Glycoconjugate Journal 20, 119–131, 2004

Synthesis and biological activities *in vitro* and *in vivo* of glycosylated human interleukin-1α, neoglyco IL-1α, coupled with *N*-acetylneuraminyl-galactose

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In order to study the effect of glycosylation on its biological activities, and to develop IL-1α with less deleterious effects, recombinant human IL-1α was chemically coupled with *N*-acetylneuraminic acid (α1-6) galactose (Neu5Ac-Gal). Glycosylated IL-1α (Neu5Ac-Gal-IL-1α) was purified by anion-exchange chromatography and average number of carbohydrate molecules introduced per molecule of IL-1α was 2.5. Neu5Ac-Gal-IL-1α exhibited reduced activities about 1/15-fold compared to IL-1α in all the activities performed *in vitro*. Binding affinities of Neu5Ac-Gal-IL-1α to Type I and Type II IL-1 receptors were decreased to 1/15 and 1/10, respectively. Neu5Ac-Gal-IL-1α exhibited reduction in activities *in vivo*, including induction of serum amyloid A and NO_x, and down-regulation of serum glucose. However, Neu5Ac-Gal-IL-1α exhibited comparable activity to IL-1α in improvement of the recovery of peripheral white blood cells from myelosuppression in 5-fluorouracil-treated mice. In addition, tissue level of Neu5Ac-Gal-IL-1α was relatively high compared to IL-1α. These results indicate that coupling with Neu5Ac-Gal enabled us to develop neoIL-1α with selective activities *in vivo*.
Published in 2004.

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 functions and physicochemical properties of glycoproteins. The advantage for neoglycoproteins is that chemically synthesized carbohydrates, not only natural but also unnatural carbohydrates, can be coupled to target proteins. Neoglycoproteins have been used for a variety of studies to induce carbohydrate-directed antibodies [1], to examine the immunogenicity of carbohydrate [2–4], to detect lectins in tissue or cell surface [5], to isolate carbohydrate-binding proteins [6], and as soluble inhibitors of glycoconjugate-mediating processes, such as cell-cell contact [7].

Sialic acid is usually present at the non-reducing position of oligosaccharide in glycoproteins and glycolipids, and plays an important role in function, stability and tissue distribution of glycoproteins [8]. Sialic acid is especially important in preventing the clearance of sialoglycoproteins from serum because asialoglycoproteins are rapidly cleared through Gal/GalNAc binding lectins present in the liver [9]. Furthermore, recent studies revealed that sialic acid is also important as a ligand for selectins [10] and Siglecs, members of the Ig superfamily. Siglecs are consisting of sialoadhesion, CD22, myelin associated glycoprotein (MAG) and CD33, present in macrophage subsets, B lymphocytes, oligodendrocytes/Schwann cells and myeloid cells, respectively [11]. In human six additional CD33-related Siglecs have been identified [12]. Therefore, it seems possible that coupling of sialic acid enables its conjugates to bind to a variety of cell types and prolong the serum level.

Interleukin 1 (IL-1) is a cytokine involved in immune and inflammatory responses, hematopoiesis, and homeostatic reactions [13]. Although IL-1 has beneficial effects in treatment of cancer patients due to its direct antiproliferative effect on some tumors and indirect effects through augmenting host defense and hematopoiesis, its therapeutic use is limited by the toxicity. Human IL-1 is nonglycosylated. To develop neoIL-1 with less deleterious effects but preserving beneficial effects, we have chemically coupled D-mannose dimmers [14–16], D-galactose [17–19] and *N*-acetylneuraminic acid (Neu5Ac), a major constituent of sialic acid family, to recombinant human interleukin-1α(rhIL-1α) [20–22] by the acyl azide method [23] and effects of the modification on biological activities of IL-1

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in vitro and *in vivo* were examined. Neu5Ac coupled IL-1α exhibited selective activities *in vivo* and enhanced tissue distribution [22]. However, in natural oligosaccharide Neu5Ac is usually conjugated with Gal. Therefore, conjugation of Neu5Ac or Neu5Ac-Gal may cause differential properties in IL-1 activities. In this study we coupled IL-1α with Neu5Ac (α1-6) Gal, which is present in natural sialyl oligosaccharide, and examined its activities *in vitro* and *in vivo*, affinity to IL-1 receptors and tissue distribution.

