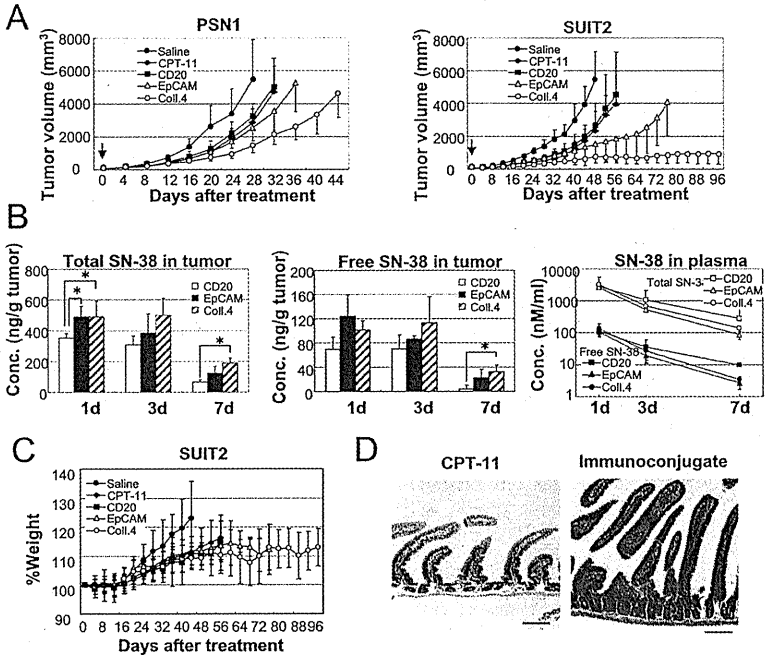

Figure 4. Antitumor effects, pharmacokinetics, and drug toxicities of anti-CD20, EpCAM, and collagen 4 immunoconjugates. (A) Antitumor activities in vivo were examined. In animal models of PSN1 and SUIT2, the three immunoconjugates or saline as control was administered to separate groups of mice by intravenous bolus injection on day 0. Arrows indicate day of administration, and the curves illustrate the effects of the treatments on tumor size. $P < 0.05$ (saline or CD20 vs EpCAM in PSN1), $P < 0.01$ (saline vs CPT11 or EpCAM in PSN1, CPT11 or CD20 vs EpCAM in SUIT2), $P < 0.001$ (saline vs CPT11 or CD20 or EpCAM in SUIT2, saline or CPT11 or CD20 or EpCAM vs collagen 4 in PSN1 or SUIT2). Bar = SD. (B) Tumor concentrations of total (bound and unbound) SN-38 (left) and free (unbound) SN-38 (center), and plasma concentrations (right) were determined using HPLC analysis. The concentrations on days 1, 3, and 7 are shown. * indicates $P < 0.05$; bar = SD. (C) Changes in the % body weight of saline, CPT-11, CD20, EpCAM, and collagen 4 in the same treated SUIT2 group (panel A). Bar = SD. (D) Pathologic mucosal change of jejunum from mouse treated with CPT11 (upper) or anti-collagen 4 immunoconjugate (lower). Scale bar = 1 mm. Coll.4, Collagen 4; Conc., concentration.

In addition there was a signal of near-infrared fluorescence at the area of lung after the treatment with anti-collagen 4 mAb (Figure 3B). We recognized same signals with some other commercially available antibodies such as anti-EGFR mAb. Although we have never observed lung toxicity in mice following administration of our immunoconjugate, we certainly need further investigation of this issue.

Antitumor Activity and Pharmacokinetic Studies in Stroma-Poor or -Rich Pancreatic Tumor Xenografts. Antitumor activities of immunoconjugates with ester bond SN-38 were evaluated in vivo. CPT-11 (clinically approved prodrug of SN-38, 66.7 mg/kg at maximum tolerance dose of this mouse, human clinical application is 100−750 mg/m^2)[24] and three immunoconjugates (administered once, at an equivalent SN-38 dose of 3 mg/kg; cytotoxic effect of SN-38 is 20 times more potent than CPT-11)[25,26] showed significant antitumor activities compared with results in mice treated with saline, in mice bearing either PSN1 (EpCAM-positive and stroma-poor) or SUIT2 (EpCAM-positive and stroma-rich) tumors. In SUIT2 tumors, while the tumor continued to increase in mice treated with CPT-11, anti-CD20 immunoconjugate, and anti-EpCAM immunoconjugate, the tumor treated in mice with anti-collagen 4 immunoconjugate stopped growing by about 1 month and never resumed up to 3 months (Figure 4A). In mice bearing PSN1 tumors (stroma poor), differences were present but less marked. Thus, anti-collagen 4−SN-38 immunoconjugate exerted the most potent antitumor activity compared with anti-CD20 or anti-EpCAM immunoconjugates and CPT-11, but the PSN1 tumor volume continued to increase slowly (Figure 4A). In both tumor models, anti-EpCAM immunoconjugate exerted superior antitumor effect compared with CPT-11 and anti-CD20 immunoconjugate, but inferior antitumor effect compared with anti-collagen 4 SN38-immunoconjugate.

A pharmacokinetic analysis in SUIT2-tumor bearing mice showed that the same high tumor concentrations of either free or total SN-38 (free and conjugated SN-38) were observed for all three immunoconjugates by day 3. On day 7, the tumor concentration of free and total SN-38 was scarcely detected in the tumor treated with anti-CD20 immunoconjugate (Figure 4B). On the other hand, significantly higher concentrations of free and total SN-38 were detected in tumor tissues of mice treated with the anti-collagen 4 immunoconjugate compared with the anti-CD20 immunoconjugate (Figure 4B). The tumor concentration of free and total SN-38 treated with anti-EpCAM immunoconjugate was intermediate among them, but not significant (Figure 4B). Regarding normal tissue distribution and elimination of antibodies and SN-38, there was no difference among immunoconjugates on day 7 after the administration. There was no significant difference in body weight changes among saline, CPT-11, and immunoconjugate groups (Figure 4C). In addition, there was no hepatotoxicity, nephrotoxicity, or bone marrow toxicity in mice treated with all three immunoconjugates compared with controls (data not shown). In the small intestinal mucosa of mice, widespread villous atrophy and decreased crypt density were observed during the treatment with free unbound CPT-11, which is well-known to have severe intestinal toxicity in

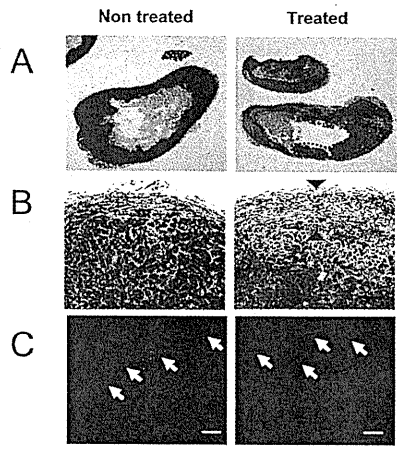

Figure 5. Histopathological features of SUIT2 tumors after anti-collagen 4 immunoconjugate treatment. (A) Hematoxylin and eosin staining of nontreated (left) and immunoconjugate-treated (right) SUIT2-tumors. A non-necrotic viable lesion in the treated tumor is enclosed by a dotted line. (B) The fibrotic capsule width in the treated tumor is indicated between black arrowheads. (C) Tumor vessels were examined by the CD31 (red)−collagen 4 (green) double-staining techniques. White arrows indicate tumor vessels or their traces in the boundary area. Scale bar = 100 μm.

clinics. On the other hand, the small intestinal mucosa of mice in groups treated with all immunoconjugates did not show any pathological change (Figure 4D).

Histopathological Features of SUIT2 Tumors after Anti-collagen 4 Immunoconjugate Treatment. The most important observation from a therapeutic standpoint was that only SUIT2 tumors treated with anti-collagen 4 immunoconjugate stopped growing about 1 month after treatment and remained dormant for more than 3 months. It is concluded that the strategy of orchestrating slow sustained release from a scaffold erected on the stable inert structural components of the tumor stroma is most effective. We compared histologically this nongrowing tumor with a size-matched, growing control tumor and found that both tumors showed central necrosis due to decreased blood flow, which is often observed in a murine xenotransplant model.[27,28] The striking difference was that large confluent necrotic zones and dense fibrotic capsule formation were observed only in the treated tumor (Figure 5A,B). In addition, CD31-positive endothelial cells, which may be tumor-feeding vessels in the peripheral part of the tumor, were never observed in the treated tumor compared with untreated control (Figure 5C). Instead, many collagen 4-positive round profiles corresponding to traces of destroyed vessels were observed in the peripheral area of the treated tumor (Figure 5C).

DISCUSSION

Cancer cells can acquire drug resistance via cellular and noncellular mechanisms.[7−9] The latter mechanisms involve various physiological barriers including excess interstitial stromal tissue, which prevents drug diffusion especially in human cancer.[7−10] Therefore, there is a rationale in overcoming this problem. In this study, we established two types of mAbs: anti-collagen 4 mAb for stroma targeting and anti-EpCAM mAb for tumor cell targeting. We then conjugated these mAbs with SN-38 using a newly designed linker assembly. Linker technology is an important part of immunoconjugate chemotherapy, and various linkers have been exploited to date. Among them, acid labile hydrazone linkage, thiol reduction of disulfide linkers, and enzymatic proteolysis of peptide linkers were favorably applied to ensure stability in plasma.[29,30] For these types of linkers, cell-mediated endocytosis and intracellular processing of the immunoconjugates were indispensable to make the active agent work. In our newly deployed linker construct, an ester bond was used for gradual cleavage in the extracellular space of the tumor. The interspaced PEG enables long survival of our immunoconjugates in the circulating plasma through a "stealth effect" permitting them to evade the RES. In addition, the immunoconjugates are too large to pass through the normal vessel wall, but they easily extravasate from leaky tumor vessels. Thus, our newly developed immunoconjugates can accumulate selectively in the tumor tissue and then exert sustained-release of SN-38. Our basic strategy for overcoming the stromal barrier as a protective shield for cancer cells is to make use of the stroma as a scaffold assembly of immunoconjugates, followed by the release of payload (anticancer agent) to the cancer cells. This free SN-38 can easily reach the cancer cells by diffusion, although the carrier mAb can hardly penetrate the stroma. Moreover, it was reported that collagen 4 in the outer layer of tumor vessels showed conspicuous structural abnormality and loose connection with both endothelial cells and pericytes, whereas that in normal tissue was well organized and tightly associated with the cells.[31] Therefore, SN-38-conjugated anti-collagen 4 mAb, which leaked out from tumor vasculature, can easily bind selectively to the collagen 4 matrix and accumulates within tumor tissues. It has been reported that tumor growth is angiogenesis-dependent and that tumors cannot grow if endothelial cell growth is limited. Moreover, there is evidence that accelerated tumor progression is only observed during the transition from the nonangiogenic stage to the angiogenic stage, a process termed "angiogenic switch".[32] Therefore, if this switch is turned off, tumors will stop growing and remain dormant indefinitely. Here, in our studies, only tumors treated with anti-collagen 4 mAb showed growth suppression and conversion to a dormant status, in keeping with reversal of the "switch". Our anti-collagen 4 immunoconjugate (mAb-bearing SN-38) can induce both tumor regression and the dormant state after a single, low-dose (SN-38, 3 mg/kg) injection. Interestingly, a dense fibrotic capsule was observed in the dormant tumor, which may be formed as a result of damage to the tumor vessels by released SN-38 and reaction of host defense in this lesion.

