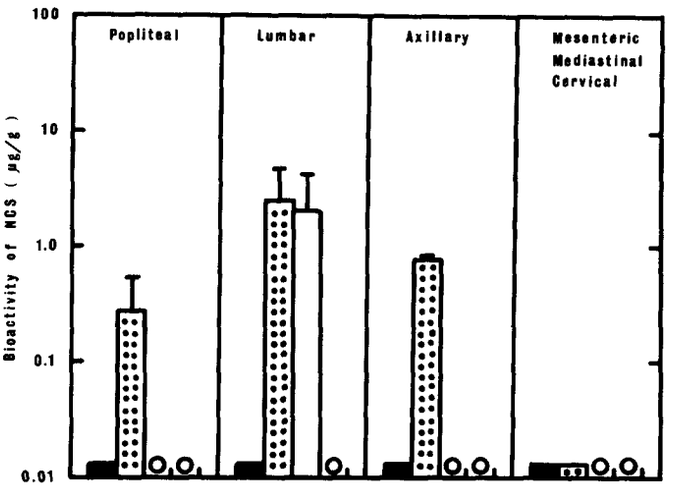

Fig. 3. Distribution of bioactivity of neocarzinostatin (NCS) in various lymph nodes (LN) after i.v. injection through tail vein. Time course and injection dose were similar to Fig. 2A.

nodes (2.0 μg/g), but undetectable in other lymph nodes. It became undetectable in all lymph nodes at 180 min.

The distribution of NCS activity in the various organs and the tissues after s.c. and i.v. injection are shown in Fig. 4 and Fig. 5, respectively. NCS accumulated to a relatively high level (about 0.02–0.03 μg/g) in the small intestine by either administration. However, that in the bladder after s.c. injection was very low and detectable only at 10 and 30 min. On the contrary, an i.v. injection resulted in an accumulation of 0.76, 1.3 and 3.5 μg/g of the bladder tissue at 10, 30 and 90 min respectively, although it became undetectable at 180 min. In the lung, more drug accumulated in 10 min after s.c. than i.v. injection. In the bone marrow, NCS was detectable at 10 and 30 min after s.c., but i.v. injection resulted in a significant level only after 10 min. In the liver, the spleen and the kidney it was detectable only at 10 min after either administration, but that in the blood was detectable only at 10 min after i.v. injection. In contrast to the above results, NCS activity in the heart, stomach, large intestine, muscle and prostate was undetectable at any time after administration in any experiments. Figure 6 shows the percentage of recovery of NCS in the urine. The recovery after i.v. injection was 23, 27, 44 and 13% at 10, 30, 90 and 180 min respectively, whereas that after s.c. injection was 0.29, 1.55, 12.0 and 4.6% at 10, 30, 90 and 180 min. The recovery at 10 min after i.v. injection was about 80 times higher than that after s.c. injection. The highest level of recovery observed at 90 min after i.v. injection was 3.7 times that after s.c. injection. In 180 min, the total recovery decreased to 30 and 40% of that at 90 min after i.v. and s.c. injection, respectively. The decreased activity may be due to inactivation during the time period.

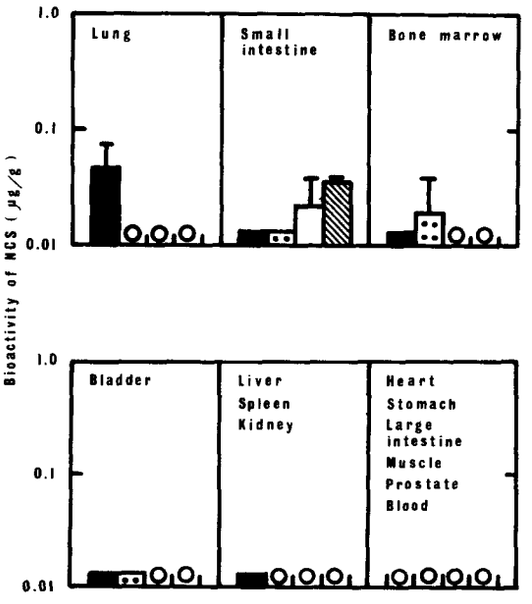

Fig. 4. Distribution of bioactivity of neocarzinostatin in organs and tissues after s.c. injection. Injection site, dose and notation were similar to Fig. 2A.