Materials and methods

Reagents

RPMI 1640 was purchased from Sigma Chemical Co. (St. Louis, MO). Eagle's MEM was from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum (FBS) was from JRH Biosciences (Lenexa, KS). Human recombinant IL-1α (rhIL-1α)(2×10^7 U/ml) was provided by Dainippon Pharmaceutical Co. (Osaka, Japan).

Coupling of NeuAc (α1-6)Gal with rhIL-1α

The synthesis of *N*-acetylneuraminic acid (α1-6) galactose (Neu5Ac-Gal) with C9 arm, 8-(Hydrazinocarbonyl)octyl o-(sodium 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-($2 \rightarrow 6$)-β-D-galactopyranoside (compound **1** in Figure 1), will be published elsewhere. Compound **1** (6.73 mg, 12.8 mmol) was dissolved in water (325 μL) and the solution chilled on ice. To the chilled solution (288 μL), cold 4 M HCl (40 μL) and 2 M sodium nitrite (20 μL) were added. After the solution was kept at room temperature for 15 min, 2 M ammonium sulfamate (20 μL) was added and the mixture was kept at room temperature for 15 min in order to inactivate excess HNO_2. This mixture (containing the acyl azide) was added to ice-cooled 0.4 M sodium borate buffer (pH 10.0, 100 μL) and PBS (400 μL) containing 1.984 mg of rhIL-1α. The pH was quickly adjusted to 9.0 with 4 N NaOH with stirring for 60 min at room temperature. The reaction mixture was then filtrated with 0.2 μm nylon membrane filter and desalted with 20 mM Tris-HCl buffer (pH 7.0) with Hi Trap Desalting column (Pharmacia). The rhIL-1α treated with the same manner without compound 1 was used as control (mock treated) IL-1α.

Purification of Neu5Ac-Gal-conjugated IL-1α

Purification was carried out at room temperature employing the FPLC system (Pharmacia). The desalted sample was loaded onto an anion-exchange chromatography column (Mono Q, Pharmacia) equilibrated with 20 mM Tris-HCl buffer (pH 7.0) at flow rate of 1 ml/min, eluted with a 30 μL linear NaCl gradient (0–0.5 M) in the same buffer, and fractions (1 ml) were collected. The buffer of fractions containing Neu5Ac-Gal-conjugated IL-1α was exchanged to PBS using HiTrap Desalting column and concentrated in Centriplus-3 (Amicon, *M*r 30,000 cut-off). The final yield of Neu5Ac-Gal-IL-1α was 43.8%.

Electrophoresis

Analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [24] on 15% polyacrylamide gel in the presence of 0.1% SDS using a vertical slab minigel apparatus. Molecular weight markers (phosphorylase b, *M*r 94,000; bovine serum albumin, *M*r 67,000; ovalbumin, *M*r 43,000; carbonic anhydrase, *M*r 30,000; soybean trypsin inhibitor, *M*r 20,100; α-lactalbumin, *M*r 14,400) were also electrohoresed. Protein bands were visualized with silver staining.