In our study, the antitumor activity of anti-EpCAM immunoconjugate was inferior to that of anti-collagen 4. We suggest that anti-EpCAM immunoconjugate may distribute unevenly within tumor cell areas and may possess less antivascular endothelial cell activity compared with anti-collagen 4 immunoconjugate.

The concentration of our immunoconjugate was much higher than that of trastuzumab−DM1 or other immunoconjugates used in previous studies.[33−35] The strategy of previous work was to reduce the dose of the mAb by selecting a very toxic agent and binding it to a mAb that can internalize into the tumor cells. Conversely, the principle of our strategy is that our immunoconjugate targets collagen 4 outside the tumor cells and allows the effective sustained release of SN-38 to attack tumor cells and vascular endothelial cells. The internalization ability of the antibody is irrelevant to our strategy. Therefore, in future we will have to exploit the linker to attach many drugs in combination with using a much more toxic agent like DM1 rather than SN-38

in order to reduce the administration dose of the antibody for clinical application.

There were a few papers describing tumor stromal targeting immunoconjugates, a mAb against a cell surface antigen FAP as fibroblast targeting therapy utilizing the internalization, or a mAb against fibronectin for the targeting of tumor vascular endothelial cells in photodynamic therapy.[14,15] In the latter, the drug directly damaged the stromal endothelial cells by the short and strong photochemical reaction. However, unlike those, our strategic concept is quite unique as follows:

(1) Newly developed SN-38-conjugated anti-collagen 4 Abs can extravasate from the leaky tumor vessels selectively and form a scaffold as it is captured by the collagen 4 network.

(2) The immunoconjugate allows the effective sustained release of SN-38 (a time-dependent anticancer agent) from the scaffold, and this released anticancer agent is distributed throughout the tumor.

(3) Consequently, the strategy described above was highly effective in causing arrest of tumor growth due to induced damage to tumor cells and tumor vessels without exerting adverse drug effects.The present method can be applied to other anticancer agents including molecular targeting agents by minor modification of chemical bonding between PEG and the drugs.

AUTHOR INFORMATION

Corresponding Author

*E-mail: yhmatsum@east.ncc.go.jp. Telephone and fax number: +81-4-7134-6857.

Notes

The authors declare that they have no conflict of interest.

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PHASE II STUDIES

Phase II study of NK105, a paclitaxel-incorporating micellar nanoparticle, for previously treated advanced or recurrent gastric cancer

Ken Kato · Keisho Chin · Takaki Yoshikawa · Kensei Yamaguchi · Yasushi Tsuji · Taito Esaki · Kenji Sakai · Masami Kimura · Tetsuya Hamaguchi · Yasuhiro Shimada · Yasuhiro Matsumura · Ryuji Ikeda

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Summary *Purpose* NK105 is a new drug delivery system formulation for paclitaxel (PTX) whose recommended dose (RD) is 150 mg PTX equivalent/m^2 administered every 3 weeks, as determined in a phase I trial. This study aimed to evaluate the efficacy and safety of NK105 in patients with advanced gastric cancer after failure of first-line chemotherapy. *Experimental design* Eligible patients had measurable disease and one chemotherapeutic regimen except taxane. NK105 (150 mg PTX equivalent/m^2) was administered by a 30-minute intravenous infusion every 3 weeks without anti-allergic premedication until disease progression, unacceptable toxicity or patient refusal. The primary efficacy endpoint was best overall response rate (ORR) post baseline. The secondary endpoints were progression-free survival (PFS), time to treatment failure (TTF) and overall survival (OS). All adverse events were reported using CTCAE v3.0. *Results* Between November 2007 and July 2009, 57 patients were enrolled and 56 were evaluable for efficacy. Two complete responses and 12 partial responses were observed for an ORR of 25%. The median PFS was 3.0 months, the median TTF was 2.8 months, and the median OS was 14.4 months. Drug related toxicity was mainly mild (grades 1–2) to severe (grades 3–4); other data: neutropenia (64.9%); leukopenia (17.5%); lymphopenia (8.8%); neuropathy-sensory (1.8%); fatigue (3.5%); and stomatitis (1.8%). There were no treatment-related deaths. *Conclusions* This study of NK105 (150 mg PTX equivalent/m^2) proves the concept for the modest activity and tolerability of a new drug delivery system formulation for PTX. A phase III trial will be evaluated to clarify survival benefit.

K. Kato (✉) · T. Hamaguchi · Y. Shimada
Gastrointestinal Oncology Division,
National Cancer Center Hospital,
5-1-1 Tsukiji, Chuo-ku,
104-0045, Tokyo, Japan
e-mail: kenkato@ncc.go.jp

K. Chin
Department of Cancer Chemotherapy,
Cancer Institute Hospital Tokyo,
Tokyo, Japan

T. Yoshikawa
Department of Gastrointestinal Surgery, Kanagawa Cancer Center,
Yokohama, Japan

K. Yamaguchi
Department of Gastroenterology, Saitama Cancer Center,
Saitama, Japan

Y. Tsuji
Department of Medical Oncology, Tonan Hospital,
Sapporo, Japan

T. Esaki
Gastrointestinal Oncology and Medical Oncology Division,
National Kyushu Cancer Center,
Fukuoka, Japan

K. Sakai
Department of Medical Oncology, Saiseikai Kumamoto Hospital,
Kumamoto, Japan

M. Kimura
Department of Surgery,
Health Insurance Hitoyoshi General Hospital,
Kumamoto, Japan

Y. Matsumura
Investigative Treatment Division,
National Cancer Center Hospital East,
Chiba, Japan

R. Ikeda
Pharmaceutical Development, Nippon Kayaku Co., Ltd.,
Tokyo, Japan

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Introduction

Gastric cancer has a high incidence rate in Asia and Eastern Europe, and is the fourth most common cancer with 933,937 new cases reported annually as of 2002 [1]. Despite a continuous decline in incidence, gastric cancer remains the second leading cause of cancer-related deaths with an estimated 700,349 deaths annually [1]. In Japan, gastric cancer is the second leading cause of cancer deaths with 50,160 new cases in 2008, accounting for 15.3% of all cancer deaths [2].

Although surgical resection is the only curative treatment for gastric cancer, two-thirds of patients are usually diagnosed in the advanced or metastatic stage. Furthermore, 50% of patients experience a relapse after curative resection. Systemic chemotherapy has been investigated for decades aiming at improving the survival time of metastatic or recurrent gastric cancer patients. Fluoropyrimidine in combination with a platinum agent was mainly used as first-line chemotherapy worldwide, and this combination has been investigated in most of the recently completed phase III clinical trials, showing a median survival time of 10–13 months [3–7]. There is little evidence of the survival benefit of second-line chemotherapy, although some reports based on retrospective analysis have shown its potential [8]. Furthermore, second-line irinotecan showed survival benefit compared with best supportive care (BSC) after failure of fluoropyrimidine-based therapy in a randomized trial [9]. However, only a small number of patients (30–60%) receive this second-line chemotherapy in every clinical practice because of their poor condition following first-line chemotherapy [10, 11]. In Japan, weekly paclitaxel (PTX) is commonly used as second-line chemotherapy for gastric cancer, showing a response rate of 16–20% and an overall survival (OS) time of 5–7 months with modest toxicity [12, 13].

PTX has a wide spectrum of antitumor activity including ovarian, breast, gastric and lung cancers [14–16]. The clinically used PTX preparation is a mixture of Cremophor EL because of PTX's poor water solubility. However, the use of Cremophor EL is associated with acute hypersensitivity reactions [17–19]. NK105 is a PTX-incorporating 'core-shell-type' polymeric micellar nanoparticle formulation [20]. The nanoparticle can be injected intravenously without using Cremophor EL as a vehicle. Therefore, NK105 is expected to possess a clinical advantage over PTX. On the other hand, macromolecular drugs, including NK105, are developed based on the characteristic macroscopic features of solid tumors such as hypervasculature, presence of vascular permeability factors stimulating extravasation within the cancer, and suppressed lymphatic clearance of macromolecules. These characteristics unique to solid tumors constitute the basis of the enhanced permeability and retention (EPR) effect [21, 22]. Notably, the in vivo antitumor activity of NK105 was significantly more potent than that of free PTX. In a phase I study, the recommended dose was 150 mg/m^2 administered every 3 weeks [23]. This was less than that of conventional PTX; however, the plasma area under the curve (AUC) of the recommended dose of NK105 was 15-fold higher than that of conventional PTX (210 mg/m^2). Moreover, hematological and non-hematological toxicities of NK105 were mild and well manageable. In particular, there was no grade 3 peripheral neuropathy even in patients who received more than 5 cycles of NK105. However, these results were only obtained from a small number of patients and therefore a larger population and longer treatment duration need to be evaluated.

This phase II clinical trial using NK105 in patients who were unresponsive to the first-line treatment of gastric cancer was therefore conducted. The specific objectives were to determine the response rate, progression-free survival (PFS), OS, time to treatment failure, and toxicity of NK105.

Patients and methods

Patient eligibility

An open-label, single-arm, multicenter, phase II study was conducted and included patients ≥20 and <75 years of age with pathologically confirmed advanced gastric carcinoma who had received one prior chemotherapy regimen except taxanes, and who had one or more measurable lesions according to the Response Evaluation Criteria in Solid Tumor (RECIST) [24]. Failure (disease progression/discontinuation due to toxicity) within 6 months of the last dose of first-line fluoropyrimidine and/or cisplatin treatment for metastatic disease or adjuvant therapy was required. Before study entry, previous therapies had to be completed for ≥2 weeks for fluoropyrimidine and an immunosuppressive agent, and ≥4 weeks for other chemotherapy, surgery or radiotherapy. Patients were required to have an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2 and adequate organ function (bone marrow function: neutrophils ≥2000/mm^3, platelets ≥10×10^4/mm^3; liver function: serum bilirubin ≤1.8 mg/dL and ALT and AST ≤2.5× the upper limit of normal; renal function: serum creatinine ≤.5 mg/dL). Exclusion criteria were as follows: already detected CNS metastasis, malignant ascites requiring invasive treatment, peripheral neuropathy of at least grade 2, or

severe uncontrolled medical conditions (e.g., impaired heart and lung function, active infection or liver disease). Written informed consent was obtained from all the patients. The study protocol was approved by the institutional review boards of the participating institutions. Financial support was provided by Nippon Kayaku Co., Ltd. (Tokyo, Japan).

Study treatment and assessment

NK105 was supplied by Nippon Kayaku Co., Ltd. (Tokyo, Japan) in 20-mL glass vials containing a dose equivalent to 30 mg of PTX. A previous phase I trial determined the recommended NK105 dose as 150 mg PTX equivalent/m^2 administered every 3 weeks [23]. NK105 was dissolved in 100 mL of 5% glucose and intravenously administered within 30 min without any pre-medication for hypersensitivity. Before each cycle, the patients had no grade ≥2 toxicity. Treatment was terminated if disease progression or severe toxicity was observed. The NK105 dose was reduced to 120 mg/m^2 or 100 mg/m^2 if patients experienced grade 4 neutropenia lasting more than 7 days, grade 4 thrombocytopenia, grade 3 non-hematological toxicity or considerable grade 2 neurosensory toxicity.

