

# *N*-acetylneuraminic acid coupled human recombinant TNF $\alpha$ exhibits enhanced anti-tumor activity against Meth-A fibrosarcoma and reduced toxicity

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**Abstract** In order to study the effect of glycosylation on its biological activities and to develop tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) with less deleterious effects, *N*-acetylneuraminic acid (NeuAc) with a C9 spacer was chemically coupled to human recombinant TNF $\alpha$ . NeuAc-coupled TNF $\alpha$  (NeuAc-TNF $\alpha$ ) exhibited reduced activities in vitro by about threefold compared to native TNF $\alpha$ . In this study, we examined a variety of TNF $\alpha$  activities in vivo. NeuAc-TNF $\alpha$  reduced activities in the up-regulation of serum levels of IL-6 and NO $_x$ , but comparable activity as native TNF $\alpha$  in the down-regulation of the serum level of glucose. However, NeuAc-TNF $\alpha$  was more potent than TNF $\alpha$  in the up-regulation of the serum level of serum amyloid A (SAA). NeuAc-TNF $\alpha$  was less toxic to mice. In addition, NeuAc-TNF $\alpha$  exhibited an augmented anti-tumor activity against Meth-A fibrosarcoma without hemorrhagic necrosis. These results indicate that coupling with NeuAc enabled us to develop neoglycoTNF $\alpha$  with selective activities in vivo, including enhanced anti-tumor activity but reduced toxicity.

**Keywords** Neoglycoprotein · Sialic acid · Tumor necrosis factor · Cytokine · Anti-tumor activity

## Introduction

Glycoproteins are widely distributed in animals, plants and microorganisms. Neoglycoproteins, proteins chemically or enzymatically coupled with carbohydrates, are quite useful to investigate the role of carbohydrates in the functions and physicochemical properties of glycoproteins. The conjugation of carbohydrates is also useful to modify the functions and stability of target proteins. The advantage to synthesize neoglycoproteins is that chemically synthesized carbohydrates, not only natural, but also unnatural carbohydrates, can be coupled to proteins [1–4].

Sialic acid is usually present at the non-reducing position of oligosaccharide in glycoproteins and glycolipids, and plays an important role in the function, stability and tissue distribution of glycoproteins [5] and as a ligand for viruses, including influenza viruses, paramyxoviruses, coronaviruses, polyomaviruses and retroviruses [6]. In glycoproteins, sialic acid is usually conjugated with galactose and plays an important role in preventing the clearance of glycoproteins from the serum because asialoglycoproteins are rapidly cleared through galactose binding lectins present in the liver [7]. Furthermore, sialic acid is also important as a ligand for selectins [8] and Siglecs [9] and is involved in cellular signaling events and the modulation of immune reactions. Therefore, it is expected that the coupling of sialic acid enables its conjugate to bind to a variety of cell types and prolong its serum level as well.

Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) is a cytokine produced mainly by macrophages and monocytes. TNF $\alpha$  exhibits a variety of biological activities and plays an important role in immunological and inflammatory reactions [10, 11]. Although TNF $\alpha$  has been expected

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to be an efficient therapeutic agent for certain tumor cells, its clinical application is limited because of its proinflammatory activity and toxicity. At high doses, TNF $\alpha$  induces acute responses such as shock, tissue injury, catabolic hormone release, vascular leakage syndrome, gastrointestinal necrosis, acute renal tube necrosis, adrenal hemorrhage, fever, and at low doses, chronic responses such as weight loss, anorexia, protein catabolism, lipid depletion, hepatosplenomegaly, sub-endocardial inflammation, insulin resistance, acute protein release and endothelial activation [11].

