N-acetylneuraminic acid coupled human recombinant TNFα exhibits enhanced anti-tumor activity against Meth-A fibrosarcoma and reduced toxicity

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Original Article

Abstract In order to study the effect of glycosylation on its biological activities and to develop tumor necrosis factor α (TNFα) with less deleterious effects, N-acetylneuraminic acid (NeuAc) with a C9 spacer was chemically coupled to human recombinant TNFα. NeuAc-coupled TNFα (NeuAc-TNFα) exhibited reduced activities in vitro by about threefold compared to native TNFα. In this study, we examined a variety of TNFα activities in vivo. NeuAc-TNFα reduced activities in the up-regulation of serum levels of IL-6 and NOx, but comparable activity as native TNFα in the down-regulation of the serum level of glucose. However, NeuAc-TNFα was more potent than TNFα in the up-regulation of the serum level of serum amyloid A (SAA). NeuAc-TNFα was less toxic to mice. In addition, NeuAc-TNFα exhibited an augmented anti-tumor activity against Meth-A fibrosarcoma without hemorrhagic necrosis. These results indicate that coupling with NeuAc enabled us to develop neoglycoTNFα 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 α (TNFα) is a cytokine produced mainly by macrophages and monocytes. TNFα exhibits a variety of biological activities and plays an important role in immunological and inflammatory reactions [10, 11]. Although TNFα has been expected
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, TNFα 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, subendocardial inflammation, insulin resistance, acute protein release and endothelial activation [11].

In the previous studies, we have synthesized neoglycohuman IL-1α by conjugating human recombinant IL-1α a variety of carbohydrate, including α-Manα(1–6)Manα[Manα(1–6)], Gal or N-acetylneuraminic acid (NeuAc), a major constituent of sialic acid [12–19]. Although these carbohydrate-conjugated IL-1α variants exhibited reduced activities in vitro, the activities in vivo were different depending on the type of carbohydrates [12, 15, 18]. Manα(1–6)-IL-1α exhibited comparable activities to nonglycosylated IL-1α 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 Manα(1–6)-IL-1α in mice differed from that of nonglycosylated IL-1α [14]. α-Gal-conjugated IL-1α exhibited a decrease in all the activities in vivo with a similar magnitude [16]. NeuAc coupled IL-1α exhibited selective activities in vivo as Manα(1–6)-conjugated IL-1α and enhanced tissue distribution [17–19].

We also synthesized NeuAc conjugated human recombinant TNFα. We obtained two glycosylated TNFαs, L NeuAc-TNFα and H NeuAc-TNFα containing 1.0 and 1.5 molecules of NeuAc per molecule of TNFα, respectively. L NeuAc-TNFα and H NeuAc-TNFα 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-kB by about 1/3 and 1/10, respectively. As a major product was L NeuAc-TNFα (the yields of L NeuAc-TNFα and H NeuAc-TNFα were 28.6 and 4.87%, respectively), in this study we examined L NeuAc-TNFα 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 TNFα (rhTNFα) was provided by Dainippon Pharmaceutical Co. (Osaka, Japan). The specific activity of rhTNFα was 6 x 10⁷ 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 x 10⁶ 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 μg/ml of streptomycin, and 10% heat-inactivated FBS) containing 1 U/ml of rhIL-6 [20].

Synthesis of glycosylated TNFα

An acyl azide derivative of NeuAc with a C9 spacer was synthesized and coupled to rhTNFα. The NeuAc-TNFα 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 spectrometry (TOF-MS) analysis. Two glycosylated TNFα were obtained and termed L NeuAc-TNFα and H NeuAc-TNFα, which contained 1.0 and 1.5 molecules NeuAc per molecule of TNFα, respectively. As a major product was L NeuAc-TNFα, we used it for in vivo experiments. For the sake of brevity, L NeuAc-TNFα was termed NeuAc-TNFα. With endotoxin test using a Limulus amoebocyte assay (sensitivity limit, 0.1 ng/ml), endotoxin contamination was negative in these TNFαs.