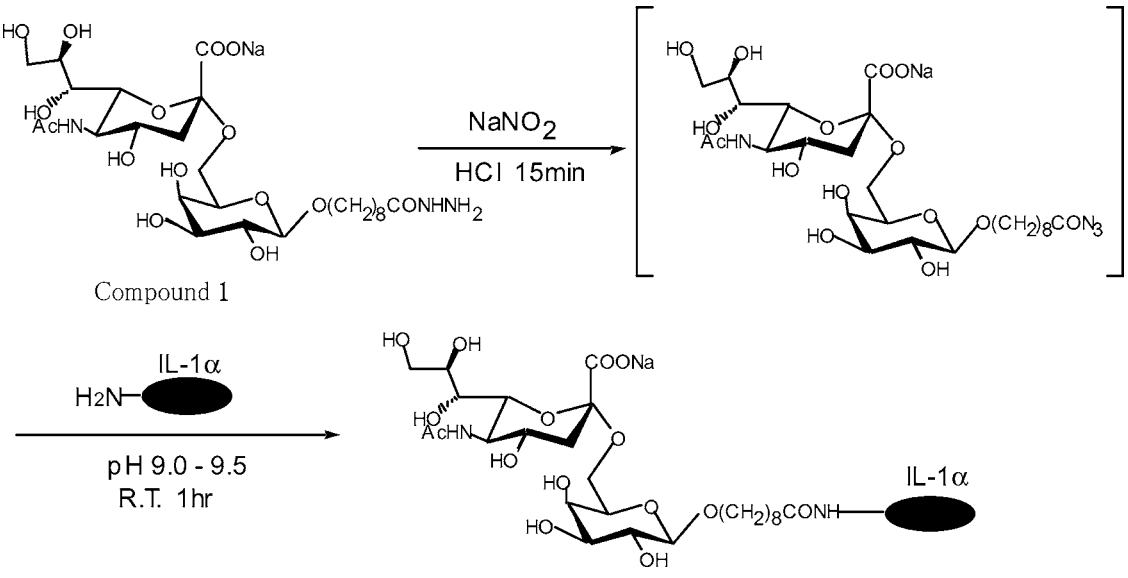

Figure 1. Synthesis of Neu5Ac-Gal-coupled IL-1α by acyl azide method.

Confirmation of Neu5Ac-Gal coupling

Size fractionated proteins were transferred from gels to immobilon polyvinylidene difluoride membrane (Milipore Corporation, Bedford, MA) using a semidry apparatus (Marysol, Tokyo, Japan) for 1 h with a current of 100 mA at room temperature. The coupled Neu5Ac-Gal was detected using biotin-hydrazide and horse-radish peroxidase (HRP)-conjugated avidin following peroxidation with $NaIO_4$ according to a protocol of G.P. Sensor kit (SEIKAGAKU CO., Tokyo, Japan).

Time-of-Flight-mass spectrometry (TOF-MS) analysis

TOF-MS analysis was performed according to the procedure of the supplier using Voyage Elite (PE Biosystems, Foster, CA).

Determination of protein content

The amount of protein was determined using a BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as a standard.

Cell culture

D10H.2 is a subclone of the mouse T cell line D10(N4)M, which had been provided by Dr. S. Hopkins (University of Manchester) [25], and its proliferation depends on IL-1 in the absence of any exogenous cytokines or mitogens. D10H.2 cells were maintained in culture medium (RPMI 1640, 100 U/ml of penicillin G, 100 μg/ml of streptomycin, 15 mM HEPES) supplemented with 5×10^{-5} M 2-mercaptoethanol, 10% heat-inactivated FBS, and 5 U/ml rhIL-1α. Murine hybridoma Murine pre-B lymphocyte 70Z/3.12 was purchased from American Type Culture Collection and maintained in culture medium supplemented with 5×10^{-5} M 2-mercaptoethanol and 10% FBS. A375-6 is an IL-1 sensitive subclone of human melanoma cell line. Mouse lymphoma cell line EL-4 6.1 C10 was provided by Dr. T. Akahoshi (University of Kitazato). Mouse myeloid cell line M1 was provided by Dr. K.S. Akagawa (National Institute of Health, Tokyo, Japan, and IL-1 sensitive clone M1-3b was obtained by limiting dilution. A375-6, EL-4 6.1 C10, and M1-3b were maintained in culture medium (RPMI1640) supplemented with 10% FBS. Human embryonic fibroblast cell line TIG-1 was provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). TIG-1 cells were maintained in culture medium (Eagle's MEM) supplemented with 10% FBS.