In the previous studies, we have synthesized neoglyco human IL-1 $\alpha$  by conjugating human recombinant IL-1 $\alpha$  a variety of carbohydrates, including D-Man $\alpha$  (1-6)Man [Man $_2\alpha$  (1-6)], Gal or N-acetylneuraminic acid (NeuAc), a major constituent of sialic acid [12–19]. Although these carbohydrate-conjugated IL-1 $\alpha$  variants exhibited reduced activities in vitro, the activities in vivo were different depending on the type of carbohydrates [12, 15, 18]. Man $_2\alpha$  (1-6)-IL-1 $\alpha$  exhibited comparable activities to nonglycosylated IL-1 $\alpha$  in the down-regulation of the serum level of glucose and the recovery of peripheral white blood cells from myelosuppression in 5-fluorouracil-treated mice, irrespective of the decrease of all other activities in vivo [13]. In addition, the tissue distribution of Man $_2\alpha$  (1-6)-IL-1 $\alpha$  in mice differed from that of nonglycosylated IL-1 $\alpha$  [14]. D-Gal-conjugated IL-1 $\alpha$  exhibited a decrease in all the activities in vivo with a similar magnitude [16]. NeuAc coupled IL-1 $\alpha$  exhibited selective activities in vivo as Man $_2\alpha$  (1-6)-conjugated IL-1 $\alpha$  and enhanced tissue distribution [17–19].

We also synthesized NeuAc conjugated human recombinant TNF $\alpha$ . We obtained two glycosylated TNF $\alpha$ s, L NeuAc-TNF $\alpha$  and H NeuAc-TNF $\alpha$  containing 1.0 and 1.5 molecules of NeuAc per molecule of TNF $\alpha$ , respectively. L NeuAc-TNF $\alpha$  and H NeuAc-TNF $\alpha$  exhibited reduction in a variety of activities in vitro, including growth inhibitory and cytotoxic activities to tumor cells, a growth stimulatory effect on normal fibroblasts, induction of IL-6 and the activation of NF- $\kappa$ B by about 1/3 and 1/10, respectively. As a major product was L NeuAc-TNF $\alpha$  (the yields of L NeuAc-TNF $\alpha$  and H NeuAc-TNF $\alpha$  were 28.6 and 4.87%, respectively), in this study we examined L NeuAc-TNF $\alpha$  for a variety of activities in vivo including anti-tumor activity and toxicity.

## Materials and methods

### Animals

ICR female mice (6 weeks old) and Balb/c female mice (6 week old) were purchased from Charles River

(Yokohama, Japan) and fed ad lib. and housed in temperature- and light-controlled (12 h/day) rooms. Mice were used in experiments after at least 1 week of acclimation. ICR mice were used for the induction of serum IL-6, serum amyloid A (SAA), NOx and the reduction of glucose. Balb/c mice were used for acute lethal toxicity and anti-tumor activity.

### Reagents

RPMI 1640 was purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was from JRH Biosciences (Lenexa, KS). Human recombinant TNF $\alpha$  (rhTNF $\alpha$ ) was provided by Dainippon Pharmaceutical Co. (Osaka, Japan). The specific activity of rhTNF $\alpha$  was  $6 \times 10^7$  U/mg based on the cytotoxic assay using L929 cells cultured with actinomycin D. Human recombinant IL-6 (rhIL-6) was provided by Ajinomoto Co. (Tokyo, Japan). The specific activity of rhIL-6 was  $5 \times 10^6$  U/mg based on the proliferative assay using MH60-BSF2 cells.

### Cell culture

Murine hybridoma clone MH60-BSF2, provided from Dr. T. Hirano (University of Osaka), was maintained in culture medium (RPMI 1640, 100 U/ml of penicillin G, 100  $\mu$ g/ml of streptomycin, and 10% heat-inactivated FBS) containing 1 U/ml of rhIL-6 [20].

### Synthesis of glycosylated TNF $\alpha$

An acyl azide derivative of NeuAc with a C9 spacer was synthesized and coupled to rhTNF $\alpha$ . The NeuAc-TNF $\alpha$  was purified by anion-exchange chromatography, and the NeuAc-coupling was confirmed by blotting with NeuAc-specific LFA lectin, the increase in its molecular weight on SDS-PAGE and time of flight mass spectroscopy (TOF-MS) analysis. Two glycosylated TNF $\alpha$  were obtained and termed L NeuAc-TNF $\alpha$  and H NeuAc-TNF $\alpha$ , which contained 1.0 and 1.5 molecules NeuAc per molecule of TNF $\alpha$ , respectively. As a major product was L NeuAc-TNF $\alpha$ , we used it for in vivo experiments. For the sake of brevity, L NeuAc-TNF $\alpha$  was termed NeuAc-TNF $\alpha$ . With endotoxin test using a *Limulus amoebocyte* assay (sensitivity limit, 0.1 ng/ml), endotoxin contamination was negative in these TNF $\alpha$ s.

