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Antimetastatic activity of neocarzinostatin incorporated into controlled release gels of CM-chitin

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6-*O*-carboxymethyl chitin (CM-chitin), a water-soluble chitin derivative, was gelled in the presence of 15 to 30 mM iron (III) chloride. At the time of gel formation, a peptidic anticancer drug neocarzinostatin (NCS), was efficiently (>50%) incorporated into the gel in the co-presence of 25 to 50 mM calcium chloride and iron (III) chloride. CM-chitin gel containing NCS was digested by lysozyme *in vitro* and NCS was released from the gel in both a time- and dose-dependent manner. In-vivo studies in mice showed that free NCS was rapidly cleared from the circulation, but NCS released from the gel was detectable in plasma even 48 h after the subcutaneous injection of the gel. Antimetastatic effects of the CM-chitin gel containing NCS were studied in the spontaneous pulmonary metastasis model using B16-BL6 melanoma; these results suggest that CM-chitin gels are useful as a sustained-release drug carrier.

INTRODUCTION

Some anticancer agents or lymphokines in general have a very short half-life in the circulation, which may result in a decrease of their therapeutic potential. Therefore, the control of drug release *in vivo* may allow more effective expression of their biological effects. For this purpose, controlled-release polymers have been used to achieve the sustained delivery of drugs, proteins and macromolecules (Langer & Folkman, 1976).

Chitin, a (1→4) linked polysaccharide composed of 2-acetamide-2-deoxy-β-D-glucopyranose residues, is distributed widely in nature, and has been reported to have some beneficial medicinal (Muzzarelli, 1978) and pharmaceutical applications (Miyazaki *et al.*, 1981). Recently, 6-*O*-carboxymethyl-chitin (CM-chitin) has been shown to induce macrophage activation for a short period *in vivo* (Nishimura *et al.*, 1984*b*), to have weak mitogenic activities on normal spleen cells (Nishimura *et al.*, 1985), and to decrease the adsorption of blood components such as serum albumin, γ-globulin and fibrinogen as compared with chitin (Nishimura *et al.*, 1984*a*). Also, no antibody induction against CM-chitin was detected (Tokura *et al.*, 1987). Chitin and CM-chitin are known to be susceptible to lysozyme in the human body (Berger & Wiser, 1957; Tokura *et al.*, 1983*a*).

We have recently reported that the addition of

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iron (III) chloride into CM-chitin solution induced gel formation and at the same time bovine serum albumin (BSA) or the anticancer drug doxorubicin (DOX) were reproducibly incorporated into the CM-chitin gel without difficulty, (Watanabe *et al.*, 1990). The release of BSA or DOX from the gels was observed to be increased by lysozyme digestion in time- and dose-dependent manners *in vitro*. In the present study, we selected the peptidic anticancer drug neocarzinostatin (NCS) to be incorporated into CM-chitin gel and examined whether NCS was released from the gel by lysozyme digestion at physiological concentrations *in vitro*. We also examined the in-vivo effect of NCS-containing gels on the spontaneous tumour metastases of B16-BL6 melanoma cells in mice and its biological characterization after administration.

MATERIALS AND METHODS

Cells

Highly metastatic B16-BL6 melanoma cells, obtained by an in-vitro selection procedure for invasion, were kindly provided by Dr I.J. Fidler, M.D. Anderson Cancer Center, Houston, Texas, USA. B16-BL6 cells, derived from C57BL/6 mice, were maintained as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 7·5% fetal bovine serum (FBS), vitamin solution, sodium pyruvate, non-essential amino acids and L-glutamine.

Animals

Inbred 7- to 10-week-old female C57BL/6 mice were purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan. The mice were maintained in the Laboratory for Animal Experiments, the Institute of Immunological Science, Hokkaido University, under laminar air-flow conditions.

Chitin derivative

Chitin was prepared from Queen Crab Shells according to the method of Hackman (1954) and powdered to 45–60 mesh before use. 6-*O*-carboxymethyl-chitin (CM-chitin) was prepared by the procedures reported previously (Nishimura *et al.*, 1984*a*). The degree of substitution was estimated to be 0·80 by potentiometric titration with 0·1 M HCl in 0·1 M NaCl under a nitrogen atmosphere at room temperature as described elsewhere (Tokura *et al.*, 1983*b*).

Reagents

Neocarzinostatin (NCS) was kindly provided from Yamanouchi Pharmaceutical Co. Ltd., Tokyo and dissolved in saline at 10 mg/ml before use. Lysozyme (egg white 50 000 U/mg) was obtained from Seikagaku Kogyo Co. Ltd., Tokyo. Bolton-Hunter reagent [*N*-succinimidyl 3-(4-hydroxy-3,5- ^{125}I-diiodophenyl) propionate, specific activity 2000 Ci/mmol] was purchased from New England Nuclear, Boston, MA. Other reagents of guaranteed grade were purchased from Wako Pure Chemical Industries Ltd., Osaka.