Assay for D10H.2 proliferation

Cultured D10H.2 cells were washed three times with IL-1 free culture medium. Fifty μl of cell suspension (2×10^5 cells/ml) was added to each flat-bottomed well of 96 well microtiter plate (Falcon, Lincoln, NJ). Fifty μl of medium containing IL-1s were added, and then the cells were cultured for 72 h at 37°C in 5% CO_2 in air. Proliferation of the cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay [26].

Assay for M1-3b growth inhibition

Cultured M1-3b cells were washed three times with culture medium. Fifty μl of cell suspension (2×10^5 cells/ml) with 5 μg/ml polymixin B were added to each flat-bottomed well of 96 well microtiter plate (Falcon, Lincoln, NJ). Polymixin B was added to the culture to prevent the effect of contaminant endotoxin which inhibits M1-3b cell proliferation. Fifty μl of medium containing IL-1s were added, and then the cells were cultured for 72 h at 37°C in 5% CO_2 in air. Proliferation of the cells was determined by MTT method [26]. The percentage of cell growth was calculated as follows:

$$\%\ \text{of control} = \frac{\text{O.D. 595 of cells cultured in medium containing samples}}{\text{O.D. 595 of cells cultured in medium alone}} \times 100$$

Assay for A375-6 growth inhibition

A375-6 cells were detached from the culture dish with 0.02% EDTA-PBS. The cells were washed with the culture medium and 100 μl of cell suspension (4×10^4 cells/ml) were added to each well of a 96 well micro titer plate. After 24 h culture at 37°C in 5% CO_2 in air, 100 μl of medium contained IL-1s were added, and the plates were incubated for another 72 h under the same conditions. The cell growth was determined by the crystal violet-staining method [27]. After solubilization of the dye-staining the absorbance at 595 nm was determined using an ELISA auto reader (Bio-Rad Laboratories, Richmond, CA). The percentage of cell growth was calculated by the same method as M1-3b.

Assay for PGE_2 released from TIG-1 cells

TIG-1 cells were cultured at 1×10^5 cells/ml for 24 h with varying concentrations of IL-1s. Prostaglandin E_2 (PGE_2) in the supernatants was quantitated by Prostaglandin E_2 Enzyme Immunoassay Kit-Monoclonal (CAYMAN CHEMICALS, Ann Arbor, MI).

^{125}I-IL-1α binding assay

(3-[^{125}I]iodotyrosyl)hrIL-1α (81.01TBq/mmol) was purchased from Amersham Pharmacia biotech (Aylesbury, UK). EL-4 6.1 C10 cells (1×10^6) or 70Z/3.12 cells (7.3×10^5) were incubated at 4°C for 1 h in a total volume of 0.2 ml RPMI 1640 containing 1 mg/ml BSA, 22.75 pg and 2.8 ng of ^{125}I-IL-1α, respectively, and varying concentrations of unlabeled rhIL-1αs. The free and bound radioactivity were separated by the binding oil column method [28] and measured with a γ-counter (Aloka, Tokyo, Japan).

Measurement of serum levels of glucose, serum amyloid A (SAA) and NO_x

Neu5Ac-Gal-IL-1α and IL-1α were diluted to the desired concentration with sterile PBS and administered to mice intraperitoneally. To rule out the influence of possibly existing lower than 0.1 ng/ml of endotoxin, polymyxin B was added at 5 μg/ml. Mice were fasted after the administration. At specified times points after the administration, mice were bled.

The glucose level in serum was determined using a glucose B-test kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

The concentration of SAA was measured by ELISA as described previously [21].