Measurement of serum levels of IL-6, glucose, serum amyloid A, and NOx

TNF $\alpha$ s were diluted to the desired concentration with sterile PBS and intraperitoneally administered to mice.

Although the samples were endotoxin negative, to prevent the effect of an undetectable amount of endotoxin, polymyxin B was added at 5 µg/ml. Mice were fasted after the administration. At the times indicated for experiment, the mice were bled.

The IL-6 activity in the serum was measured by proliferation assay with IL-6-dependent MH60·BSF2 cells [20]. The amount of IL-6 was expressed as the equivalent amount of rhIL-6. The glucose level in the serum was determined by using a glucose B-test kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

The concentration of SAA in the serum was measured by ELISA, as described previously [21].

The serum nitrite/nitrate (NO<sub>x</sub>) level was measured by the method described by Misko et al. [22]. Briefly, 301 of each sample were incubated for 15 min at 37°C with 10 µl of the nitrate reductase (2.5 U/ml; Boehringer Mannheim) and 10 µl nicotinamide-adenine dinucleotide phosphate (2 mM; Sigma Chemical Co.). After incubation, 50 µl of Griess reagent and 50 µl of TCA (10% aqueous solution) were added. The protein precipitates were removed by centrifugation at 15,000 rpm for 5 min and 50 µl of each supernatant was transferred to 96-well plate (Falcon) and the O.D. 595 nm was measured using an ELISA autoreader (Bio-Rad Laboratories, Richmond, CA).

#### Evaluation of the toxicity of TNF $\alpha$ to mice

D-galactosamine (18 mg/0.2 ml in PBS) was intraperitoneally injected into 9-week-old female Balb/c mice. Ten minutes after the injection, test samples were administered intraperitoneally into the mice. Every 2 h after the injection of the samples, the mortality of the mice was determined.

#### Evaluation of the anti-tumor effect of TNF $\alpha$

Meth-A fibrosarcoma cells ( $4 \times 10^5$  cells) were inoculated intradermally into the abdomen of 9-week-old female Balb/c mice. Eight days later after the confirmation of tumor establishment (tumor size, 5–8 mm in diameter), test samples were intravenously administered twice a week for 2 weeks. The tumor size was determined based on the formula of Haranaka et al. [23]. At 24 h after the first injection of TNF $\alpha$ , a score of tumor hemorrhagic necrosis, from 1 to 4, was determined according to the method of Carswell et al. [24].

#### Determination of protein content

The amount of protein was determined using a Protein Assay kit (Bio-rad, Richmond, CA) with bovine serum albumin as the standard.

#### Statistical analysis

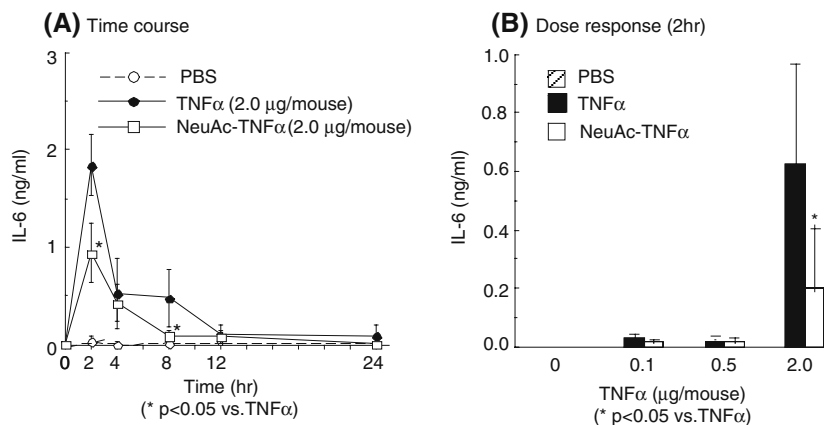
Differences between group means were assessed using the *t* test.

## Results

#### Ability of TNF $\alpha$ to induce serum IL-6 in mice

Mice were injected intraperitoneally with native TNF $\alpha$  (termed TNF $\alpha$ ) or NeuAc-TNF $\alpha$  and the serum IL-6 level was determined. Mice injected with TNF $\alpha$  exhibited a sharp increase in the IL-6 level with a maximum level 2 h after TNF $\alpha$  treatment (Fig. 1a). The elevation of serum IL-6 at 2 h was not observed at the injection of 0.5 µg TNF $\alpha$ /mouse (Fig. 1b). NeuAc-TNF $\alpha$  exhibited a significant decrease in its activity.