Tumor assessment by computed tomography was performed within 4 weeks before registration to this study and repeated every 6 weeks. Overall response rates (ORRs) were evaluated in accordance with RECIST [24]. If the response was observed, CT scans were taken 4 weeks later to confirm the response. Tumor responses were confirmed by an external review committee (ERC). PFS was defined as the duration from the date of the first dose of the study drug to the date of the first confirmation of disease progression as determined by the ERC, or death from any cause, and censored at the last tumor assessment. Time to treatment failure (TTF) was defined as the time from the administration of the first dose of the study drug to the discontinuation of the protocol treatment. OS was defined as the duration from the first dose of the study drug to death.

Adverse events were evaluated weekly in accordance with the Common Terminology Criteria for Adverse Event, version 3.0 [25]. All adverse events were evaluated until 21 days after the last dose of the study drug.

For pharmacokinetics (PK) analysis, blood samples were collected from the first 6 patients before the first dose and 15 and 30 min, 1, 3, 6 and 24 h, 3 days, and 1 week after the first dose in the first cycle. Plasma level of PTX (both micelle-entrapped and released) was measured by liquid chromatography/tandem mass spectrometry. A non-compartmental model using the WinNonlin Professional Ver. 5.2 program (Pharsight Corp., St. Louis, MO, USA) was used to determine the PK parameters of AUC, maximum plasma drug concentration (Cmax), time to reach maximum concentration after drug administration (Tmax) and half-life of the terminal phase ($t_{1/2}$).

Statistical analysis

The primary study endpoints were overall response rate (ORR) as assessed by the ERC. The standard Southwest Oncology Group phase II design was adopted [26], selecting an alpha error of 0.05 and a beta error of 0.10. The minimum activity required for this experimental treatment was 16%, while the alternative hypothesis was to obtain a 33% response rate. Therefore, the accrual had to be stopped if there were less than 4 responses with the first 30 patients, in which an additional 25 patients were planned to be enrolled. Fifty-five patients were required to test the null hypothesis. The secondary endpoints were PFS, TTF, OS, and safety. PFS, TTF, and OS were estimated using the Kaplan-Meier (product-limit) method with 95% confidence interval (CI).

The study was performed in accordance with the Declaration of Helsinki and Good Clinical Practice. Written informed consent was obtained from all patients who participated. The protocol was approved by the independent ethics committee or institutional review board at each site.

Results

Patient characteristics

All 57 patients enrolled between November 2007 and July 2009 were included in the safety analysis; however, 1 was excluded from the efficacy analysis because this patient had no measurable lesion. Therefore, 56 patients were included in the efficacy evaluation of NK105 (Fig. 1). At the data cut-off date (January 7, 2010), 54 patients had discontinued the protocol treatment for the following reasons: disease progression (n=50), adverse events (n=3), and patient

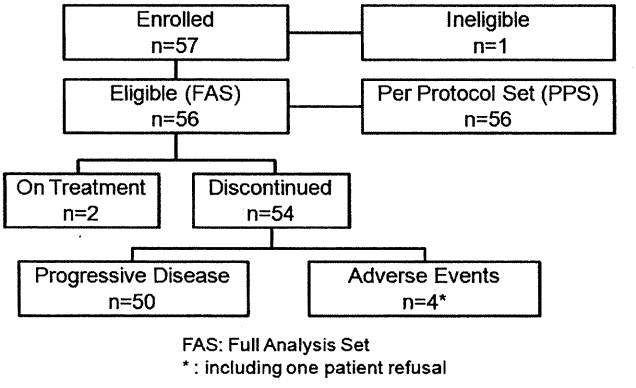

Fig. 1 CONSORT diagram of 57 patients

Table 1 Baseline demographic characteristics

Characteristic	Number of patients	Percentage
Sex		
Male	39	69.6
Female	17	30.4
Age (years)		
Median	63.5	
Range	25-74	
ECOG performance status		
0	43	76.8
1	12	21.4
2	1	1.8
Histologic type		
Intestinal	30	53.6
Diffuse	25	44.6
Unknown	1	1.8
Gastrectomy		
Yes	35	62.5
No	21	37.5
Adjuvant chemotherapy		
S-1	9	16.1
S-1/CDDP	2	3.6
others	2	3.6
Prior chemotherapy		
S-1	9	16.1
S-1/CDDP	24	42.9
Others	10	17.9

ECOG, Eastern Cooperative Oncology Group

refusal (n=1). The baseline demographic characteristics of the enrolled study patient population are shown in Table 1. Most patients were men (69.6%) with a median age of 63.5 years (range, 25–74). The ECOG PS was 0 in 43 patients, 1 in 12, and 2 in 1. Twenty-one (37.5%) patients had primary unresectable advanced gastric cancer and 35 (62.5%) had recurrence after gastrectomy. All patients received prior chemotherapy, namely, an S-1-containing regimen (78.6%) and others (21.4%). The median progression free survival of first line treatment was 6.4 month.

Treatment duration

The median treatment duration was 2.8 months (range, 0.5–18.5) with a median treatment cycle of 4 (range, 1–28). After NK105 discontinuation, 46 patients (82.1%) received chemotherapy. with irinotecan-based regimens (37), whereas 5 continued to receive the protocol treatment or were lost to follow up. Five patients have had BSC rather than chemotherapy. The mean relative dose intensity (ratio of the dose received to the dose planned) was 95.1% for NK105.

Efficacy

At the final data cut-off date (January 7, 2010), the median follow-up duration was 11.3 months (range, 6.0–22.0).

The results of efficacy analysis are shown in Table 2. The ORR was 25% (95% CI: 14.4–38.4%). The median time to response and the duration of response were 1.8 and 3.7 months, respectively. The median PFS, median OS, and median TTF were 3.0 months (95% CI: 2.6–5.3), 14.4 months (95% CI: 12.6–15.9), and 2.8 months (95% CI: 2.5–4.9), respectively (Figs. 2 and 3).

Safety

The major adverse events observed with NK105 were grade 1 or 2 in severity (Table 3). The most common adverse events were alopecia (87.7%), peripheral neuropathy (64.9%), fatigue (57.9%), myalgia (59.6%), anorexia (47.4%), rash (49.1%), althralgia (45.6%), stomatitis (31.6%), diarrhea (22.8%), and nausea (28.1%). The most common grade 4 hematological toxicities were neutropenia (24.6%), anemia (1.8%), leukocytopenia (1.8%), and lymphopenia (1.8%). Grade 3/4 non-hematological toxicities were infrequent, with fatigue (3.5%) and peripheral neuropathy (1.8%). Neutropenic fever was not seen. Although NK105 was administered without any premedication, there were no patients who experienced grade 3/4 hypersensitive reaction.

Dose reductions to reduce toxicity were performed in 7 patients (12.3%). There were 6 serious adverse events

Table 2 Overall response rate

	n	CR	PR	SD	PD	NE	ORR (CR+PR) (95%CI)	DCR (CR+PR+SD) (95%CI)
All patients	56	2	12	17	25	0	25.00% (14.4–38.4)	55.4% (41.5–68.7)
Histologic type								
Intestinal	30	0	6	11	13	0	20.00%	56.70%
Diffuse	25	2	6	6	11	0	32.00%	56.00%
Unknown	1	0	0	0	1	0	0.00%	0.00%

CR complete response
PR partial response
SD stable disease
PD progressive disease
NE not evaluable
ORR overall response rate
DCR disease control rate

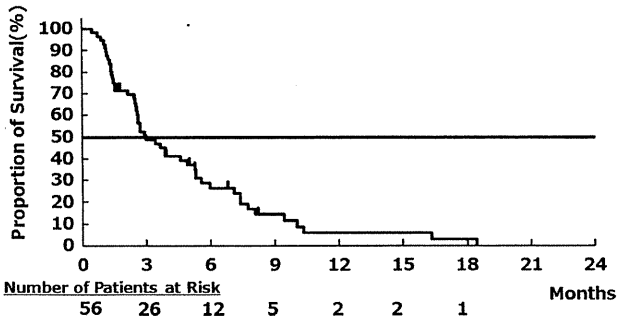

Fig. 2 Progression-free survival of 56 patients

related to NK105 in 2 patients: pneumonia (n=1) and fever (n=1). There were no treatment-related deaths.

Pharmacokinetics

Plasma PTX concentration after the intravenous infusion of NK105 was determined in each of the first 6 patients enrolled in this study. The AUC of NK105 at 150 mg/m^2 was 214±10 μg hr/mL, which was about 9-fold larger than that of conventional PTX at dose of 210 mg/m^2 (conventional dose for every 3 weeks) [27]. The V_{ss} and CL_{tot} of NK105 were significantly lower than those of conventional PTX. These PK parameters were almost similar to those observed in the phase I study.

Discussion

In the present study, we showed that NK105 has activity against advanced gastric cancer with an ORR of 25%, including previously treated gastric cancer with 2 CR cases. However the ORR which is the primary endpoint of this study did not meet the hypothesis, 64.3% of 14 responders in the present trial showed no response to the first-line chemotherapy. Response rates do not always correlate with improved survival as an ultimate goal, particularly for intractable cancers (e.g., gastric cancer). Therefore, the clinical benefit observed in this trial in which 30.4% of the

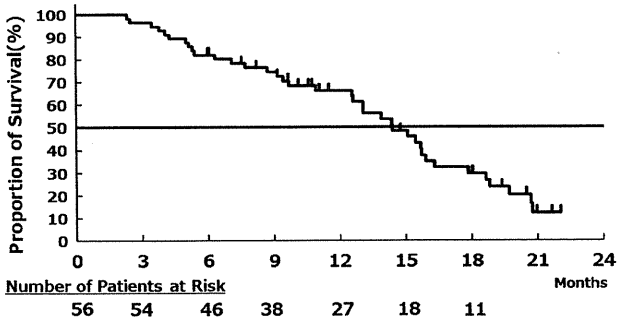

Fig. 3 Overall survival of 56 patients

Table 3 Incidence of common adverse event

Adverse Drug Reaction	Safety analysis set (N=57)					
	Number of patients				All	Grade 3/4
	Grade 1	Grade 2	Grade 3	Grade 4	%	%
Leukocytepenia	12	27	9	1	86	17.5
Neutropenia	5	10	23	14	91.2	64.9
Anemia	5	19	6	1	54.4	12.3
Lymphopenia	16	8	4	1	50.9	8.8
Platelet	15	1	0	0	28.1	0
Anorexia	22	5	0	0	47.4	0
Nausea	15	1	0	0	28.1	0
Diarrhea	11	2	0	0	22.8	0
Stomatitis	15	2	1	0	31.6	1.8
Fatigue	24	7	2	0	57.9	3.6
Arthralgia	22	4	1	0	45.6	1.8
Myalgia	32	2	0	0	59.6	0
Neurosensory toxicity	29	7	1	0	64.9	1.8
Alopecia	34	16	–	–	87.7	0
Rash	23	4	1	0	49.1	1.8
ALT	13	1	0	0	24.6	0
AST	16	2	0	0	31.6	0
G-GTP	9	3	0	0	21.1	0

enrolled patients achieved stable disease for several months is remarkable. Consequently, the disease control rate was 55.4%, which is promising for previously treated patients with advanced gastric cancer. Therefore, the median OS (14.4 months; 95% CI: 12.6–15.9 months) in this study is also reasonably promising.