The effect of protease inhibitors were tested for the stabilization of bioactivity of NCS in the various homogenates. Data obtained for the lung and the kidney indicate that the effect was obvious in which $t_{\frac{1}{2}}$ became about 4–6 times longer (Fig. 7A, B). Whereas the effect for the muscle, lymph nodes, bladder

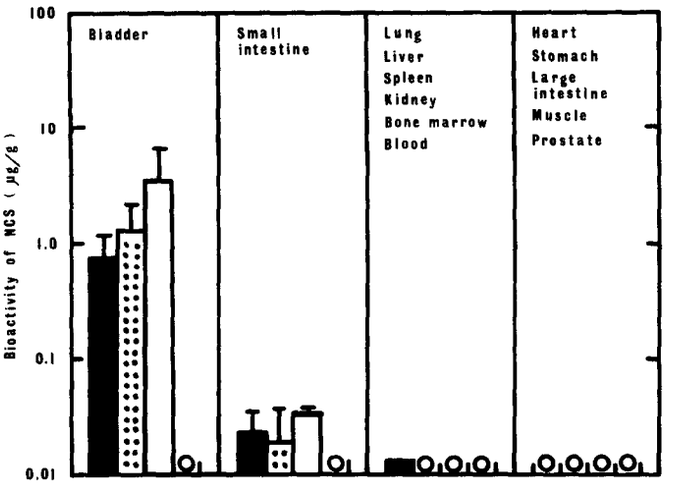

Fig. 5. Distribution of bioactivity of neocarzinostatin in organs and tissues after i.v. injection. The dose and notation were similar to Fig. 2.A.

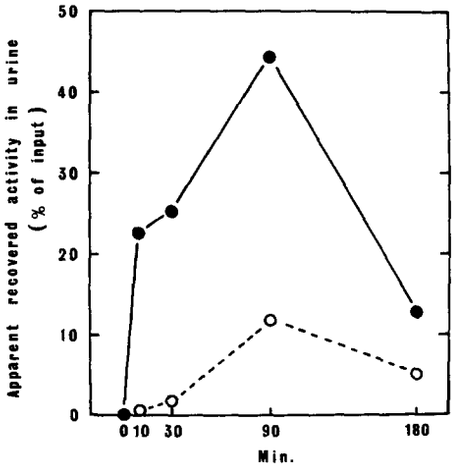

Fig. 6. Urinary excretion of neocarzinostatin as determined by bioactivity. The solid line with closed circles shows recovery (%) after i.v. injection, and the dotted line with open circles shows recovery (%) after s.c. injection. Each circle shows the mean value of 4 rats in cumulative time period.

and urine were not apparent (not shown). They were the tissues or specimen with very mild inactivation rates, and *Ki** values with or without the inhibitors are in the range of 0.075 ± 0.02 min. Their values are close to those obtained for the lung and the kidney in the presence of the inhibitors. On the contrary, the liver homogenates exhibited an extremely rapid inactivation rate. The inhibitors exhibited a definite effect but only a limited extend: $t_{\frac{1}{2}}$ became 50% longer and *Ki* of 0.46/min was obtained with the inhibitors.

DISCUSSION

The metastasis of tumors is a very serious problem in cancer therapy. It is known to progress frequently via the lymphatic system. In surgical operations of malignant tumors, the regional lymph nodes may be removed completely [22]. However, it is a very formidable task to remove completely such micrometastases in the remote lymph nodes. In chemotherapy using liposome emulsion, a few drugs such as 5-fluorouracil (5-FU) and bleomycin have been tried to treat metastatic tumors in lymph nodes and appeared to be effective. Since the assessment of the biological activity of the emulsion in lymph nodes is very difficult due to limited bioavailability of the drug from lamella structure of the drug-emulsion to tumor tissue, an explicit efficacy was not well evaluated [23–27]. In lymphology it is known that once large proteinaceous molecules enter the interstitial void (tissue fluid), they do not re-enter the blood capillaries directly but are taken up by the lymphatic vessels and returned to the blood stream indirectly [28].