**Fig. 1** Effect of TNF $\alpha$ s on the serum IL-6 level in mice. PBS or TNF $\alpha$ s were intraperitoneally administered and the mice were bled at the time indicated (a) or 2 h later (b). The serum IL-6 level was determined by MH60·BSF-2 bioassay. Mice were used in groups of five (a) and seven (b). Each point represents the mean  $\pm$  SD



### Ability of TNF $\alpha$ to induce SAA in mice

Mice were injected intraperitoneally with native TNF $\alpha$ , and their ability to induce SAA, an acute phase protein produced by hepatocytes in response to TNF $\alpha$ , was examined. TNF $\alpha$  increased the SAA level after 4 h treatment, which exhibited a peak at 8 h and the level was sustained for up to 24 h. NeuAc-TNF $\alpha$  exhibited a more potent activity in the induction of SAA (Fig. 2a). Although the data were not significant, the dose-response experiment at 8 h indicated that NeuAc-TNF $\alpha$  at 2  $\mu$ g/mouse is more potent than TNF $\alpha$  (Fig. 2b).

### Effect of TNF $\alpha$ treatment on the serum glucose level in mice

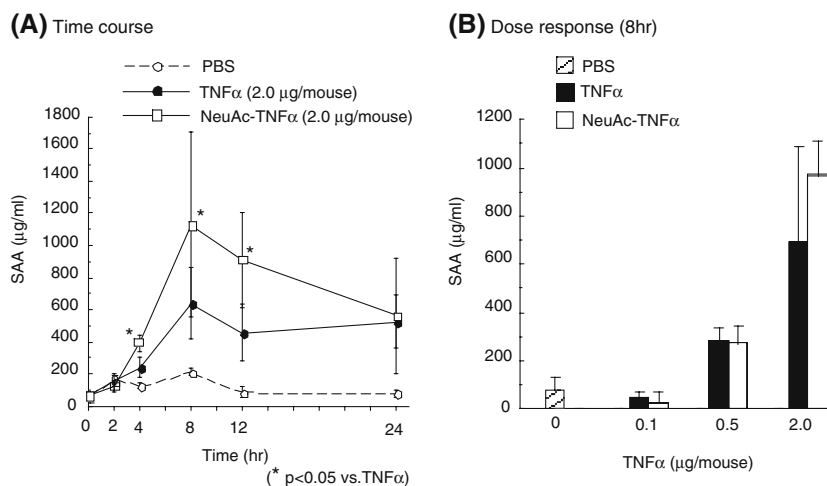
The effect of TNF $\alpha$  on the serum glucose level was examined. The mice were injected intraperitoneally with native TNF $\alpha$  or NeuAc-TNF $\alpha$ , and then fasted. In the control mice the serum glucose level decreased

with the duration of fasting up to 24 h (Fig. 3a). TNF $\alpha$  caused a significant reduction at 2 h after treatment, and the decrease continued to 24 h. NeuAc-TNF $\alpha$  also caused a reduction for up to 24 h after treatment. A dose-response experiment at 8 h indicated that NeuAc-TNF $\alpha$  exhibited comparable activity to TNF $\alpha$  (Fig. 3b).

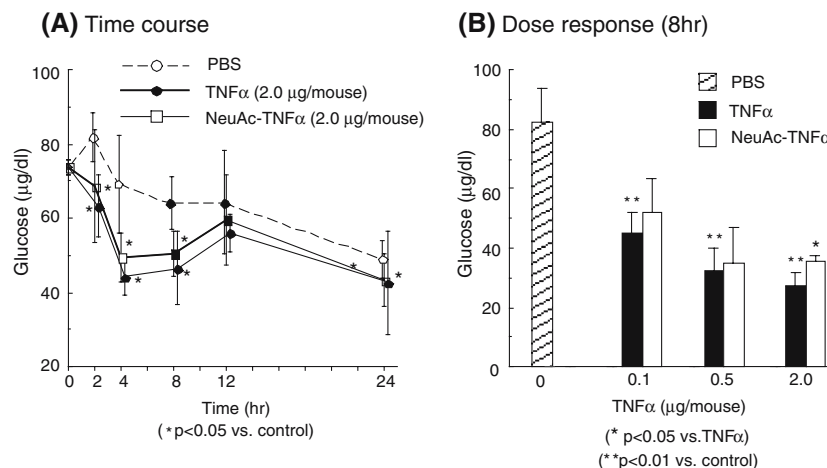
### Ability of TNF $\alpha$ to induce serum NO $_x$ in mice