Although there are several active regimens in the second-line therapy for gastric cancer [11], there is no standard therapy for advanced gastric cancer that has progressed after the initial fluoropyrimidine and cisplatin-based treatment. There is, therefore, an urgent need to develop new regimens that can provide a significant benefit to patients. Several drugs have been investigated in phase II studies for the second-line treatment of gastric cancer, with response rates ranging between 12.5% and 31.8% [11].

NK105 was generally well tolerated and its toxicity features were similar to those of conventional PTX. The most common hematological toxicity was neutropenia. Grade 3 or 4 neutropenia and anemia accounted for 64.9% and 12.3%, respectively, but all hematological toxicities were manageable throughout this study. Non-hematological toxicities were mild and grade 3 at worst. During the entire study, only 1 patient experienced grade 3 neuropathy (1.8%). The incidence rates of grade 3 or 4 neuropathy of other PTX formulations, namely, conventional PTX [27], Opaxio [28], a PTX conjugating poly-L-

glutamic acid, and Abraxane [29, 30], an albumin bound PTX, were 10%, 15%, and 11%, respectively, in each phase II setting. The incidence rates of other major grade 3 non-hematological toxicities were also low, which included 1 stomatitis (1.8%), 2 fatigue (3.6%), 1 arthralgia (1.8%), and 1 rash (1.8%). As patients with advanced gastric cancer suffer from marked anorexia, fatigue, constipation, weight loss, and other conditions, a favorable feature of NK105 is that its induces a lower incidence of gastrointestinal toxicity.

The PK analysis of NK105 in both phase II and the previous phase I studies suggests that the distribution of PTX-incorporating micelles is mostly restricted to the plasma and, in part, to extracellular body fluids. When compared with conventional PTX at a dose of 210 mg/m^2 (conventional 3-week regimen dose), NK105 at a dose of 150 mg/m^2 exhibited more than 9-fold larger plasma AUC and a 26-fold lower CL_{tot}. Specifically, the increased AUC and prolonged release of active paclitaxel from the micelle formulation might produce good efficacy. It is speculated that NK105 preferentially accumulated in tumor tissue utilizing leaky tumor vessels. On the other hand, the Vss of NK105 was more than 10-fold lower than that of conventional PTX, suggesting that PTX may have a relatively lower distribution in normal neural tissue following NK105 administration. Lower distribution in normal tissue caused less toxicity of NK105 than the other paclitaxel formulations. On the other hands, modest anti-tumor activity of NK105, in spite of the larger AUC, may be caused by the hypo-vascular environment of gastric cancer cell.

Formulations categorized in DDS have been developed with some already approved for clinical use or are under clinical evaluation. These preparations include Opaxio (polyglutamate-conjugated PTX) [28] and Abraxane (albumin-coated PTX) [29, 30]. Since PTX is highly water-insoluble, a mixture of Cremophor EL and ethanol must be used to dissolve PTX for injection. However, the use of Cremophor EL is associated with acute hypersensitivity reactions [17–19]. The common advantage of these formulations categorized in DDS is that they are injectable intravenously without the mixture of Cremophor EL and ethanol. The present trial showed that NK105 could be dissolved in 5% glucose solution without Cremophor EL and ethanol, and administered safely at a short infusion time (30 min) without any anti-allergic agents.

In conclusion, NK105 appeared to be very efficacious without compromising the antitumor activity of PTX in patients with previously treated advanced gastric cancer. While we understand at the moment that it is challenging to make direct comparisons between our data of NK105 and the published reports of other PTX formulations, some observations can be made and we believe that the present results warrant further clinical evaluation including a comparison with other clinically available taxanes.

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Cancer stromal targeting (CAST) therapy☆

Yasuhiro Matsumura *

Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, 6-5-1, Kashiwanoha, Kashiwa, 277–8577, Japan

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ABSTRACT

Despite great advances in cell and molecular biology, pharmacology and medicine, there is to date no antitumor drug available which can specifically kill tumor cells in the human body without damaging normal tissue, because it has not been possible to find a truly cancer specific molecule to target.
Low molecular weight (MW) anticancer drugs extravasate easily from normal vessels in the body causing drug adverse effects. Conversely, high MW anti-tumor agents including antibodies against cancer cell antigens, accumulate selectively in tumors because of their leaky vasculature. However, most human solid tumors possess abundant intercellular connective tissue, hindering diffusion of such macromolecules. That is why immunoconjugate therapy for stroma rich common solid cancer has not yet proved successful in clinics. In this review, I describe a successful new strategy that overcomes the above contradictory drawbacks by conjugating a small MW cyototoxic drug with an antibody against particular components of tumor stroma. Stroma-targeting immunconjugates bound to the stroma to create a scaffold, from which sustained release of cytotoxic agent occurred and subsequently diffused throughout the tumor tissue to damage both tumor cells and tumor vessels. Cancer-stroma targeting (CAST) therapy was thus validated as a new modality of oncological therapy, especially for refractory, stromal-rich cancers.

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Contents

1. Preface

Conventional low molecular weight (LMW) anticancer agents (ACA) including molecular targeting agents can easily extravasate from normal blood vessels and are distributed throughout the whole body leading to adverse effects of the drugs (Fig. 1A). In order to overcome such off-target effects caused by LMW ACAs, formulations categorized in drug delivery system (DDS) have been developed to achieve selective delivery of ACAs to cancer tissue at an effective concentration for the appropriate duration of time, so that we may be able to reduce the adverse effects of a LMW drug and simultaneously enhance the antitumor effect.

There are two main concepts in DDS, active targeting and passive targeting. Active targeting involves monoclonal antibodies (mAb) or

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* Tel.: +81 4 7134 6857; fax: +81 4 7134 6866.
E-mail address: yhmatsum@east.ncc.go.jp.

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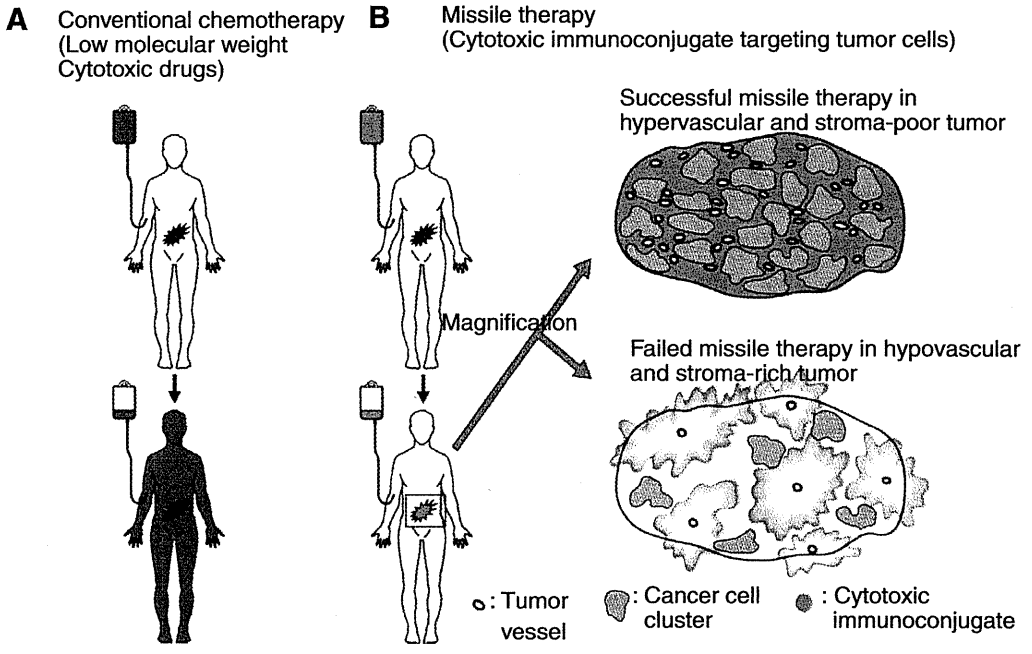

Fig. 1. Drawback of so-called missile therapy. (A) LMW anticancer agents (black) can distribute throughout whole normal body resulting in serious side effects. (B) Cytotoxic immunoconjugates (blue) accumulate selectively in the tumor tissue. Successful (upper) and failed (lower) immunoconjugate therapy were shown.

ligands to tumor related receptors which can target the tumor by utilizing the specific binding ability between the antibody and antigen or between the ligand and its receptor. The passive targeting system can be achieved by the EPR effect, that is, the enhanced permeability and retention effect [1–3]. Small molecules easily leak from normal vessels in the body, which gives small molecules a short plasma half life. On the other hand, macromolecules have a long plasma half life because they are too large to pass through the normal vessel walls, unless they are trapped by the reticuloendothelial system (RES) in various organs. In the solid tumor tissues, it was found that solid tumors generally possess the several pathophysiological characteristics: hypervasculature, secretion of vascular permeability factors stimulating extravasation of macromolecules within the cancer, and absence of effective lymphatic drainage from tumors that impedes the efficient clearance of macromolecules accumulated in solid tumor tissues.

Macromolecules and lipids in the interstitial tissue are known to be recovered via the lymphatics in normal tissues [4]. The limited recovery from the lymphatic system in tumor tissues may be attributed to poor development of the lymphatics in tumor tissues, which has been demonstrated by using lipid lymphographic agents [5].

Although there is no clear anatomical proof that tumor lymphogenesis is implicated in the drainage of extravasated macromolecules in human, some studies have indicated that the growth of lymphatic vessels is actively involved in tumor dissemination [6].

This inconsistency regarding tumor lymphogenesis may be due to differences between mice and humans, or differences among tumor types. These characteristics of solid tumors are the basis of the enhanced permeability and retention effect, the EPR effect (Fig. 2). Based on the EPR effect, several formulations categorized in passive targeting have been developed and some of them such as doxil [7] and abraxane [8] have been approved in clinical use and ACAs incorporating micelles and polymer conjugated ACAs are now under preclinical and clinical evaluation [9–11].

Meanwhile, mAb is also too large to pass through the normal vessel wall but easily extravasate from leaky tumor vessels and long retained in the solid tumor tissue by utilizing the EPR effect. Moreover, mAb can target the tumor cell actively. Therefore, to date, numerous mAbs recognizing molecules on tumor cell surfaces have been developed and conjugated with ACAs, radioisotopes, or toxins to create so-called missile therapy ([12–14] and Table 1). Initially, the missile therapy strategy was expected to be highly successful.