NCS, which is a proteinaceous antitumor antibiotic with a mol. wt of 10,700, was expected to be recovered via the lymphatic system and accumulate in the lymph and

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**Ki* means inactivation rate constant which can be obtained as follows:

$$Ki = \frac{\ln 2}{t_{\frac{1}{2}}} = \frac{0.693}{9 \pm 2 \text{ min}} \fallingdotseq 0.075 \pm 0.02$$

where $t_{\frac{1}{2}}$ is the biological half-life of the NCS in the homogenates.

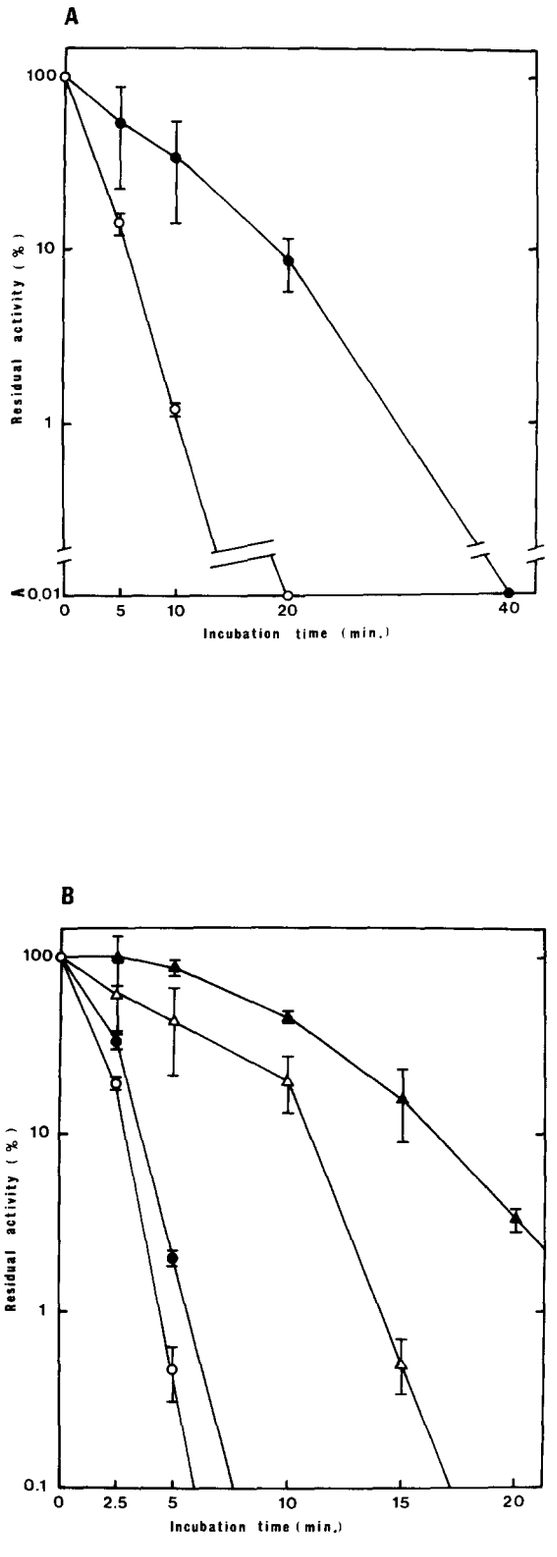

Fig. 7. Effect of protease inhibitors on the inactivation of NCS by the tissue homogenates.

(A) lung homogenated; control without inhibitor (○—○), *with inhibitors* (●—●).

(B) liver; without inhibitor (○—○), *with inhibitors* (●—●). *Kidney; without inhibitor* (△—△), *with inhibitors* (▲—▲).

The data show the mean value and S.D. *of triplicate assays. Incubation with the homogenates was at* 37°C.

lymph nodes when injected s.c. or i.v. As described in this report, this point was clarified. When injected s.c., NCS accumulated at particularly high levels in the primary regional lymph nodes (popliteal) of the injection area (midpoint between the ankle and the knee of the hind leg), and also at a lower level in the secondary lymph nodes (lumbar) (Fig. 2). Therefore, when NCS is administered to the tumor tissues directly, its concentration at the metastatic tumor cells in the regional lymph nodes will be high as well as in the tumor cells *in situ*.