Preparation of CM-chitin gel containing NCS

CM-chitin gel was prepared by the procedure and conditions described previously (Watanabe *et al.*, 1990). Briefly, CM-chitin for injection (5 mg) was dissolved in 500 μl of saline in a 1·5 ml microcentrifuge tube. To make a uniform viscous solution this solution was vigorously stirred for 30 min at room temperature. Next, 30 μl of NCS (10 mg/ml) and 100 μl of 375 mM calcium chloride solution were added to the solution, and the mixture was stirred for 30 min at 4°C. Then, 100 μl of 225 mM iron (III) chloride solution was dropped into the reaction mixture, and the mixture was stirred for an additional 30 min. The resultant gel of CM-chitin was sonicated at 40% duty cycle for 15 s with a Sonicator W-375, (Ultrasonic Inc., New York), to make fine particles. The resulting gel suspension was centrifuged at 15 000 rpm for 5 min, washed once with saline by the centrifugation, and diluted with saline to the appropriate concentration before use.

Determination of the amounts of CM-chitin gel and NCS incorporated into gel

The supernatant and washing fluids were collected during the preparation of CM-chitin gel containing NCS, to measure the amounts of residual CM-chitin and incorporated NCS. The amount of CM-chitin was determined by a modified ninhydrin method (Yemm & Cocking, 1955). The amount of NCS incorporated into the gel was calculated by measuring the decrease in absorbance of the supernatant and washing fluids at 290 nm. Briefly, 200 μl of 5 N NaOH was added into 500 μl of washing fluids containing unincorporated NCS in a microcentrifuge tube, and the mixture centrifuged at 15 000 rpm for 5 min to remove the residual iron (III) and calcium ions. The amount of unincorporated NCS in the supernatant was determined by measuring the absorbance at 290 nm. A similar process was repeated in the absence of iron (III) chloride. Finally, the amount of the NCS incorporated into the gel was calculated by comparing both absorbances.

Preparation of radiolabeled NCS

NCS was iodinated by Bolton-Hunter reagent according to the conventional procedure as described previously

(Saiki *et al.*, 1989). Briefly, NCS (2 mg) was dissolved in 200 μl of 0·1 M borate buffer, pH 8·5 and added to 1 mCi Bolton-Hunter reagent (specific activity, 2000 Ci/mmol) freshly dried from a solution in benzene. After agitation of the mixture at 4°C overnight, the reaction was quenched by addition of 35 μl of cold 0·2 M glycine in 50 mM phosphate buffer, pH 7·5. Iodinated NCS was separated from by-products by gel filtration on Sephadex G-25 which was equilibrated and eluted with 50 mM phosphate buffer, pH 7·5. ^{125}I-labeled NCS thus obtained was confirmed by the absorbance at 290 nm in a spectrophotometer. The specific activity of the labeled NCS was 10^7 cpm/μg.

Release of ^{125}I-labeled NCS from CM-chitin gel by lysozyme digestion *in vitro*

The preparation of ^{125}I-labeled NCS-containing gel was the same as described above. The suspension of radiolabeled NCS-containing gel in saline ($1·5 \times 10^8$ cpm/15 μg) in a microcentrifuge tube was incubated with 500 or 5000 U/ml of egg white lysozyme (EC 3.2.1.17) at 37°C for 7 days. At various time points, the mixture was centrifuged at 10 000 rpm for 5 min and the amount of ^{125}I-labeled NCS released into the supernatant was determined by measuring the radioactivity in a gamma counter. Saline with fresh lysozyme was then added to the remaining gel pellets and incubation was continued at 37°C. This process was repeated at each time point. The result was expressed as accumulative amounts of released NCS at various time points.

Release of NCS from CM-chitin gel in plasma

C57BL/6 female mice were administered subcutaneously (s.c.) with 10 μg NCS or NCS-containing gel (10 μg NCS/330 μg gel) in a volume of 100 μl/mouse. At given intervals, a 200 μl blood sample was withdrawn from the retro-orbital plexus and diluted with 800 μl of PBS. The plasma samples were separated by centrifugation at 2800 rpm for 10 min, followed by filtration through a 0·22 M Millipore filter. The amount of released NCS in plasma was determined by growth inhibition of tumor cells. Briefly, the diluted plasma (0·1 ml) was added to a monolayer of B16-BL6 melanoma cells (5×10^3/0·1 ml/well) in 96-well tissue culture plate (Falcon No. 3072, Becton Dickinson Labware, Lincoln Park, NJ) and the cells were incubated for 48 h at 37°C. The culture was pulsed with 0·5 μCi/well of [^{3}H]-thymidine ([^{3}H]-TdR, specific activity 23 Ci/mmol, Amersham International, Buckinghamshire, UK) for the last 4 h before the termination of culture, and collected on a glass filter by an automatic cell harvester. The radioactivity was measured in a liquid scintillation counter. NCS concentration in plasma was calculated from the cumulative radioactivity at each time point based on the standard curve of growth inhibition of B16-BL6 cells.