TNF $\alpha$  induces nitric oxide (NO) synthesis in a variety of cell types. The generated NO reacts with molecular oxygen and water, and subsequently, nitrite and nitrate (NO $_x$ ) were accumulated in the biological fluids. To examine the ability of TNF $\alpha$  to induce NO, serum nitrate was converted to nitrite by nitrate reductase, and then the amount of total nitrite was determined. Native TNF $\alpha$  injected mice exhibited an increase in the serum NO $_x$  level after 4 h treatment with a maximum level at 8 h, and the level decreased, but still remained high as compared to control at 24 h (Fig. 4a). NeuAc-TNF $\alpha$  also up-regulated the serum NO $_x$  level. The time course experiment

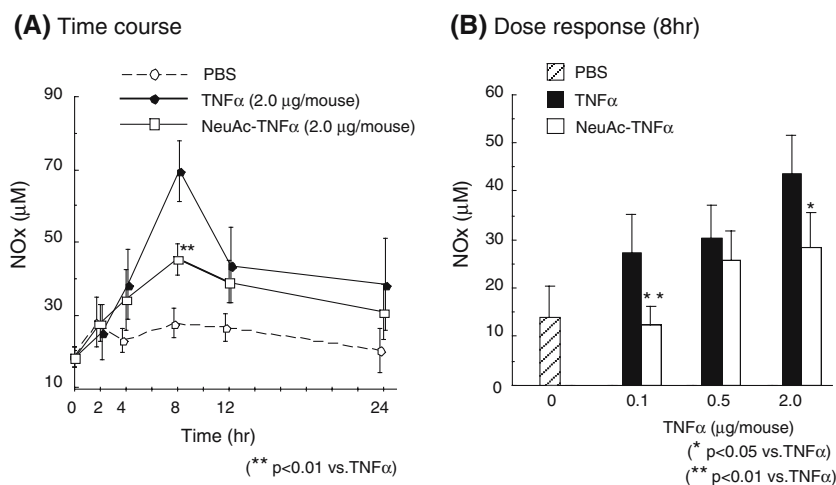
**Fig. 2** Effect of TNF $\alpha$ s on the SAA level in mice. PBS or TNF $\alpha$ s were intraperitoneally administered and mice were bled at the time indicated (a) or 8 h later (b). The SAA level was determined with ELISA. Mice were used in groups of five (a) and seven (b). Each point represents the mean  $\pm$  SD



**Fig. 3** Effect of TNF $\alpha$ s on the serum glucose level in mice. PBS or TNF $\alpha$ s were intraperitoneally administered and mice were bled at the time indicated (a) or 4 h later (b). The serum glucose level was determined using the glucose B-test (Wako). Mice were used in groups of five (a) and seven (b). Each point represents the mean  $\pm$  SD



**Fig. 4** Effect of TNF $\alpha$ s on the serum NOx level in mice. PBS or TNF $\alpha$ s were intraperitoneally administered and mice were bled at the time indicated (a) or 8 h later (b). The serum NOx level was determined as described in [Materials and methods](#). Mice were used in groups of five (a) and seven (b). Each point represents the mean  $\pm$  SD



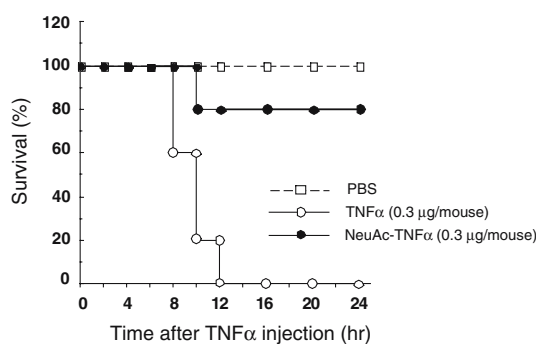
and the dose-response experiment at 8 h showed that NeuAc-TNF $\alpha$  is less active than TNF $\alpha$  (Fig. 4b).