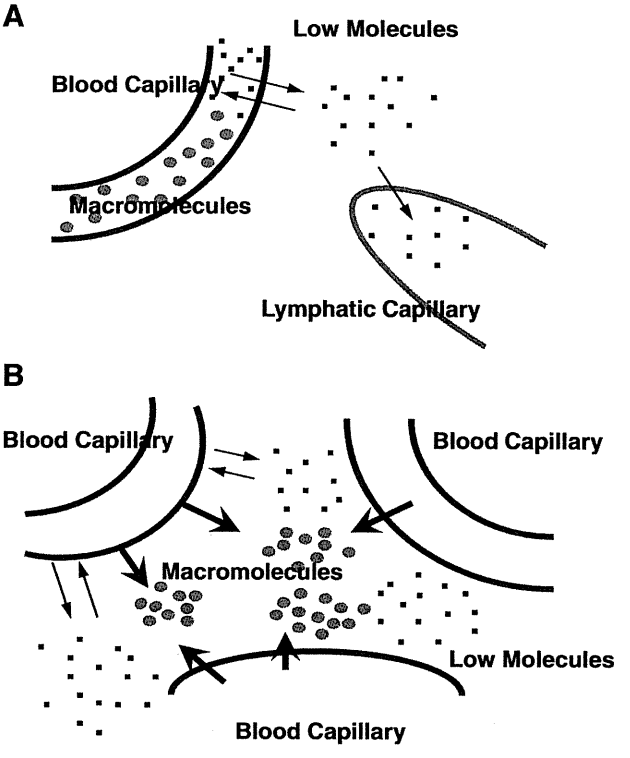

Fig. 2. Enhanced Permeability Retention (EPR) effect.Diagramatic presentation of normal tissue (A) and tumor tissue (B). In tumor (B), macromolecules are extravasated from tumor blood vessels and retained in tumor tissue for a long time because of absence of the lymphatic capillary.Low molecular substances (including a conventional anticancer drug) traverse freely between the interstitial space and the blood capillary.

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Table 1
Immunoconjugates in American Society of Clinical Oncology (ASCO) 2011.

Name	Target molecule	Payload	Cancer	Clinical stage	Supplement
^{131}I-chTNT-1/b	DNA/Histon H1	^{131}I	Glioma	P2	Convection enhanced
NGR-hTNF	CD13	TNF	Ovary	P2	+ Doxorubicin
Ac-lintuzumab	CD33	^{225}Actinium	AML	P2	RadioactiveT1/2 10 days
PSMA-ADC	PSMA	MMAE	Prostate	P1	4 MMAE/Ab
EC145	Folate	Deacetylvinblastine	Ovary	r-P2	Doxil vs. D/EC145
IMGN901	CD56	Maytasinoid	Multiple myeloma	P1	+ Lenaliomide/Dexa
EMD273066	EpCAM	IL2	Solid tumor	P1b	+ Cyclophosphamide
SGN-75	CD70	MMAE	Renal, NHL	P1	4.5 mg/kg/3w
CMC544	CD22	Calicheamycin	ALL	P1	
^{177}Lu-J591	PSMA	177Lu	Prostate	r-P2	^{177}Lu-J591b vs. ^{111}I-J591
Myelotag	CD33	Calicheamycin	AML	P2	Cytarabine, fludarabine
SGN-35	CD30	MMAe	HL	P2	1.8 mg/kg/3w
^{131}ILI9SIR	Tenascin-c	^{131}I	NHL	P1/2	2.05 GBq/m^2
T-DM1	HER2	DM1	Breast	P2	plts
T-DM1	HER2	DM1	Breast	P3	1st line
T-DM1	HER2	DM1	Breast	P3	Previous chemo. Trastuzumab/Taxotere

However, the use of HMW agents presents a dilemma for cancer therapy because the very properties that favor their high accumulation in the lesion also cause low diffusion of these macromolecules within a tumor [15]. Most human solid tumors possess abundant stroma that prevents immunoconjugate diffusion and, consequently, becomes a barrier preventing immunoconjugate from directly attacking cancer cells [15–18].

2. Success and failure of active targeting

Recent successful examples of immunoconjugates include anti-CD33 antibody-calicheamicin conjugate for acute myeloid leukemia and radiolabeled anti-CD20 mAbs for B-cell non-Hodgkin's lymphoma treatment [19]. On the other hand, in most human solid tumors, there are abundant stroma that prevents mAb diffusion and, consequently, becomes a barrier preventing mAb from directly attacking cancer cells. In fact, immunoconjugate therapies for common solid tumors (e.g., colorectal, lung and pancreatic cancers) have not yet proved successful in clinical practice because of heterogeneity of target antigens and low uptake and retention of chemotherapeutic drugs in these types of cancer (Fig. 1B). This contrasts with the results seen in treatment of human hematologic malignancies and of tumor xenografts in mice, which usually have less interstitial tissue within the neoplasm [19]. The kinetics of drug distribution within tumors are considered to be functions of interstitial conductivity, which is determined by the quantity and density of the extracellular matrix (ECM e.g., proteoglycan, fibronectin) and fibrosis (e.g., collagen fiber) in the stroma [15–18]. Such compact assembles of various tissue constituents in solid tumors cause reduced drug penetration and also act as a stromal barrier to prevent the diffusion of mAbs (HMW proteins) [20,21].

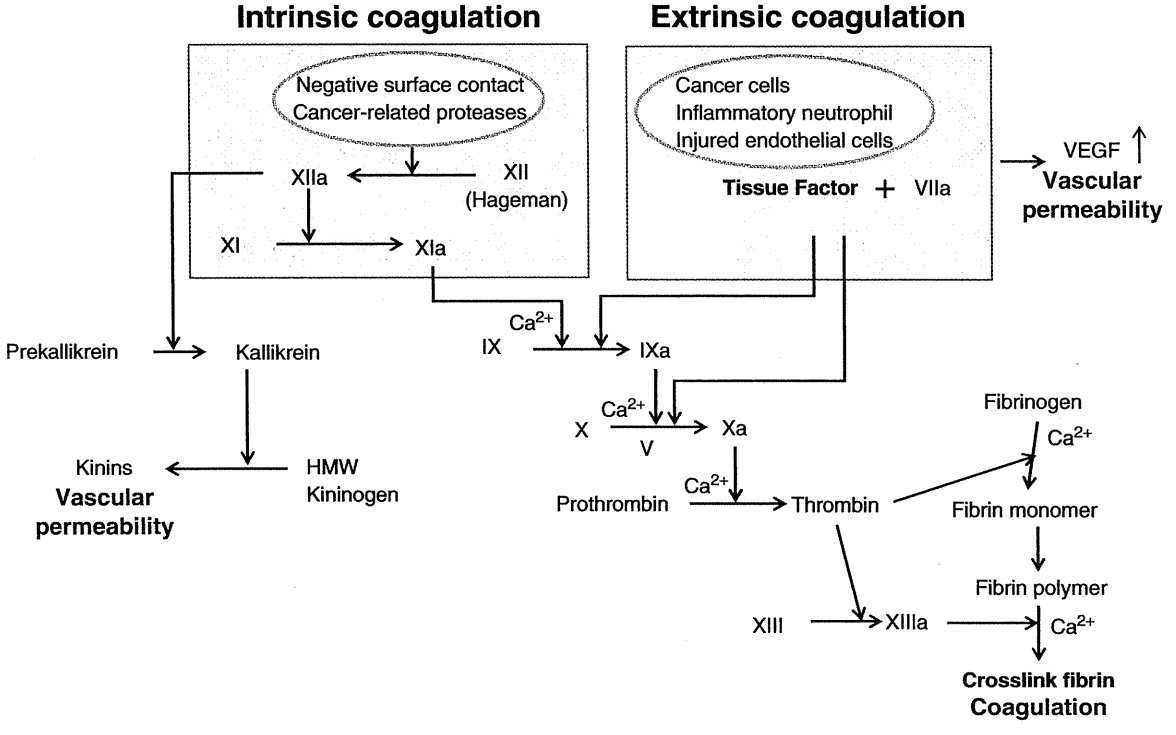

Fig. 3. Cancer and blood coagulation. Both intrinsic and extrinsic coagulation pathway may be involved in tumor vascular permeability.

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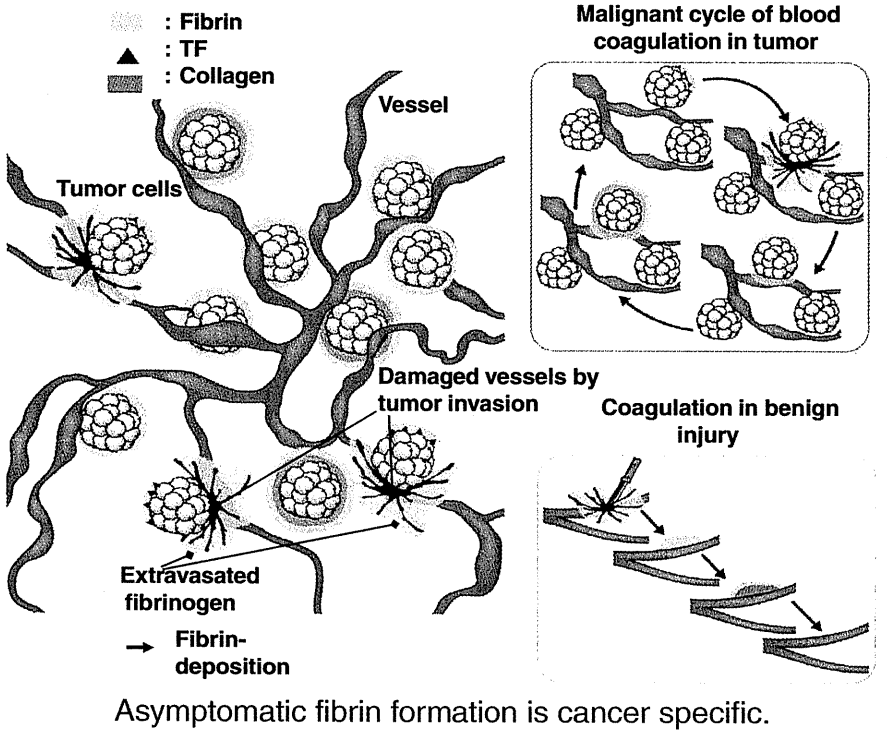

Fig. 4. Diagram of the 'malignant cycle of blood coagulation' in cancer tissue. Although the tissue factor on tumor cells may cause fibrin clot formation, most fibrin clots occur when and where fibrinogen leaks from a damaged vessel wall caused by tumor cell invasion. Fibrin clots are subsequently replaced by collagenous fibers. The malignant cycle of blood coagulation consists of four steps, namely (1) tumor cell invasion into vessel; (2) hemorrhage; (3) fibrin clot formation; and (4) replacement with collagenous tissue. There are many points in common between cancer stroma and wound healing. The absolute difference between cancer stroma and wound healing is that such conditions in cancer last for as long as cancer cells survive.

3. Cancer stroma and blood coagulation

The increased tumor vascular permeability is the most important event for the EPR effect. At the time we proposed the EPR effect, we also succeeded in purifying two types of kinin (bradykinin and hydroxyprolyl^3-bradykinin) from the ascitic fluid of a patient with gastric cancer [1,22].

We also clarified that this kinin generation system was triggered by the activated Hageman factor, an intrinsic coagulation factor XII [23].

Meanwhile, Dvorak et al. discovered that vascular permeability factor (VPF) was involved in tumor vascular permeability [24]. Later, it was found that VPF was identical to vascular endothelial growth factor (VEGF) [25]. Recently, an extrinsic coagulation factor, namely a tissue factor (TF), appeared to activate VEGF production [26]. So, both intrinsic and extrinsic coagulation factors may be involved in tumor vascular permeability as well as tumor-induced blood coagulation (Fig. 3).