NO_x levels in serum were measured according to the method described by Misko *et al.* [29]. Briefly, 30 μl of each sample was 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. Protein precipitates were removed by centrifugation at 15,000 rpm for 5 min and 50 μl of each supernatant were transferred to 96-well plate (Falcon) and the O.D. 595 nm was measured using an ELISA auto reader (Bio-Rad Laboratories, Richmond, CA).

Hematology

Peripheral blood samples (10 μl) of mice obtained through the orbital vein were added to 90 μl of Turk solution (Wako Pure Chemical Industries, Ltd, Osaka, Japan). The white blood cell (WBC) number was counted microscopically using a hemacytometer.

Radiolabeling of IL-1

rhIL-1α and Neu5Ac-Gal-IL-1α were labeled with ^{125}I using RADIATION KIT[I-125] (ICN Pharmaceuticals Inc., Costa Mesa, CA). The specific radioactivities of IL-1α and Neu5Ac-Gal-IL-1α were 1.53×10^6 cpm/μg and 1.32×10^6 cpm/μg, respectively. The labeled IL-1αs were diluted with unlabeled IL-1α or Neu5Ac-Gal-IL-1α to achieve a radioactive concentration of 1.5×10^5 cpm/μg before intraperitoneally administration (3×10^5 cpm/2 μg/mouse) to mice to investigate their tissue distribution.

Determination of protein content

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

Statistical analysis

Differences between group means were assessed by unpaired *t* test.

Results

Purification of NeuAc-Gal coupled IL-1α

The Neu5Ac-Gal coupled IL-1α was purified employing FPLC system using an anion-exchange chromatography column. As shown in Figure 2A and B, untreated IL-1α and treated IL-1α

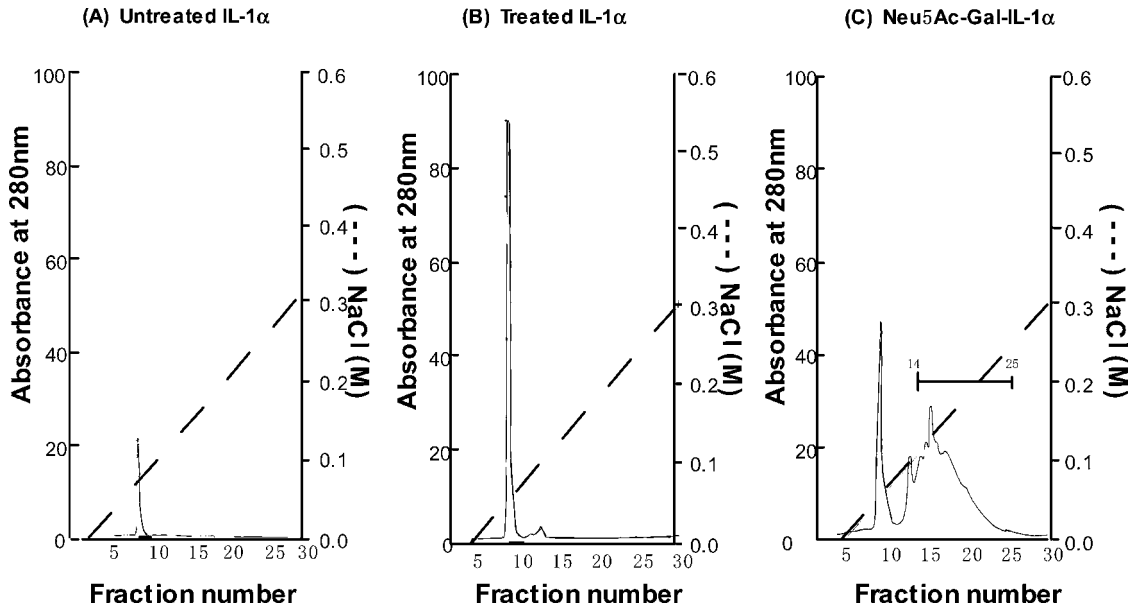

Figure 2. Purification of Neu5Ac-Gal-IL-1α by anion exchange column chromatography. (A) Untreated IL-1α (99 μg), (B) treated IL-1α (497 μg) or (C) Neu5Ac-Gal-IL-1α (1.49 mg) after reaction were applied to the anion-exchange chromatography column (Mono Q, Pharmacia) with FPLC system. The starting buffer was 20 mM Tris-HCl (pH 7.0), and the elution buffer was the starting buffer containing 0.5 M NaCl with a linear gradient. Fractions (1 ml) were collected at a flow rate of 1 ml/min.