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

TNFαs 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 (NOx) level was measured by the method described by Misko et al. [22]. Briefly, 30 l 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 TNFα 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 TNFα

Meth-A fibrosarcoma cells (4 × 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 TNFα, 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 TNFα to induce serum IL-6 in mice

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

Mice were injected intraperitoneally with native TNFα, and their ability to induce SAA, an acute phase protein produced by hepatocytes in response to TNFα, was examined. TNFα 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-TNFα 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-TNFα at 2 µg/mouse is more potent than TNFα (Fig. 2b).

Effect of TNFα treatment on the serum glucose level in mice

The effect of TNFα on the serum glucose level was examined. The mice were injected intraperitoneally with native TNFα or NeuAc-TNFα, and then fasted. In the control mice the serum glucose level decreased with the duration of fasting up to 24 h (Fig. 3a). TNFα caused a significant reduction at 2 h after treatment, and the decrease continued to 24 h. NeuAc-TNFα also caused a reduction for up to 24 h after treatment. A dose-response experiment at 8 h indicated that NeuAc-TNFα exhibited comparable activity to TNFα (Fig. 3b).

Ability of TNFα to induce serum NOx in mice

TNFα 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 (NOx) were accumulated in the biological fluids. To examine the ability of TNFα to induce NO, serum nitrate was converted to nitrite by nitrate reductase, and then the amount of total nitrite was determined. Native TNFα injected mice exhibited an increase in the serum NOx 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-TNFα also up-regulated the serum NOx level. The time course experiment...

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Fig. 2 Effect of TNFαs on the SAA level in mice. PBS or TNFαs 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 ± SD

Fig. 3 Effect of TNFαs on the serum glucose level in mice. PBS or TNFαs 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 ± SD
Acute lethal toxicity of TNFα to mice

We determined the lethal toxicity of TNFαs to mice. Mice were sensitized to LPS by the injection of α-galactosamine intraperitoneally, and then the TNFαs (0.3 μg/mouse) were administered intraperitoneally. As shown in Fig. 5, all the mice injected with native TNFα died after up to 12 h. In contrast, 4/5 of the mice injected with NeuAc-TNFα 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-TNFα decreased to 1/3, as compared to TNFα (Table 1).

Anti-tumor activity of TNFα for Meth-A tumor bearing mice

In order to compare the anti-tumor activity of TNFαs, TNFαs were injected intravenously into the mice twice 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 TNFαs at either 2.0 or 0.5 μg per injection. However, the anti-tumor effect of NeuAc-TNFα was more potent as compared to native TNFα (Fig. 6b). In another experiment, NeuAc-TNFα at 2 μg 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-TNFα at 0.5 μg per injection did so in 2 and 3 per 7 mice on day 19 and 21, respectively, while native TNFα at either 2 or 0.5 μg per injection could not up to 32 days, though it inhibited the tumor growth. In addition, while native TNFα induced hemorrhagic necrosis, NeuAc-TNFα 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 TNFα- or NeuAc-TNFα treated control and tumor bearing mice, as compared to the PBS control (data not shown).

Discussion

In the present study, we demonstrated that NeuAc-TNFα 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-κB activation in hepatocytes, NeuAc-TNFα exhibited similarly reduced activities by about 1/3, as compared to native TNFα. It is interesting as to why the effects of NeuAc coupling to TNFα activities in vivo differed according to the activities. The activities of TNFα in vitro could be mainly determined by its affinity for the receptor. In contrast, the in vivo system
is more complex, and the potency of TNFα activity cannot be determined solely by the affinity to its receptor. In our previous studies, NeuAc-IL-1α similarly exhibited reduced activities in all the assays performed in vitro, including its receptor binding affinity, as compared to native IL-1α, 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-TNFα in its distribution into tissues, accessibility to target cells, retardation in the serum and receptor binding activity.