#### Acute lethal toxicity of TNF $\alpha$ to mice

We determined the lethal toxicity of TNF $\alpha$ s to mice. Mice were sensitized to LPS by the injection of D-galactosamine intraperitoneally, and then the TNF $\alpha$ s (0.3  $\mu$ g/mouse) were administered intraperitoneally. As shown in Fig. 5, all the mice injected with native TNF $\alpha$  died after up to 12 h. In contrast, 4/5 of the mice injected with NeuAc-TNF $\alpha$  survived up to 24 h. Three out of five and two out of five of the mice were still survived after 48 and 72 h injection, respectively. The dose-response experiment showed that the toxicity of NeuAc-TNF $\alpha$  decreased to 1/3, as compared to TNF $\alpha$  (Table 1).

#### Anti-tumor activity of TNF $\alpha$ for Meth-A tumor bearing mice

In order to compare the anti-tumor activity of TNF $\alpha$ s, TNF $\alpha$ s were injected intravenously into the mice twice



**Fig. 5** Acute lethal toxicity of TNF $\alpha$  PBS or TNF $\alpha$ s (0.3  $\mu$ g/mouse) were intraperitoneally administered into the mice 10 min after the injection of D-galactosamine. Each group consisted of five mice

a week 8 days after the inoculation with Meth-A fibrosarcoma, at this time, tumor establishment was confirmed. As shown in Fig. 6a, tumor growth was significantly reduced by the injection of TNF $\alpha$ s at either 2.0 or 0.5  $\mu$ g per injection. However, the anti-tumor effect of NeuAc-TNF $\alpha$  was more potent as compared to native TNF $\alpha$  (Fig. 6b). In another experiment, NeuAc-TNF $\alpha$  at 2  $\mu$ g per injection resulted in the complete disappearance of tumors in 1, 2, 3 and 4 per 7 mice on day 13, 18, 20 and 32, respectively, and NeuAc-TNF $\alpha$  at 0.5  $\mu$ g per injection did so in 2 and 3 per 7 mice on day 19 and 21, respectively, while native TNF $\alpha$  at either 2 or 0.5  $\mu$ g per injection could not up to 32 days, though it inhibited the tumor growth. In addition, while native TNF $\alpha$  induced hemorrhagic necrosis, NeuAc-TNF $\alpha$  induced necrosis without hemorrhage (Fig. 7). During the course of the treatment up to 32 days, no significant body weight loss was observed in either the native TNF $\alpha$ - or NeuAc-TNF $\alpha$  treated control and tumor bearing mice, as compared to the PBS control (data not shown).

#### Discussion

In the present study, we demonstrated that NeuAc-TNF $\alpha$  exhibits selective activities in vivo. This is in contrast to its in vitro activities, in all the assays performed in vitro, including antiproliferative or cytotoxic activity to tumor cells, a proliferative effect on fibroblast cells, a stimulatory effect on IL-6 production by melanoma cells and NF- $\kappa$ B activation in hepatocytes, NeuAc-TNF $\alpha$  exhibited similarly reduced activities by about 1/3, as compared to native TNF $\alpha$ . It is interesting as to why the effects of NeuAc coupling to TNF $\alpha$  activities in vivo differed according to the activities. The activities of TNF $\alpha$  in vitro could be mainly determined by its affinity for the receptor. In contrast, the in vivo system



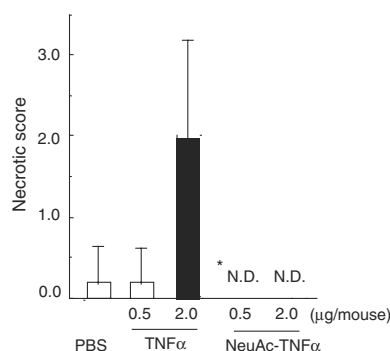
**Table 1** Survival rate of mice injected with TNF $\alpha$