In the 19th century, French surgeon Armand Trousseau described thrombophlebitis in patients with stomach cancer for the first time

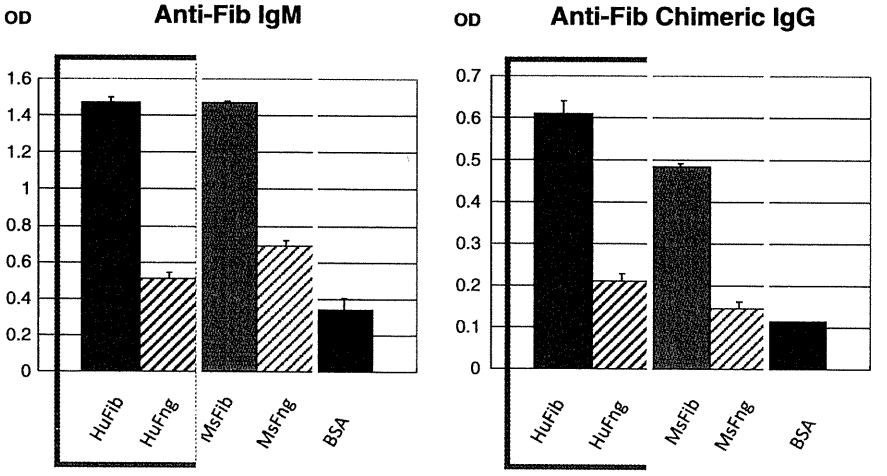

Fig. 5. Anti-fibrin IgM and its chimeric IgG recognize both human and mouse fibrin but not their fibrinogen. ELISA showed that anti-fibrin (Fib) IgM and its chimeric IgG recognize both human (Hu) and mouse (Ms) fibrin but not their fibrinogen (Fng). OD, optimal density [29].

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[27]. Today, a large body of clinical evidence supports the conclusion that abnormal coagulation occurs in a variety of cancer patients [28]. It is now known that TF is highly expressed on the surface of almost all human tumor cells and alternatively spliced soluble TF is also produced by many types of tumor [26]. Therefore, TF may be involved in tumor related abnormal blood coagulation.

Above all, any malignant tumor can erode the surrounding normal tissue, and the more erosive types of cancer have more destructive actions. If these cancer clusters erode adjacent normal or tumor vessels, microscopic haemorrhage may occur at any place and at any time within or adjacent to cancer tissues, and fibrin clots immediately form in situ to stop the bleeding. The fibrin clots are subsequently replaced by collagenous stroma in a process similar to that in normal wound healing and other non-malignant diseases. Fibrin clots formation in non-malignant disorders such as cardiac infarction, brain infarction, injuries, and active rheumatoid arthritis should form only at the onset or active state of disease and subsequently disappear by plasmin digestion or replacement with collagen within a few weeks and is accompanied by some symptoms. On the other hand, the fibrin clot formation in cancer lasts for as long as the cancer cells survive in the body and occurs silently. Therefore, we call this 'malignant cycle of blood coagulation' (Fig. 4). In fact, tumor invasion and metastasis progress without symptoms (which is why imaging instruments are needed). When any symptoms accompanying cancer such as pain, intestinal obstruction, or macroscopic bleeding occur, the cancer is likely to involve sensory nerves and destruction of bones and larger blood vessels, and to occupy the whole lumen of a particular place of the intestine. Usually, patients with an advanced stage of cancer receive chemotherapy and it is worth noting that oncologists never treat such patients if they suffer from existing acute thrombotic complications, bleeding by injury, or active inflammation. Therefore, we conclude that growth factors and tyrosine kinases never become tumor-specific molecules but that fibrin clots in cancer tissues of patients who can receive chemotherapy are actually tumor-specific [29].

4. Monoclonal antibodies targeting cancer stroma

Although there are a few papers describing tumor stromal targeting using immunoconjugates, the target molecule was still a surface antigen on cells in tumor stroma, or the ECM (proteoglycan, fibronectin) was used for targeting the tumor vascular endothelial cell [30,31]. The principle of our strategy is that our newly developed immunoconjugates selectively extravasated from leaky tumor vessels, bound to the extracellular molecules in the stroma and created a scaffold from which effective sustained release of the LMW ACA occurred. This free LMW ACA can easily reach the cancer cells by diffusion through the stroma barrier. To date we have developed mAbs against collagen 4 [32], fibrin (not fibrinogen) [29], and TF that are abundantly found in the stroma of solid tumors, especially invasive tumors. In the case of anti-fibrin mAb, following extensive screening using two ELISA sets, one for human fibrinogen (physiologically existing precursor of fibrin), the other for human fibrin (being only formed at several abnormal conditions), we successfully developed a mAb that reacted only with human fibrin, not with human fibrinogen. However, since the obtained mAb was IgM, it was converted into human IgG format for clinical application using an antibody engineering technique. Another advantage of the anti-fibrin IgM and the chimeric IgG was that they cross-reacted with mouse fibrin but not with mouse fibrinogen (Fig. 5).

5. Drug design, anti-tumor activity, and PK study of immunoconjugates

5.1. Anti-mouse collagen 4 mAb conjugated with SN-38 in human pancreatic tumor xenografts

SN-38 is a topoisomerase 1 inhibitor, and an active component of CPT-11 which is used clinically for colorectal, lung and other cancers. For the mAb conjugation to phenol-OH in SN-38, an ester bond was

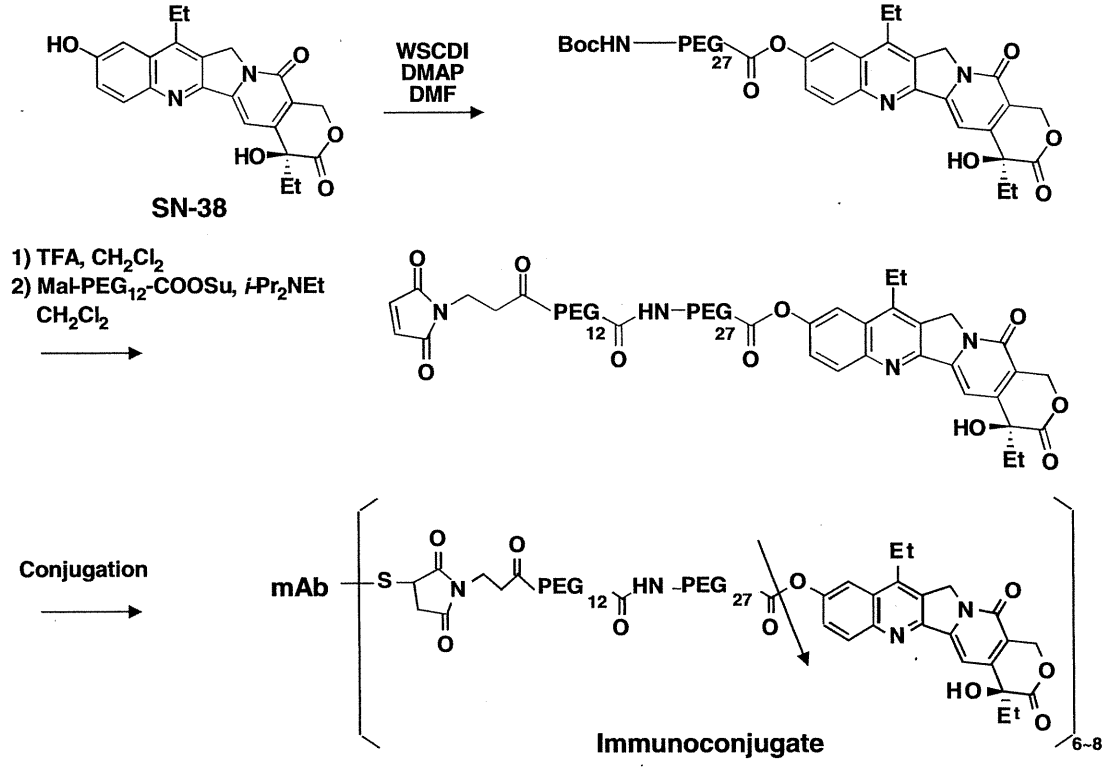

Fig. 6. Structure and drug-release of anti-mouse collagen 4 immunoconjugate. Synthetic scheme of the immunoconjugate. The arrow indicates the cleavage site for releasing free active SN-38. PEG, Polyethylene glycol [32].

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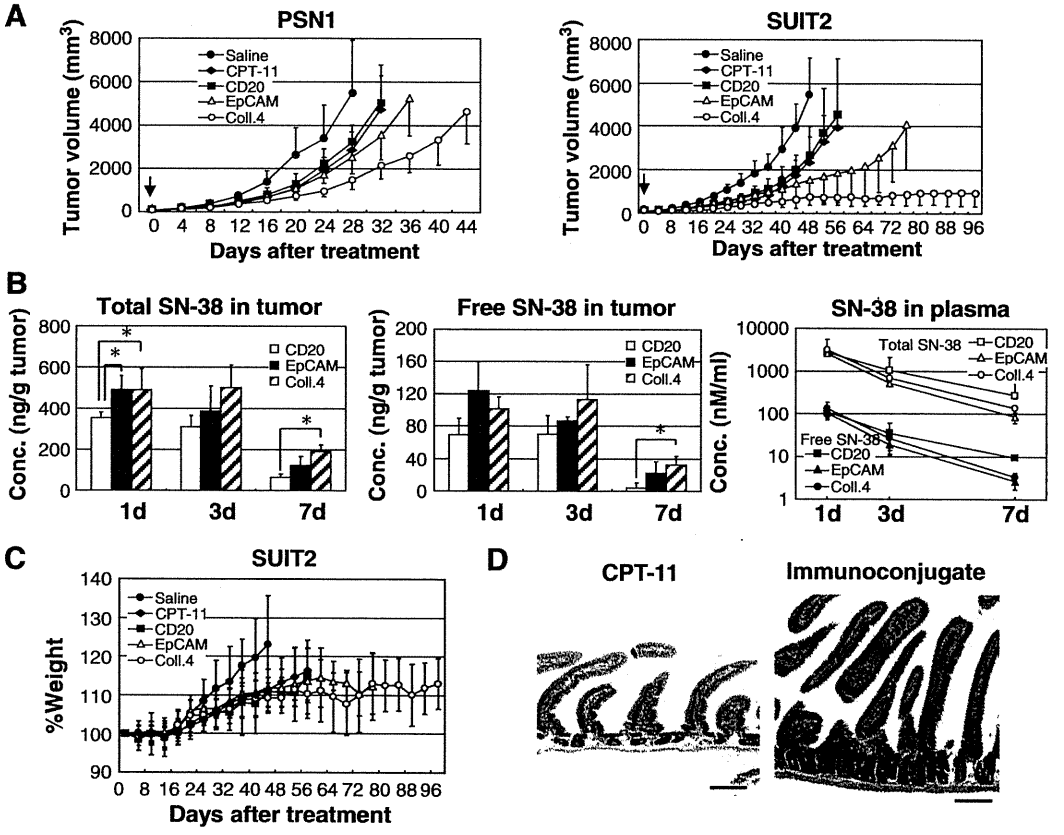

Fig. 7. Anti-tumor effects, pharmacokinetics and drug toxicities of anti-CD20, EpCAM and collagen 4 immunoconjugates. (A) Anti-tumor activities in vivo were examined. In animal models of PSN1 and SUIT2, the 3 immunoconjugates or saline as control, were administered to separate groups of mice by intravenous bolus injection on day. Arrows indicate day of administration and the curves illustrate the effects of the treatments on tumor size. $P<0.05$ (Saline or CD20 vs. EpCAM in PSN1), $P<0.01$ (Saline vs. CPT11 or EpCAM in PSN1, CPT11 or CD20 vs. EpCAM in SUIT2), $P<0.001$ (Saline vs. CPT11 or CD20 or EpCAM in SUIT2, Saline or CPT11 or CD20 or EpCAM vs. Collagen 4 in PSN1 or SUIT2). Bar = SD. (B) Tumor concentrations of total (bound and unbound) SN-38 (upper) and free (unbound) SN-38 (middle), and plasma concentrations (lower) were determined using HPLC analysis. The concentrations on days 1, 3 and 7 are shown. *$P<0.05$, Bar = SD. (C) Changes in the % body weight of saline, CPT-11, CD20, EpCAM and Collagen 4 in the same treated SUIT2 group (A) were shown Bar = SD. (D) Pathologic mucosal change of jejunum from mouse treated with CPT11 (upper) or anti-collagen 4 immunoconjugate (lower). Scale bar: 1 mm [32].

selected. In our design, polyethylene glycol (PEG) was combined close to the bond (Fig. 6). PEG is known to evade non-specific capture by RES. The steric structure around the bond protects against immunoconjugate degradation in the blood. PEG has already been used for this purpose [2,3,11,33]. The drug (SN-38)/mAb ratio (the number of drugs attached to a mAb) of each immunoconjugate ranged from 6.7 to 8.4.