eluted in Fr. 9–10. The small peak at Fr. 13 of treated IL-1α seemed to be due to the conformational changes caused by the reaction condition. In contrast, glycosylated IL-1α eluted in Fr. 7 to 25 (Figure 2C). As Fr. 13 may contain nonglycosylated IL-1α as demonstrated in Figure 1B, Fr. 14 to 25 were collected as glycosylated IL-1α. The yield of Neu5Ac-Gal-IL-1α was 43.8%.

SDS-PAGE analysis of Neu5Ac-Gal-IL-1α and confirmation of glycosylation

Untreated, treated (mock treated) and Neu5Ac-Gal-IL-1s were analyzed on SDS-PAGE. As shown in Figure 3A, untreated IL-1α and treated IL-1α migrated at 17.6 kDa, and Neu5Ac-Gal-IL-1α migrated at higher molecular weight. The M.W.

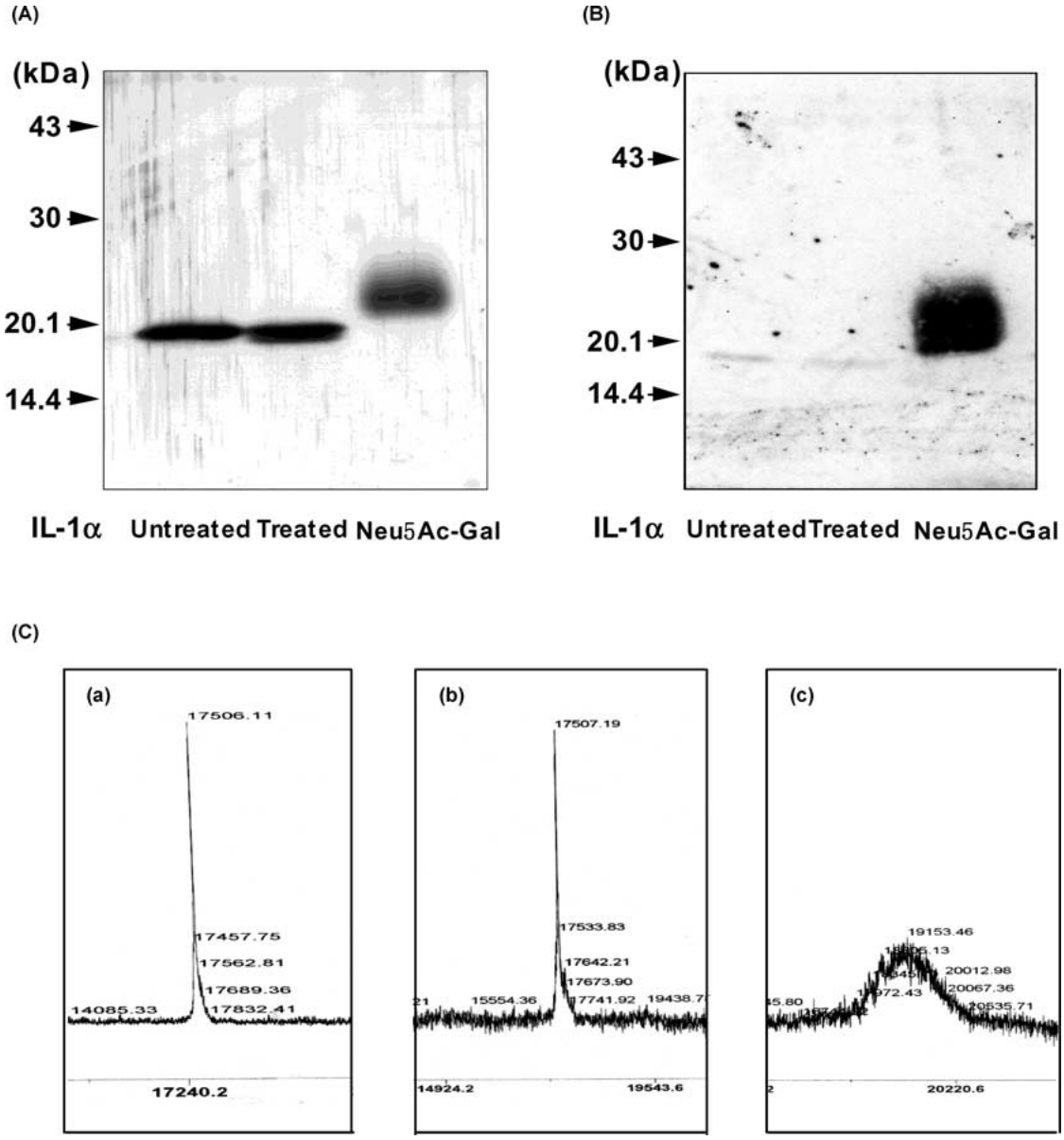

Figure 3. Characterization of glycosylated IL-1. 0.75 μg of IL-1αs were analyzed on SDS-containing 15% polyacrylamide gel under reduced condition. (A) IL-1αs were visualized with silver staining. (B) After electrophoresis, IL-1αs were transferred onto a PVDF membrane. The membrane was stained with biotin-hydrazide and horse-radish peroxidase (HRP)-conjugated avidin following peroxidation with $NaIO_4$. Molecular weight standards electrophoresed in parallel are indicated to the left in kilo Daltons. (C) Molecular weight of IL-1αs were determined by TOF-MS analysis. (a) Untreated IL-1α, (b) Treated IL-1α, (c) Neu5Ac-Gal-IL-1α. Representative data of three independent experiments are shown.