Dose ( $\mu\text{g}/\text{mouse}$ )	Time after TNF $\alpha$ injection (h)										
	0	2	4	6	8	10	12	16	20	24	
PBS	100 <sup>a</sup> (5/5)	100 (5/5)	100 (5/5)	100 (5/5)	100 (5/5)	100(5/5)	100 (5/5)	100 (5/5)	100 (5/5)	100 (5/5)	100 (5/5)
Untreated TNF $\alpha$											
3.0	100 (5/5)	100 (5/5)	100 (5/5)	80 (4/5)	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)
1.0	100 (5/5)	100 (5/5)	100 (5/5)	80 (4/5)	20 (1/5)	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)
0.3	100 (5/5)	100 (5/5)	100 (5/5)	100 (5/5)	60 (3/5)	20 (1/5)	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)
NeuAc-TNF $\alpha$											
3.0	100 (5/5)	100 (5/5)	100 (5/5)	100 (5/5)	20 (1/5)	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)
1.0	100 (5/5)	100 (5/5)	100 (5/5)	100 (5/5)	60 (3/5)	20 (1/5)	20 (1/5)	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)
0.3	100 (5/5)	100 (5/5)	100 (5/5)	100 (5/5)	100 (5/5)	80 (4/5)	80 (4/5)	80 (4/5)	80 (4/5)	80 (4/5)	80 (4/5)

<sup>a</sup> Percentage of survival after each dose of TNF $\alpha$  injection. Number of dead mice/total mice given in parentheses

is more complex, and the potency of TNF $\alpha$  activity cannot be determined solely by the affinity to its receptor. In our previous studies, NeuAc-IL-1 $\alpha$  similarly exhibited reduced activities in all the assays performed in vitro, including its receptor binding affinity, as compared to native IL-1 $\alpha$ , but in vivo, it exhibited selective activities, such as reduction in the activity to induce serum IL-6, moderate reduction in the activities to induce serum SAA and NOx, comparable activity to reduce the serum glucose level and augmented activity to improve the recovery of peripheral white blood cells from myelosuppression in 5-FU treated mice [18, 19]. There are many proteins or glycoproteins interacting with sialic acid in the serum, tissues and on the cell surface, thus they will influence NeuAc-TNF $\alpha$  in its distribution into tissues, accessibility to target cells, retardation in the serum and receptor binding activity.

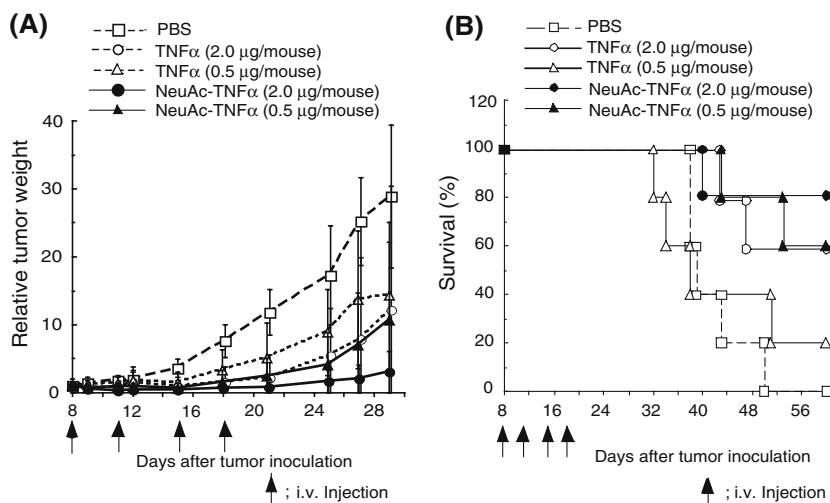
TNF $\alpha$  exerts pleiotropic effects in vivo by acting on many cell types. IL-6 is produced from many cell types by TNF $\alpha$  alone and in synergy with IL-1. IL-6 plays an important role in acute and chronic inflammatory reactions, including differentiation and proliferation of T



**Fig. 7** Induction of hemorrhagic necrosis of solid tumor in Meth-A bearing mice by TNF $\alpha$ s. PBS or TNF $\alpha$ s (2.0 or 0.5  $\mu\text{g}/\text{mouse}$ ) was intraperitoneally administered into mice after Meth-A fibrosarcoma inoculation. After 24 h of a single injection necrotic score, the grade of response (0–3) was determined according to Carswell et al. Mice were used in a group of five. Each value is the mean  $\pm$  SE. Asterisk not detected

and B cells and the induction of acute phase proteins from hepatocytes [25]. NeuAc-TNF $\alpha$  exhibited a significant decrease in the IL-6 induction activity.