Spontaneous metastasis assay

Mice were inoculated s.c. with B16-BL6 melanoma cells (5×10^5) into the right hind footpad. The primary tumors were surgically removed on day 21 after tumor inoculation. The treatments with NCS-containing gel began one day after amputation of primary tumors. Mice were killed 14 days after the amputation, the lungs were fixed in Bouin's solution, and the lung tumor colonies were counted under a dissecting microscope.

Assay of organ and tissue retention of ^{125}I-labeled NCS

^{125}I-labeled NCS-containing gel (9·8 μg NCS/$9·8 \times 10^7$ cpm/115 μg gel) or ^{125}I-labeled NCS (9·8 μg) in a volume of 0·1 ml were injected intravenously (i.v.) or s.c. into the lateral tail vein or into the left flank, respectively. Mice were exsanguinated at the indicated times. The lungs, liver, spleen, kidneys, injection site (in the case of s.c. injection) and 100 μl of blood were collected from each mouse, and rinsed in 70% ethanol. The radioactivity in each organ was measured in a gamma counter.

Statistical analysis

The statistical significance of the differences between the groups was determined by applying Student's two-tailed *t*-test.

RESULTS

Preparation of CM-chitin gel containing NCS

We first tried to prepare CM-chitin gel containing NCS according to the method described previously (Watanabe *et al.*, 1990). Iron (III) chloride at a final concentration of 15 or 30 mM was added to the mixture of CM-chitin (10 mg) and NCS (500 μg). As shown in Table 1, a high degree (80–90%) of gel formation was observed by the addition of iron chloride alone, but the amount of NCS incorporated into the gel was only 3 μg (6%). The addition of calcium chloride alone at final concentrations ranging from 25 to 50 mM, in place of iron (III) chloride, into the mixture caused no gel formation. When both iron (III) chloride and calcium chloride were added to the mixture of CM-chitin and NCS, more than 80% gel formation was observed with the increased incorporation of NCS into the gel. In the following experiment, we used 30 mM iron (III) chloride and 50 mM calcium chloride for gel preparation because more than 90% of CM-chitin was recovered as gel and more than 50% of NCS was incorporated into the gel. We next examined whether NCS can be quantitatively incorporated into the CM-chitin gel formed by the presence of 30 mM iron (III) chloride and 50 mM calcium chloride (Fig. 1). Various amounts of NCS ranging from 250 to 2000 μg were added to 20 mg

Table 1. Effect of calcium chloride and iron chloride on the formation of CM-chitin gel and the incorporation of NCS into gel

Addition[a]		Gel formation[b]	Incorporation of NCS[c]	
$FeCl_3$ (mM)	$CaCl_2$ (mM)	(%)	(μg/mg gel)	(%)
0	0	—		
	25	—		
	50	—		
15	0	78	1·5 ± 0·3	2·9
	25	82	5·4 ± 1·7	10·7
	50	84	7·2 ± 2·2	14·4
30	0	91	3·0 ± 1·3	6·0
	25	92	25·7 ± 1·1	51·4
	50	90	27·1 ± 2·2	54·1

[a]Various concentrations of $FeCl_3$ and $CaCl_2$ solution were added to the mixture of CM-chitin (10 mg) and NCS (500 μg).
[b]Results are expressed as the amount of incorporated NCS into the gel measured by OD at 290 nm.
[c]Recovery is expressed as the amount of incorporated NCS per the amount of initially added NCS.

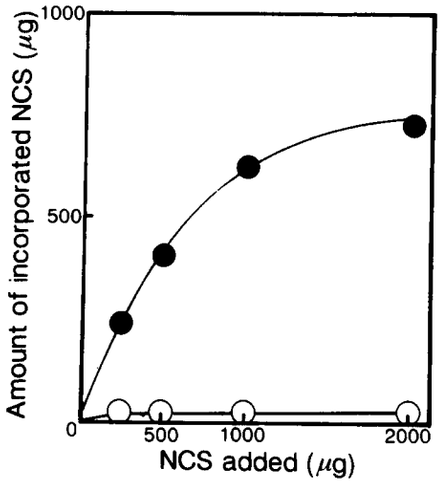

Fig. 1. Incorporation of NCS into CM-chitin gel. Various amounts of NCS were added to 20 mg CM-chitin in distilled water. The CM-chitin gel was formed in the presence (●) or absence (○) of 750 mM calcium chloride and 125 mM iron (III) chloride and centrifuged at 15 000 rpm for 5 min. The incorporated NCS was calculated by measuring the unincorporated NCS in the supernatant using a spectrophotometer at 290 nm.

of CM-chitin solution. Figure 1 shows that NCS was incorporated into the gel effectively when less than 500 μg of NCS was initially added to the mixture (more than 80%). However, when 2 mg of NCS was initially added, the incorporation of NCS into the gel was only 30% (720 μg). In any case, more than 80% of CM-chitin was recovered as gel irrespective of the increasing amounts of NCS. The results shown in Table 1 and Fig. 1 indicate that about 30 μg of NCS were able to be incorporated into 1 mg of the CM-chitin gel (about 50% recovery of NCS initially added). The gel thus prepared was smaller on average than human platelets (2–3 μm in diameter) and showed a homogenous distribution in gel size. The average size of a gel particle was estimated to be 0·695 μm by coulter multisizer (data not shown).