TNFα exerts pleiotropic effects in vivo by acting on many cell types. IL-6 is produced from many cell types by TNFα 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 and B cells and the induction of acute phase proteins from hepatocytes [25]. NeuAc-TNFα exhibited a significant decrease in the IL-6 induction activity.

| Dose (µg/mouse) | Time after TNFα injection (h) |
|-----------------|-------------------------------|
|                 | 0    | 2    | 4    | 6    | 8    | 10   | 12   | 16   | 20   | 24   |
| PBS             | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 100  |
| Untreated TNFα  | 100  | 100  | 100  | 100  | 80   | 0    | 0    | 0    | 0    | 0    |
| 3.0             | 100  | 100  | 100  | 100  | 80   | 20   | 0    | 0    | 0    | 0    |
| 1.0             | 100  | 100  | 100  | 100  | 60   | 20   | 0    | 0    | 0    | 0    |
| 0.3             | 100  | 100  | 100  | 100  | 80   | 80   | 80   | 80   | 80   | 80   |
| NeuAc-TNFα      | 100  | 100  | 100  | 100  | 60   | 20   | 0    | 0    | 0    | 0    |
| 3.0             | 100  | 100  | 100  | 100  | 80   | 80   | 80   | 80   | 80   | 80   |
| 1.0             | 100  | 100  | 100  | 100  | 60   | 20   | 20   | 0    | 0    | 0    |
| 0.3             | 100  | 100  | 100  | 100  | 100  | 80   | 80   | 80   | 80   | 80   |

* Percentage of survival after each dose of TNFα injection. Number of dead mice/total mice given in parentheses.

**Fig. 7** Induction of hemorrhagic necrosis of solid tumor in Meth-A bearing mice by TNFαs. PBS or TNFαs (2.0 or 0.5 µg/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 ± SE. Asterisk not detected.

**Fig. 6** Anti-tumor effect of TNFα. PBS or TNFαs (2.0 or 0.5 µg/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 TNFα. b Days of complete regression after tumor inoculation. Mice were used in groups of five. Each value is a mean ± SE.
TNFα 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-TNFα exhibited a more potent activity in the induction of SAA.

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

NO is an important effector molecule in neurotransmission, vasodilatation and host defense against microorganisms and tumor cells. TNFα alone or in synergy with IL-1α 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 TNFα, which is a serious deleterious effect in the application of TNFα to patients [11]. NeuAc-TNFα was weak in the induction of serum NOx level. Therefore, it is beneficial for the therapeutic use of NeuAc-TNFα. A disadvantageous aspect is that Neu-TNFα may be weak in augmenting the host defense against infection of microorganisms and tumors.

The toxic effect of TNFα contributes to endotoxin shock [24], which is also a major factor for the limited use of TNFα to patients. NeuAc-TNFα exhibited reduced acute lethal toxicity in D-galactosamine sensitized mice. This is also beneficial for the therapeutic use of NeuAc-TNFα. When native TNFα or NeuAc-TNFα 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 TNFα exerts its anti-tumor effect on the fibrosarcoma Meth-A in mice [10, 11, 29]. NeuAc-TNFα inhibited the tumor growth more rapidly and efficiently than native TNFα. The rate of complete disappearance of the tumor was also higher in NeuAc-TNFα as compared to native TNFα. As has been known, TNFα caused hemorrhagic necrosis. Quite interestingly, however, NeuAc-TNFα 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 TNFα-injected mice, but not the NeuAc-TNFα injected mice, became rowdy. Probably the TNFα-injected, but not the NeuAc-TNFα injected mice, felt pain. TNFα exerts its anti-tumor effect through its direct cytotoxic effect, necrosis or apoptosis, on tumor cells and also indirectly through host cells. TNFα 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 TNFα 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-TNFα caused necrosis without hemorrhage remained to be elucidated, our study indicated that conjugation of NeuAc to TNFα 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 TNFα in future.

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