**Fig. 6** Anti-tumor effect of TNF $\alpha$ . PBS or TNF $\alpha$ s (2.0 or 0.5  $\mu\text{g}/\text{mouse}$ ) were intraperitoneally administered into the mice twice a week four times after Meth-A fibrosarcoma inoculation. **a** Changes in the relative tumor weights by TNF $\alpha$ . **b** Days of complete regression after tumor inoculation. Mice were used in groups of five. Each value is a mean  $\pm$  SE



TNF $\alpha$  alone or in synergy with IL-1, IL-6 or glucocorticoid induces the synthesis of acute phase proteins by hepatocytes, and the acute phase proteins are implicated in the reduction of inflammation and the recovery of damaged tissues. SAA is the representative produced by hepatocytes in response to cytokines [26]. NeuAc-TNF $\alpha$  exhibited a more potent activity in the induction of SAA.

In response to TNF $\alpha$  or IL-1, the serum level of glucose decreases due to insulin-dependent and independent manners, the latter mechanism involves the reduction of gluconeogenesis in the liver and glucose-uptake by tissues [27]. NeuAc-TNF $\alpha$  exhibited a comparable activity to native TNF $\alpha$ .

NO is an important effector molecule in neurotransmission, vasodilatation and host defense against microorganisms and tumor cells. TNF $\alpha$  alone or in synergy with IL-1 $\alpha$  and interferon augments the production of NO from many cell types, including macrophages, hepatocytes, vascular endothelial cells and smooth muscle cells [28]. In particular, NO produced by smooth muscle cells is implicated in the hypotension caused by TNF $\alpha$ , which is a serious deleterious effect in the application of TNF $\alpha$  to patients [11]. NeuAc-TNF $\alpha$  was weak in the induction of serum NO $x$  level. Therefore, it is beneficial for the therapeutic use of NeuAc-TNF $\alpha$ . A disadvantageous aspect is that Neu-TNF $\alpha$  may be weak in augmenting the host defense against infection of microorganisms and tumors.

The toxic effect of TNF $\alpha$  contributes to endotoxin shock [24], which is also a major factor for the limited use of TNF $\alpha$  to patients. NeuAc-TNF $\alpha$  exhibited reduced acute lethal toxicity in D-galactosamine sensitized mice. This is also beneficial for the therapeutic use of NeuAc-TNF $\alpha$ . When native TNF $\alpha$  or NeuAc-TNF $\alpha$  was injected into the control or tumor-bearing mice that were not sensitized with D-galactosamine, there was no obvious toxicity.

It is well established that TNF $\alpha$  exerts its anti-tumor effect on the fibrosarcoma Meth-A in mice [10, 11, 29]. NeuAc-TNF $\alpha$  inhibited the tumor growth more rapidly and efficiently than native TNF $\alpha$ . The rate of complete disappearance of the tumor was also higher in NeuAc-TNF $\alpha$  as compared to native TNF $\alpha$ . As has been known, TNF $\alpha$  caused hemorrhagic necrosis. Quite interestingly, however, NeuAc-TNF $\alpha$  caused necrosis without hemorrhage. The tumor lesion was pale; subsequently, the tumor regressed and disappeared. It is of note that when the necrotic tumor lesion was touched by hand, the TNF $\alpha$ -injected mice, but not the NeuAc-TNF $\alpha$  injected mice, became rowdy. Probably the TNF $\alpha$ -injected, but not the NeuAc-TNF $\alpha$  injected mice, felt pain. TNF $\alpha$  exerts its anti-tumor effect

through its direct cytotoxic effect, necrosis or apoptosis, on tumor cells and also indirectly through host cells. TNF $\alpha$  induces inflammatory exudates by inducing chemokines and cytokines, and augments the tumor cell killing activity of monocytes, macrophages, neutrophils and NK cells [10, 11]. It is also reported that TNF $\alpha$  selectively stems blood flow in newly formed microcapillaries in the tumor lesion by inducing thrombus formation, leading to the autolysis of tumors [29]. Although the mechanism by which NeuAc-TNF $\alpha$  caused necrosis without hemorrhage remained to be elucidated, our study indicated that conjugation of NeuAc to TNF $\alpha$  rendered the conjugate more potent in anti-tumor effect and less toxic. The study also suggests that the site-directed conjugation of NeuAc will enable us to develop a more ideal TNF $\alpha$  in future.

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