Release of ^{125}I-labeled NCS from CM-chitin gel by lysozyme digestion

CM-chitin is known to possess increased sensitivity to lysozymic degradation as compared with chitin (Berger & Wiser, 1957; Tokura *et al.*, 1983*a*). We therefore investigated whether or not NCS can be released from CM-chitin gel containing NCS by lysozyme digestion *in vitro*. CM-chitin gel containing NCS (15 μg NCS/0·6 mg gel) was incubated with 500 and 5000 U/ml lysozyme (Fig. 2). Within two hours after incubation, about 30% of NCS was released from the gel irrespective of the presence or absence of lysozyme. The release of NCS from the gel in the presence of 500 or 5000 U/ml lysozyme was observed to increase in both a time- and concentration-dependent manner. On day 7 after the incubation, about 80% of NCS was released from the gel by lysozyme treatment at the concentration of 500 U/ml, which is approximately equivalent to the physiological concentration in the human plasma. Approximately 50% of the NCS was released from the gel in the absence of lysozyme during 4 days of incubation and thereafter was scarcely released. This suggests spontaneous or nonspecific release of NCS from the gel. However, when 500 U/ml of lysozyme was newly added to such a gel suspension on day 4

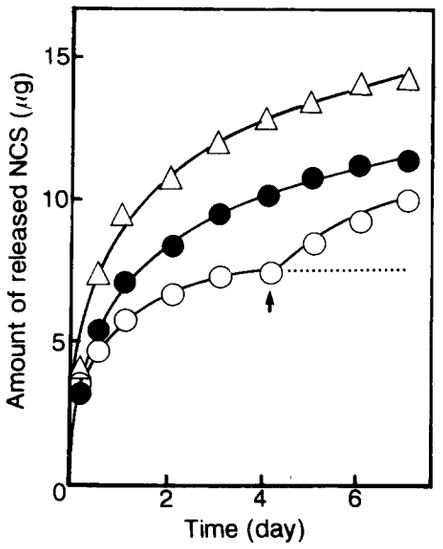

Fig. 2. Release of ^{125}I-labeled NCS from CM-chitin gel by lysozyme digestion. CM-chitin gel containing ^{125}I-labeled NCS ($1·5 \times 10^8$ cpm/15 μg NCS/45 μg gel) was incubated without (○) or with lysozyme, 500 U/ml (●) or 5000 U/ml (△) for the indicated days. At each time point, the reaction mixture was centrifuged at 3000 rpm for 10 min and the released radiolabeled NCS in the supernatant was counted in a gamma counter. An arrow within the figure represents the addition of 500 U/ml lysozyme into the gel suspension.

(indicated by an arrow in Fig. 2), 20% of NCS was additionally released from the gel during the last 3 days. Fig. 3 shows photographs using a 200-power scanning electron microscope on CM-chitin gel treated with or without 500 U/ml of lysozyme for 3 days. Lysozyme-treated gel was shown to be rough and contain large spaces in the matrices of the gel as compared with untreated gel, indicating clearly that the gel is digested with lysozyme. We also confirmed that when CM-chitin gel was incubated with 500 U/ml of lysozyme for 4 days, the amount of reducing groups on the sugar residue increased in a time-dependent manner and was approximately 5% of CM-chitin on day 4 as determined by the method of Park and Johnson (1949) with *N*-acetylglucosamine as a reference standard.

Release of NCS from CM-chitin gel containing NCS *in vivo*

To further study the potential of CM-chitin gel as a sustained-release dosage form, we measured NCS levels in the plasma at various time points after the s.c. administration of CM-chitin gel containing NCS into mice. Figure 4 demonstrates that the clearance of NCS from the circulation was very rapid over a 6 h period after the s.c. injection of NCS. At 24 h after the injection, NCS in the plasma was undetectable (less than 1 ng/ml) as assessed by the growth inhibition of B16-BL6 melanoma cells by the plasma. On the other hand, NCS-containing gel gave a maximum plasma concentration at 6 h after the s.c. injection and