of Neu5Ac-Gal-IL-1α was determined by TOF-MS analysis (Figure 3C). IL-1α and control IL-1α exhibited the same peak at 17.5 kDa. In contrast, Neu5Ac-Gal-IL-1α exhibited a major peak at 19.2 kDa. Therefore, average number of Neu5Ac-Gal coupled to IL-1α was 2.5 mole per molar IL-1α. To confirm that Neu5Ac-Gal was coupled to IL-1α, IL-1-transferred membrane was analyzed by oxidation with $NaIO_4$, the method to detect carbohydrate. As shown in Figure 3B, only Neu5Ac-Gal-IL-1α was markedly stained indicating that Neu5Ac-Gal was coupled to IL-1α. The faint staining of untreated and control IL-1s was nonspecific because nonglycosylated molecular weight markers were also stained in the same density (data not shown).

IL-1 proliferative effect on T cells

Biological activities of Neu5Ac-Gal-IL-1α were compared to those of untreated IL-1α, and control (mock treated) IL-1α. T cell proliferation-stimulating activity was determined by using the IL-1-dependent mouse T cell clone D10H.2 [25]. The cells were cultured for 4 days with or without IL-1s, and then the cell proliferation was determined. As shown in Figure 4A, control IL-1α exhibited the activity comparable to untreated IL-1α. In contrast, Neu5Ac-Gal-IL-1α exhibited about 1/15 activity of untreated IL-1α.

IL-1 antiproliferative effect on melanoma cells and myeloid leukemic cells

IL-1 inhibits the growth of human melanoma cells A375-6 and mouse myeloid leukemic cells M1 [30,31]. To determine the IL-1 antiproliferative activity, A375-6 cells and M1 cells were treated with or without IL-1s for 3 and 4 days, respectively, and then cell proliferation was determined. As shown in Figure 4B and C, control IL-1α exhibited the antiproliferative activity comparable to untreated IL-1α for these two cell types. The activity of Neu5Ac-Gal-IL-1α decreased to about 1/15 of untreated IL-1α.