Anti-tumor activities of immunoconjugates with ester bond SN-38 were evaluated in mice bearing human pancreatic tumor genografts. CPT-11 and three immunoconjugates showed significant anti-tumor activities compared to results in mice treated with saline, in mice bearing either PSN1 (EpCAM positive and stroma poor) or SUIT2 (EpCAM positive and stroma rich) tumors. In SUIT2 tumors, while the tumor continued to increase in mice treated with CPT-11, anti-CD20 immunoconjugate (as a non-specific control) and anti-EpCAM immunoconjugate, the tumor treated in mice with anti-collagen 4 immunoconjugate stopped growing by about one month and never resumed up to 3 months (Fig. 7A). In mice bearing PSN1 tumors (stroma poor), differences were present but less marked. Thus, anti-collagen 4 SN-38-immunoconjugate exerted the most potent antitumor activity as compared with, anti-CD20 or anti-EpCAM immunoconjugates, and CPT-11 (Fig. 7A). In both tumor models, anti-EpCAM immunoconjugate exerted superior antitumor effect compared to CPT-11 and anti-CD20 immunoconjugate, but inferior anti-tumor effect to anti-collagen 4 SN38-immunoconjugate.

Significantly higher concentrations of free and total SN-38 were detected in tumor tissues of mice treated with the anti-collagen 4 immunoconjugate compared to the anti-CD20 immunoconjugate (Fig. 7B). The tumor concentration of free and total SN-38 treated with anti-EpCAM immunoconjugate was intermediate among them, but not significant (Fig. 7B). There was no significant difference in body weight changes among saline groups, CPT-11 and immunoconjugate groups (Fig. 7C). In the small intestinal mucosa of mice, widespread villous atrophy and decreased crypt density were observed by the treatment of free unbound CPT-11 which is well-known to have severe intestinal toxicity in clinics. On the other hand, the small intestinal mucosa of mice in groups treated with all immunoconjugates did not show any pathological change (Fig. 7D).

The most important observation from a therapeutic standpoint was that only SUIT2-tumors treated with anti-collagen 4 immunoconjugate stopped growing about one month after treatment and remained dormant for more than 3 months. It may be concluded that the strategy of orchestrating slow sustained release from a scaffold erected on the stable inert structural components of the tumor stroma is most effective. We histologically compared this non-growing tumor with a size-matched, growing, control tumor and found that both tumors showed central necrosis due to decreased blood flow, which is often observed in a murine xenotransplant model [34,35]. The striking difference was that large confluent necrotic zones and dense fibrotic capsule formation

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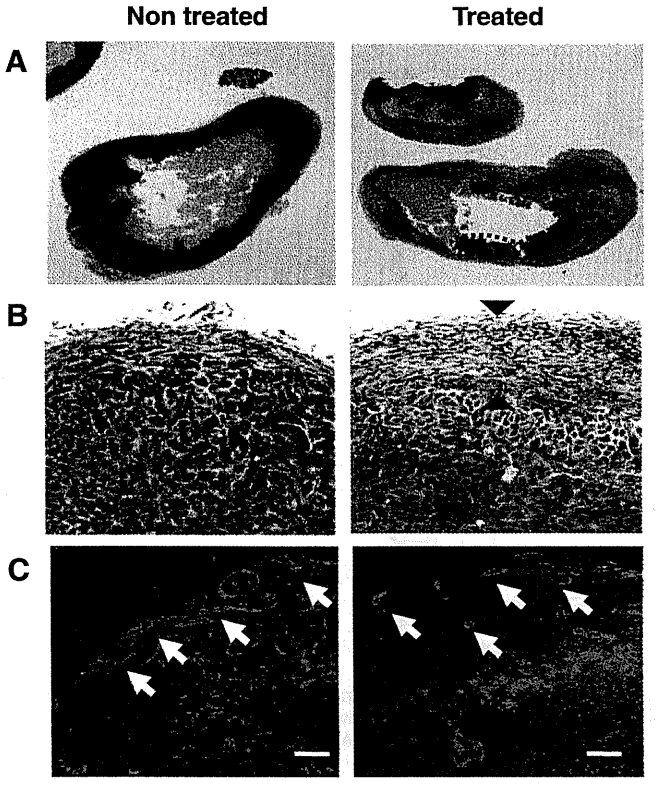

Fig. 8. Histopathological features of SUIT2 tumors after anti-collagen 4 immunoconjugate treatment. (A) Hematoxylin and eosin staining of non-treated (left) and immunoconjugate-treated (right) SUIT2-tumors. A non-necrotic viable lesion in the treated tumor is enclosed by a dotted line. (B) The fibrotic capsule width in the treated tumor is indicated between black arrowheads. (C) Tumor vessels were examined by the CD31 (red) collagen 4 (green) double-staining techniques. White arrows indicate tumor vessels or their traces in the boundary area. Scale bar: 100 μm [32].

were observed only in the treated tumor (Fig. 8A and B). In addition, CD31-positive endothelial cells, which may be tumor-feeding vessels in the peripheral part of the tumor, were never observed in the treated tumor compared with untreated control. Instead, many collagen 4-positive round profiles corresponding to traces of destroyed vessels were observed in the peripheral area of the treated tumor (Fig. 8C).

5.2. Anti-mouse (human) fibrin mAb conjugated with SN-38 in chemically induced tumor

Chemically induced mouse cutaneous cancer was selected as an appropriate experimental model for evaluating the therapeutic effects of our immunoconjugate chemotherapy, because this spontaneous carcinogenetic model has remarkable fibrin deposition and abundant interstitial tissue as in human cancer (Fig. 9A), unlike human tumor xenografts, which have less fibrin clots and interstitial tissue [36,37]. In addition, the spontaneous tumor is very slow in tumor growth that is also more similar to general human cancer as compared to the xenografts. Using systemic in vivo imaging, anti-fibrin IgM, anti-fibrin chimeric IgG and anti-fibrinogen IgG were delivered and retained in the tumor until Day 3, utilizing leaky tumor vessels [1–3]. However, accumulation of anti-fibrin IgM and anti-fibrinogen IgG was weak and was eliminated by Day 7, but the chimeric IgG was still highly retained (Fig. 9B). The use of human-chimera is benefit for clinical application to avoid human anti-mouse neutralizing antibodies (HAMA) and allergic reaction in human. In addition, because of the rapid blood clearance and low penetration of IgM compared with IgG [38], IgM is not suitable as a drug delivery vehicle. The branched composition had one maleimide for attachment of mAb, one PEG_{12} spacer and three PEG_{27} ester bonds for attachment of three SN-38 molecules (Fig. 10A). There were approximately eight thiol residues able to react with the maleimide in the reduced mAb. The calculated drug (SN-38)/mAb ratio of the immunoconjugate was about 24. This immunoconjugate exerted significantly stronger anti-tumor activity compared with CPT-11 (Fig. 10B). Although treatment-related body weight loss was observed in mice treated with each drug, there was no significant difference between control groups and CPT-11 or the immunoconjugate treatment group. After injection of the immunoconjugate, the concentration of total SN-38 (antibody bound and unbound form) and free SN-38 (unbound form) in plasma gradually declined within a week, whereas CPT-11 showed rapid clearance (Fig. 11A). Significantly high concentrations

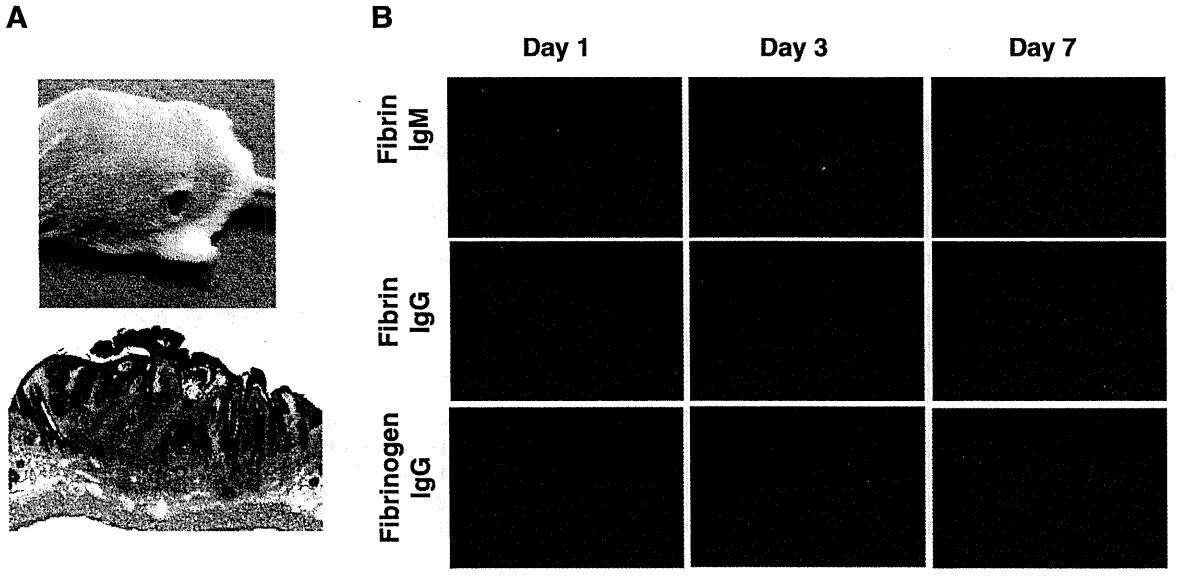

Fig. 9. In vivo systemic imaging analysis of Alexa-647-labeled anti-fibrin IgM, chimeric IgG or anti-fibrinogen mAb. (A) Chemically induced FVB/N mouse cutaneous tumor model. These spontaneous tumors have remarkable fibrin deposition and abundant interstitial tissue as in clinical human cancer,unlike human tumor xenografts in mice. (B) In vivo systemic imaging analysis of Alexa-647-labeled anti-fibrin IgM (upper), chimera IgG (middle) or anti-fibrinogen mAb (lower) on Days 1, 3 and 7 after injection. Arrows indicate each tumor position [29].