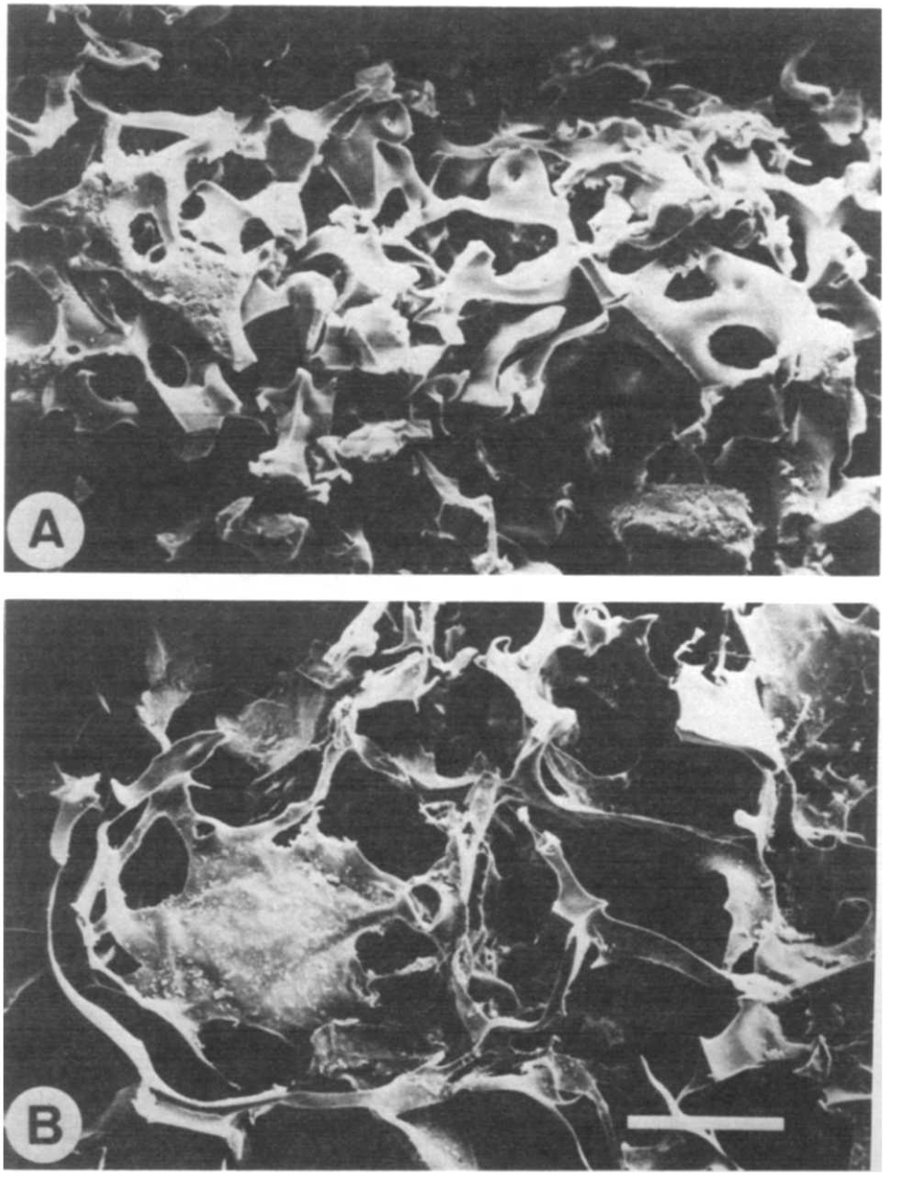

Fig. 3. Scanning electron microscopy of CM-chitin gel treated with or without lysozyme. CM-chitin gel was incubated with (A) or without (B) lysozyme (500 U/ml) for 3 days at 37°C. Bar = 1 μm.

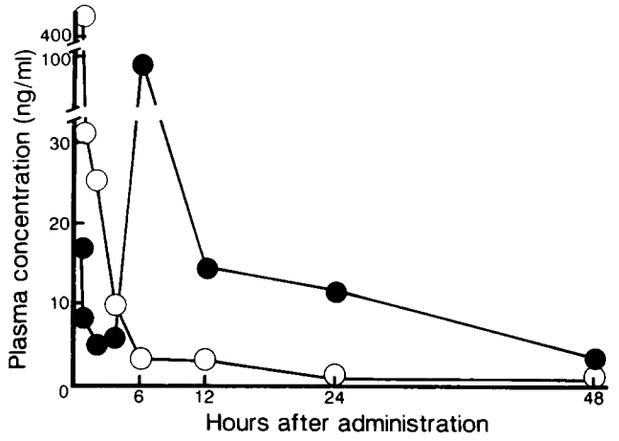

Fig. 4. Plasma concentration of NCS after the s.c. administration of NCS or NCS-containing gel. Mice were administered s.c. with NCS (10 μg) (●) or CM-chitin gel containing NCS (10 μg NCS/330 μg gel) (○), and plasma samples were obtained at various time points. NCS concentration in plasma was calculated from the standard curve by the growth inhibition of B16-BL6 melanoma cells *in vitro*.

thereafter NCS was gradually cleared from the circulation. NCS in the plasma was still detectable 48 h after administration.