Furthermore, the high lymphotropic character of NCS after an i.v. injection was observed similarly (Fig. 3). Namely, the drug concentration in lymph nodes was about the same as in the bladder (Fig. 5). The bladder was known to be one of the target organs of NCS [13], which was re-confirmed in the present study. Therefore, NCS is used successfully for treatment of bladder cancer [14, 15]. According to the study of bladder cancer treated with an i.v. injection of NCS, the metastatic recurrence rate has been shown very low [15]. This may be explained by the fact that the high accumulation of NCS in lymph nodes (Fig. 3) resulted in a low recurrence rate via the lymphatic system.

As represented in the result of Fig. 2B, the recovery of NCS in regional lymph nodes was relatively rapid after s.c. administration (e.g., about 10 min), but slow after i.v. administration (Fig. 3). These results may be interpreted as follows. Upon i.v. injection (Fig. 3), NCS was first distributed systemically, and subject to rapid renal excretion as well as leaking out of the capillaries into the tissue fluid, from which it was then recovered in lymph nodes [28]. On the contrary, s.c. injection of NCS resulted in direct accumulation in the regional lymph nodes with a consequently rapid and very high rate of recovery (Fig. 2B), but a very low rate of recovery in the urine (Fig. 6), due to proteolytic degradation, was interesting.

Contrary to the high accumulation of NCS in the bladder after i.v. injection, the s.c. injection resulted in only detectable tissue concentration of the drug at 10 and 30 min only (Fig. 4). This may be due to the fact that urinary accumulation of NCS after s.c. injection was much slower and less extensive than that after i.v. injection (Fig. 6). Furthermore the urinary NCS is known to traverse to the bladder epithelium [29]. The drug concentration in the bladder tissue and the recovery in the urine increased up until

90 min, but became undetectable in the bladder tissue, it also decreased in the urine at 180 min (Figs. 5 and 6). These results indicate inactivation of NCS in the tissues, blood and urine between 90 and 180 min as reported [29].

In addition to the bladder and lymph nodes, the small intestine, bone marrow, lung, liver, spleen and kidney were revealed to accumulate NCS after either an i.v. or s.c. injection. In the blood, NCS activity was detected only during the initial 10 min after i.v. injection (Fig. 5) and the excretion of NCS from the blood into the urine was rapid (Fig. 6). In contrast to these organs, NCS was not detected in the heart, stomach, large intestine, muscle and prostate in the present experiment.

The present assay using protease inhibitors is at least 10 times more sensitive (Fig. 1) than that by Fujita *et al.* [16]. The accumulation of NCS reported previously in the stomach, the heart, and the muscle shows a disagreement with present data. Our previous data utilizing [^{14}C] succinyl-NCS [13] and the present result (Figs. 4 and 5) are in fair agreement despite rapid proteolytic degradation of such as the liver, the lung, and the kidney (Fig. 7A, B).

In all the above organs and tissues, the bioactivity of NCS was lower than anticipated from the data of [^{14}C] succinyl-NCS [13]. At least two reasons may explain the difference of distributions between the bioactivity of NCS and radioactivity of [^{14}C] succinyl-NCS: (i) The radioactivity does not decrease during the assay, while the bioactivity is readily inactivated *in vivo* and also *in vitro* during the assays even in the presence of inhibitors (Fig. 7A, B) [16, 17]; (ii) The biological activity of succinyl-NCS is more stable than that of NCS *in vivo* and *in vitro* [17, 30]. If succinyl-NCS is used instead of NCS, bioactivity will be detected at higher levels than for NCS.

In cancer chemotherapy, the prime requisite is the accessibility of the drug to the target organs followed by drug penetration into the tumor tissue. The second requisite is the susceptibility of the tumor cells to the drug. NCS is shown to penetrate more readily into tumor cells than into normal cells [31–33]. Therefore, the accessibility of NCS to lymph nodes as described above, is highly advantageous in the control of lymphatic metastasis.

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