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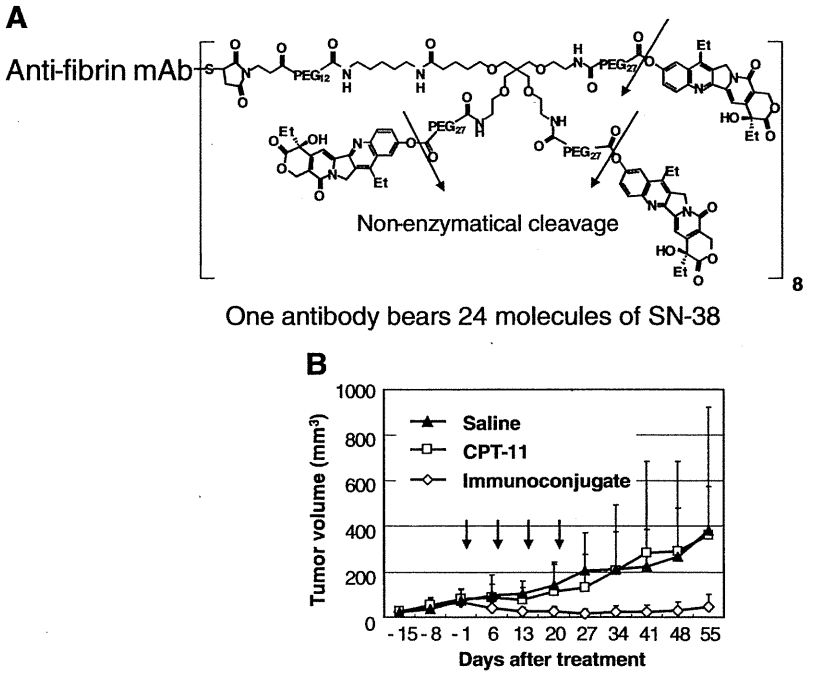

Fig. 10. Drug design and anti-tumor effect of anti-fibrin immunoconjugate. (A) Drug design of immunoconjugate; mAb-PEG-three branched PEG-$(SN\text{-}38)_3$ via ester bond. One antibody bears 24 molecules of SN-38. The arrow indicates the cleavage site for releasing free active SN-38. (B) Anti-tumor activity in vivo was examined. Immunoconjugates, CPT-11 or saline, were administered to mice bearing chemical-induced cutaneous cancer via intravenous injection on Day 0, 7, 14, and 21. Arrows indicate day of administration and the curves illustrate the effect of treatment on tumor size. $P=0.0005$ (CPT-11 vs. immunoconjugate), $P<0.0001$ (saline vs. immunoconjugate). Bar = SD [29].

of total and free SN-38 were detected in tumor tissues treated with the immunoconjugate for a long time compared to CPT-11 (Fig. 11B). The second significant observation of the treatment was a change in the gross tumor color from reddish to white (Fig. 11C). There was no clear change of fibroblast or macrophage, which play an important role for tumor progression [39,40]. It was found that discontinuation and irregularity comprising a mixture of narrowness and enlargement of the tumor vessels were manifested after treatment with the immunoconjugate (Fig. 12D and E).

6. Proposal of cancer stromal targeting (CAST) therapy and future prospects

Although there have been numerous reports of genetic and phenotype changes in tumors, a large body of pathological and clinical evidence indicates that there are no pivotal changes in tumor cells that distinguish them from normal dividing cells. Unlike in the case of using antibiotics against bacterial infection, therefore, ACAs need to be delivered selectively to tumor tissues and should be kept there long enough to reproduce the concentrations they reach in the Petri dish, which is a closed space where the cytocidal effects of any ACAs including molecular targeting agents are very strong. In the body, however, administered ACAs are cleared with the passage of time. Furthermore, as described in the main part of this topic, most human cancers possess abundant stroma that hinder the penetration of DDS including ACA-conjugated antibodies specific to surface antigens on cancer cells. I am now concerning that current studies mainly based on molecular and cellular biology while ignoring pathophysiology and pharmacology may be leading the development of antitumor drugs in the wrong direction. In our proof of concept studies using chemically induced spontaneous tumors (similar to general human cancers in terms of abundant tumor stroma and slow growing feature), we have made clear that our newly developed tool is not a simple cytotoxic immunoconjugate. Our strategic concept of cancer stromal targeting (CAST) therapy is unique as follows.

1) Newly developed cytotoxic immunoconjugate can extravasate from the leaky tumor vessels selectively, and forms a scaffold as it is captured by tumor stromal network.
2) The immunoconjugate allows the effective sustained release of anti-cancer agent from the scaffold, and this released anti-cancer agent is distributed throughout the tumor.
3) Consequently, the strategy described above was highly effective in causing arrest of tumor growth due to induced damage to tumor cells and tumor vessels without exerting the drug adverse effect (Fig. 12).

The present discovery by a hybrid of tumor stromal biology and physiology with organic chemistry may open a new field of drug design and produce many potentially useful treatment modalities especially for stromal rich and refractory cancer such as pancreatic cancer, stomach cancer, and glioma.

Disclosure statement

The author has no conflict of interest in this article.

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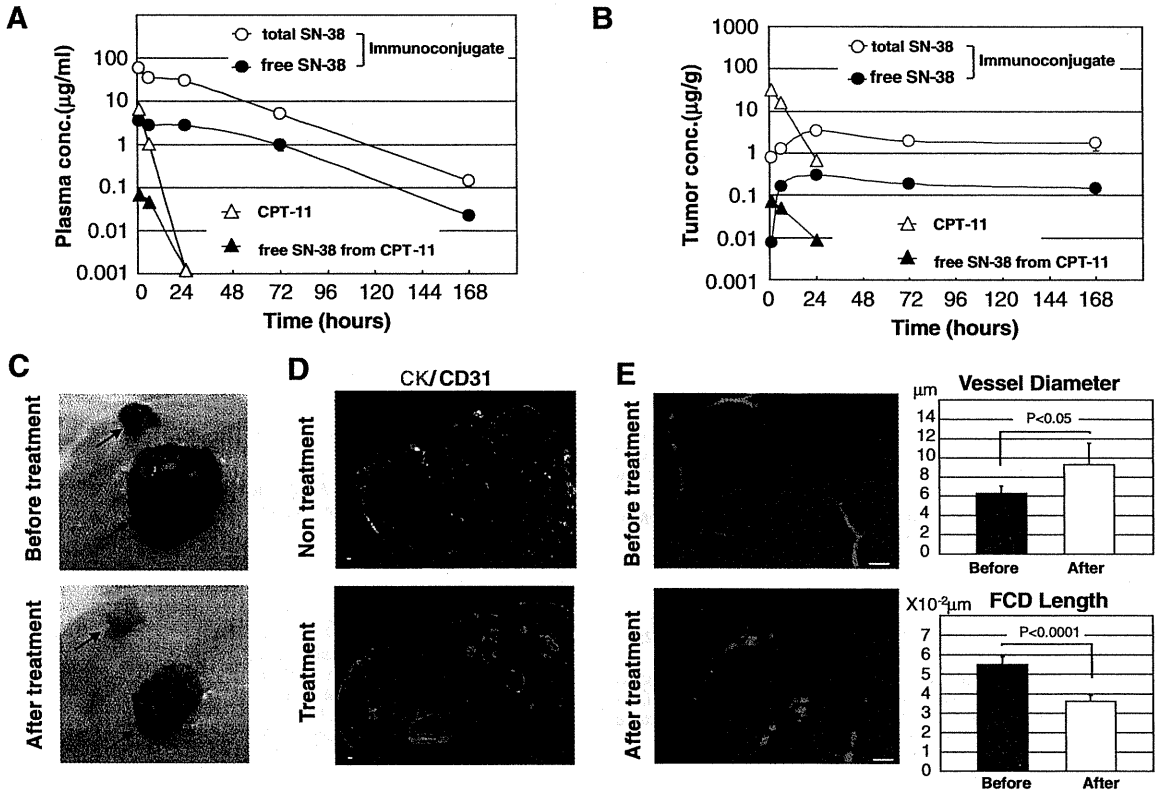

Fig. 11. Drug distribution and anti-vascular activity of the immunoconjugate. (A) Plasma concentration of total SN-38 (bound and unbound form) or CPT-11 and free SN-38 (unbound form) released from the immunoconjugate or converted from CPT-11 was determined using HPLC 1, 6, 24, 72, and 168 h after the injection. (B) Tumor concentration of total SN-38 (bound and unbound form) and free SN-38 (unbound form) released from the immunoconjugate, CPT-11 and free SN-38 converted from CPT-11 was determined. (C) Tumor color changed from reddish to white at 5 days after injection of the immunoconjugate but not CPT-11. Arrows indicate each tumor position. (D) Tumor vessels after the injection of the immunoconjugate were examined using CD31 (red) and CK (cytokeratin, green). Untreated mouse was used as control. Bar: 100 μm. (E) Tumor vessels before and after the injection were visualized using FITC-dextran by in vivo fibered confocal fluorescence microscopy (left). Quantified vessel diameter and functional capillary density (FCD) length are shown (right). Bar: 20 μm [29].

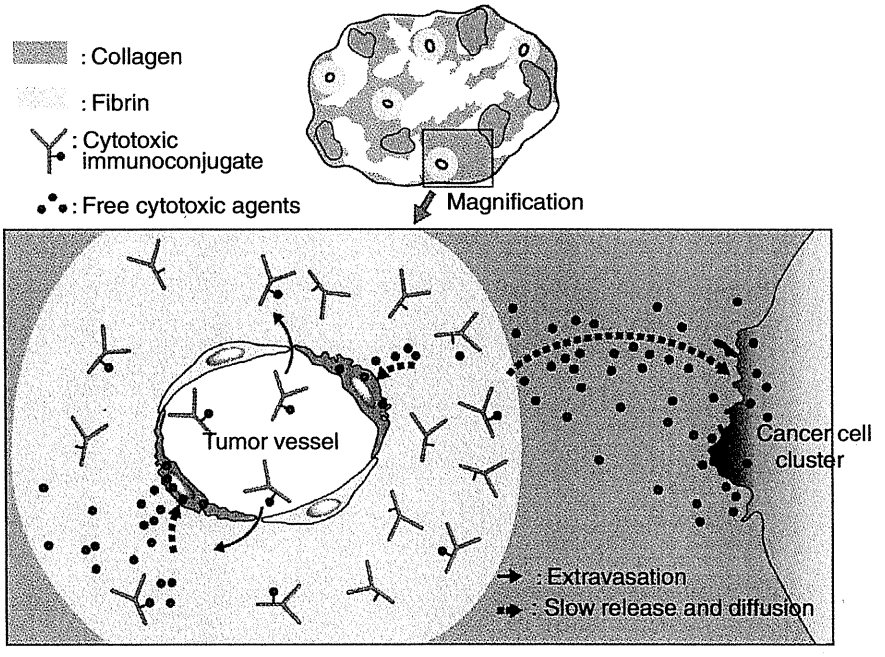

Fig. 12. New strategy of drug delivery using cytotoxic immunoconjugate directing tumor stroma in hypovascular and stroma-rich tumor. Newly developed immunoconjugates extravasate selectively from leaky tumor vessels, bind specifically to the fibrin network around the tumor vessels to create a scaffold, and then allow the effective sustained release of SN-38, a time-dependent anti-cancer agent, from the scaffold. Since this released anti-cancer agent is LMW, it is subsequently distributed over the entire tumor-stroma barrier and induces damage not only to tumor cells but also to tumor vessels [29].

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