Organ localization and retention of ^{125}I-NCS containing CM-chitin gel

We next analyzed the organ distribution and retention of ^{125}I-labeled NCS or gel containing ^{125}I-labeled NCS by s.c. or intravenous (i.v.) administrations. Mice were killed 8 h after the injections, and their visceral organs were collected and monitored for radioactivity in a gamma counter. The data of a representative experiment are shown in Fig. 5. In the case of the s.c. administrations, significantly higher values were found in the blood and at the injection site of mice at 8 h after injection with CM-chitin gel containing NCS. However, there are no discernible differences between NCS and NCS-containing gel injected mice in the arrest and retention of labeled NCS in lung, liver, spleen and kidneys. When NCS-containing gel was injected i.v. into mice, significantly higher levels of NCS were observed in the lung, liver and spleen of mice as compared with NCS alone. Also we histologically observed the injected site in the skin at 10 days after s.c. administration of NCS (Fig. 6(A)) or NCS-containing gel (Fig. 6(B)). Figure 6(A) shows the regenerative phase of the degenerated changes which were caused by acute inflammatory reaction with high concentrations of NCS in the locale of the injection at an early stage. Drastic ulceration was still observed in the skin. There are newly formed capillaries and collagen fibers around the degenerated collagenous tissue. NCS appears to disappear from the injection site 10 days after administration in soluble form. In the case of NCS-containing gel (Fig. 6(B)), on the other hand, small necrotic foci and degenerated areas were scattered in the subcuticular and subcutaneous fat tissue and massive infiltration of macrophages, which engulfed brown pigments (iron-containing gel), were seen around these areas. NCS incorporated in the gel caused more mild damage and persistent inflammatory reaction to the tissues around the inoculated gel and showed longer retention in the injected sites.

Inhibition of spontaneous lung metastasis by CM-chitin gel containing NCS

We next examined the effect of gel containing NCS on lung metastasis of B16-BL6 melanoma in the spontaneous metastasis model (Table 2). Three intermittent administrations of NCS, NCS-incorporated gel or a mixture of NCS and empty gel, or six administrations of NCS significantly reduced the number of lung tumor colonies. In particular, NCS-containing gel inhibited tumor metastasis more effectively than either

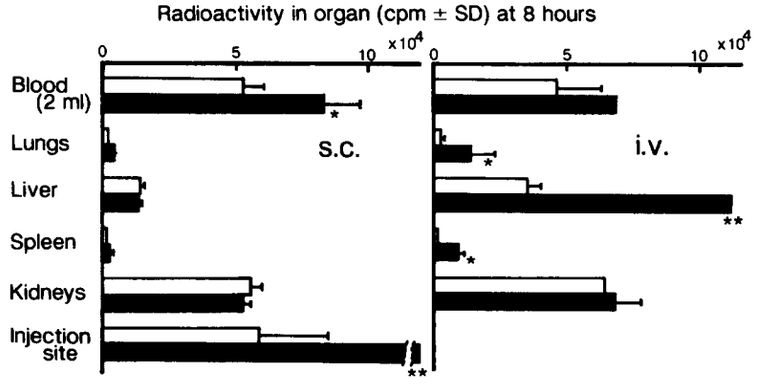

Fig. 5. Organ and tissue distribution of ^{125}I-labeled NCS injected i.v. or s.c. into mice. Labeled NCS (9·8 μg, □) or gel containing labeled NCS (9·8 μg/9·8 × 10^7 cpm/115 μg gel, ■) were injected s.c. into the left flank or i.v. into the lateral tail vein of mice. At 8 h after the injection, mice were killed and radioactivity retained in the organs were measured. Results are mean cpm ± SD of three mice per group. * $P < 0.05$; ** $P < 0.005$.

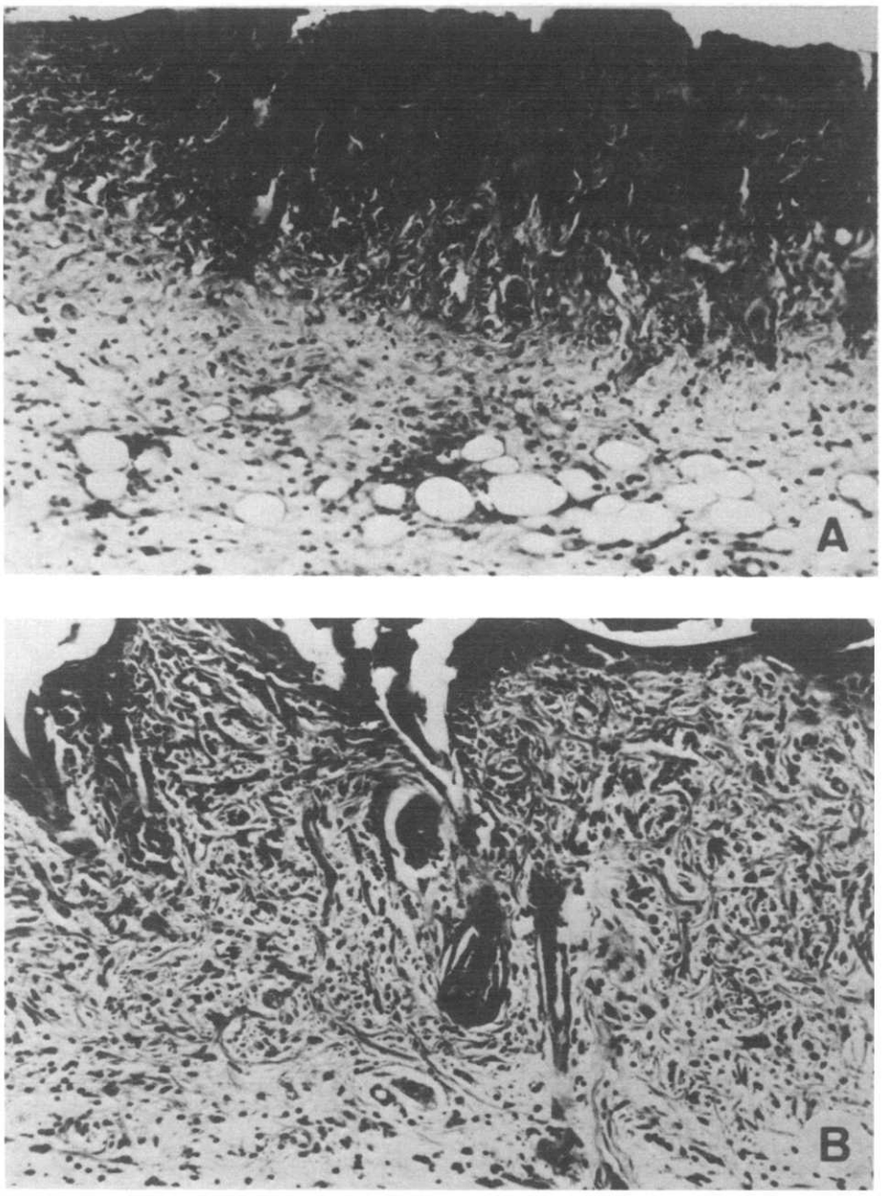

Fig. 6. Photomicroscopy of the NCS-injected site of skin. A: NCS (10 μg)-injected. The skin undergoes ulceration and the hyaline degenerated collagenous tissue is exposed. In the surrounding tissue newly formed capillaries and collagen fibers are seen. Haematoxylin eosin staining (H.E.) 160×. B: NCS-containing gel (10 μg/330 μg gel)-injected. The epidermal layer is thin and the hair follicles show destructive changes. The cutis and subcutaneous tissue show diffuse infiltration of mononuclear cells, including gel-phagocytized macrophages and polymorphonuclear cells. H.E. 100×.

NCS or a mixture of NCS and empty gel by three s.c. administrations after the amputation ($P < 0{\cdot}001$, respectively). However, no reduction of lung tumor colonies was observed by the administration of CM-chitin gel alone.

DISCUSSION

The applicability of natural polysaccharides in the design of dosage forms for sustained release has been examined as follows: some of these polysaccharides are known to form gels in which drugs may be incorporated by the formation of matrices. Agar in the form of beads, konjac in the form of cylinders and natural gums in the form of compressed tablets have been examined as matrices for sustained release of drugs by Nakano *et al.* (Nakano *et al.*, 1979*a*,*b*,*c*,*d*, 1980*a*,*b*). Takahashi *et al.* (1978) have reported that pectin was shown to form water-insoluble complexes with nonsteroidal anti-inflammatory drugs such as benzydamine hydrochloride and ketoprofen and

Table 2. Effect of NCS-containing CM-chitin gel on spontaneous lung metastases caused by intrafootpad injection of B16-BL6 melanoma cells

S.c. administration	Amount	Day	No. of lung metastases on day 35 mean ± SD (range)	P[a]	
Control (PBS)	—	22,25,28	84 ± 27 (57–120)		
NCS-containing gel	10 μg / 330 μg	22,25,28	12 ± 3 (9– 18)	<0·001	<0·001
NCS + Gel	10 μg + 330 μg	22,25,28	34 ± 4 (30– 39)	<0·001	
Gel alone	330 μg	22,25,28	79 ± 22 (82–100)		<0·001
NCS	10 μg	22,25,28	35 ± 7 (24– 42)	<0·05	
NCS	10 μg	22,24,26 28,30,32	15 ± 6 (9– 17)	<0·001	

Five C57BL/6 mice per group were administered s.c. with NCS or NCS-containing CM-chitin gel at various days after surgical excision of primary tumor on day 21 (average tumor size: 11 ± 3 mm in diameter). Mice were sacrificed 2 weeks after tumor excision.
[a]Compared with control by Student's two-tailed *t*-test.

resulted in sustained release. Miyazaki *et al.* (1981) reported that sustained release of indomethacin and papaverine hydrochloride was obtained from dried gels of chitin and chitosan. The applicability of chitosan as a vehicle for sustained release of propranolol hydrochloride was examined by Sawayanagi *et al.* (1982). We have previously demonstrated that CM-chitin gel was easily prepared by the addition of 15 to 30 mM iron (III) chloride into a CM-chitin (0·5–0·8 degree of substitution) solution (Watanabe *et al.*, 1990). BSA or anticancer drug doxorubicin (DOX) were able to be incorporated into the gel under these conditions, and BSA or DOX were released from the gel by lysozyme treatment in a time-dependent fashion (Watanabe *et al.*, 1990).

In the present study, we selected the peptidic anticancer drug NCS to prepare the sustained releasing CM-chitin gel, and examined *in vivo* antimetastatic effects in comparison with NCS alone. The addition of 15 to 30 mM iron (III) chloride to the mixture of CM-chitin and NCS solution caused CM-chitin gel formation, but NCS was slightly incorporated into the resultant gel (less than 6%). However, when 25 to 50 mM calcium chloride, as well as iron (III) chloride, were added to the mixture, NCS was efficiently incorporated into the gel (approximately 50%). The reason for the difference in the incorporation of NCS is still unclear. It may be partially due to low affinity between NCS and the hydrophilic conditions in the gel, or high diffusibility of NCS from the matrix because NCS consists of richly nonpolar or hydrophobic amino acids such as Ala, Val, Leu, Ile, Trp and Phe, and is much smaller in molecular weight than BSA. It has been reported that the interaction of CM-chitin with fibrinogen was enhanced in the presence of calcium ion (Nishimura *et al.*, 1984*a*), and CM-chitin-Ca complex is expected to interact tightly with the hydrophobic domain (especially Phe) of fibrinogen (Uraki & Tokura, 1988). Therefore, the addition of calcium ion is likely to facilitate the incorporation of NCS into CM-chitin gel. Further study will be needed to analyze these points.

The gel thus prepared was much smaller on average (0·695 μm in diameter) than human platelet and red blood cells, and showed homogeneous size distribution. It may be practical to deliver the gel by intradermal or i.v. administrations rather than surgical implantation. In addition, since CM-chitin gel is biodegradable (i.e. lysozyme-susceptible), it may not be necessary to remove the gel carrier from the inoculated sites. NCS was shown to be continuously released from the NCS-incorporated gel by lysozyme digestion at physiological concentrations *in vitro* (Fig. 3). 80% of NCS initially added was released during 7-day incubation with lysozyme. However, 50% of NCS was released in the absence of lysozyme during the same incubation time, indicating that it is due to the spontaneous release of NCS from the gel. In the case of BSA-containing gel, BSA was observed to be slightly released from the gel in the absence of lysozyme (approximately 5%). This may be partially associated with the low affinity between NCS and CM-chitin as mentioned above.

To analyze the controlled release of NCS from the gel *in vivo*, we compared the NCS level in plasma after the s.c. administration of NCS-containing gel or NCS. The administration of 10 μg NCS showed that NCS concentration in the plasma increased within 1 h and thereafter rapidly decreased up to 24 h (Fig. 4). In contrast, a maximum plasma concentration of NCS was observed 6 h after the s.c. administration of NCS-containing gel, and thereafter NCS was slowly cleared from the circulation (Fig. 4). When ^{125}I-labeled NCS or gel containing ^{125}I-labeled NCS was administered s.c. into mice, the radioactivity in blood or at the injection site of gel-treated mice was higher than that of NCS-treated mice at 8 h after the injection (Fig. 5). These results suggest that CM-chitin gel containing NCS is useful for sustaining release of the short half-life NCS in the circulation, which may result in the prolongation

of the drug action. When the gel containing ^{125}I-labeled NCS was injected i.v. into mice, higher values of NCS was observed in lung, spleen and liver (Fig. 5). This result may be due to the arrest of the gel in the chosen organs and subsequent release of ^{125}I-labeled NCS from the gel. As shown in Table 2, three intermittent administrations of NCS-containing gel inhibited the spontaneous lung metastasis of B16-BL6 melanoma as effectively as six administrations of NCS alone. This result indicates that the administration of NCS-containing gel may lead to the reduction of the injection frequency or the total dosage of NCS as compared with unincorporated NCS. In a separate experiment, there are no significant differences between NCS-containing gel and NCS injected mice in the prolongation of survival time, whereas the administrations of NCS caused a marked decrease in body weight prior to death as compared with those of NCS-containing gel (data not shown). Further study will be needed to examine the dosage schedules in detail for prevention of spontaneous metastasis and prolongation of survival time. It is, however, expected that the application of CM-chitin gel as a sustained release-carrier for drugs might provide a promising modality for the therapy of some diseases including cancer. We are also applying gel preparations containing various drugs such as cytokines, and regulating the release of drugs from the gel.

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