IL-1 stimulation of PGE_2 production by fibroblast cells

To evaluate the stimulating activity of PGE_2 production by fibroblast cells [32], human fibroblast cell line TIG-1 cells were cultured with or without IL-1s for 24 h, and then the amount of PGE_2 in the culture supernatants was determined (Figure 4D). The activity of control IL-1α was the same as that of untreated IL-1α. Neu5Ac-Gal IL-1α exhibited the activity about 1/15 of untreated IL-1α.

Competitive binding of ^{125}I-IL-1α to T cells and B cells with unlabeled IL-1s

T cells and B cells preferentially express Type I and Type II IL-1 receptor (IL-1R), respectively [33,34]. To determine the ability of IL-1s to bind Type I IL-1R the competitive binding of ^{125}I-IL-1α with unlabeled IL-1s to mouse T cell line cells (EL-4) was examined. As shown in Figure 5A, control IL-1α exhibited binding activity comparable to untreated IL-1α. The binding activity of Neu5Ac-Gal-IL-1α was about 1/15. Using mouse preB cell line 70/3.12 cells, the binding activity of IL-1α to Type II IL-1R was examined. As shown in Figure 5B, the binding activity of control IL-1α to Type II IL-1R was again comparable to untreated IL-1α. In contrast, the activity of Neu5Ac-Gal-IL-1α was about 1/10 of untreated IL-1.

Ability of IL-1 to induce SAA in mice

IL-1α and NeuAc-Gal-IL-1α were injected intraperitoneally into mice to investigate their ability to induce SAA, a major acute phase protein produced by hepatocytes in response to IL-1 [35]. Control IL-1α was not examined in an *in vivo* study because its biological activities *in vitro* and physicochemical property were not changed from IL-1α. IL-1α increased SAA level after 4 h treatment with maximum level at 8–12 h, and then decreased at 24 h. In contrast, Neu5Ac-Gal-IL-1α increased SAA level with a peak at 8 h, and then decreased at 12 h (Figure 6A). A dose-response experiment at 8 h indicated that Neu5Ac-Gal-IL-1α exhibited about 1/20 activity of IL-1α. (Figure 6B).

Ability of IL-1 to induce serum NO_x in mice

IL-1 induces NO synthesis in a variety of cell types. Nitric oxide (NO_x) reacts with molecular oxygen and water to generate nitrite and nitrate that accumulate in biological fluids [36]. To measure the level of NO_x, serum nitrate was converted to nitrite by nitrate reductase, and then the amount of total nitrite was determined. The serum NO_x level of IL-1α-injected mice began to increase at 4 h with a peak level at 8–12 h and decreased but still remained high compared to control at 24 h (Figure 4A). Neu5Ac-Gal-IL-1α induced the similar time-dependent increase of serum NO_x level up to 8 h, and then decreased at 12 h (Figure 7A). A dose-response experiment at 8 h showed that Neu5Ac-Gal-IL-1α exhibited about 1/5–1/10 activity of IL-1α (Figure 7B).

Effect of IL-1 treatment on serum glucose level in mice

The effect of IL-1 on serum glucose level was examined. Mice were injected intraperitoneally with IL-1α or Neu5Ac-Gal-IL-1α, and then fasted. In control mice the serum glucose level slightly decreased up to 12 h, and was further decreased at 24 h (Figure 8A). IL-1α caused a reduction of serum glucose level at 2 h after treatment and the decrease continued to 12 h. Neu5Ac-Gal-IL-1α also caused the reduction for up to 12 h after treatment. A dose-response experiment at 8 h indicated that Neu5Ac-Gal-IL-1α exhibited about 1/5 activity of IL-1α (Figure 8B).

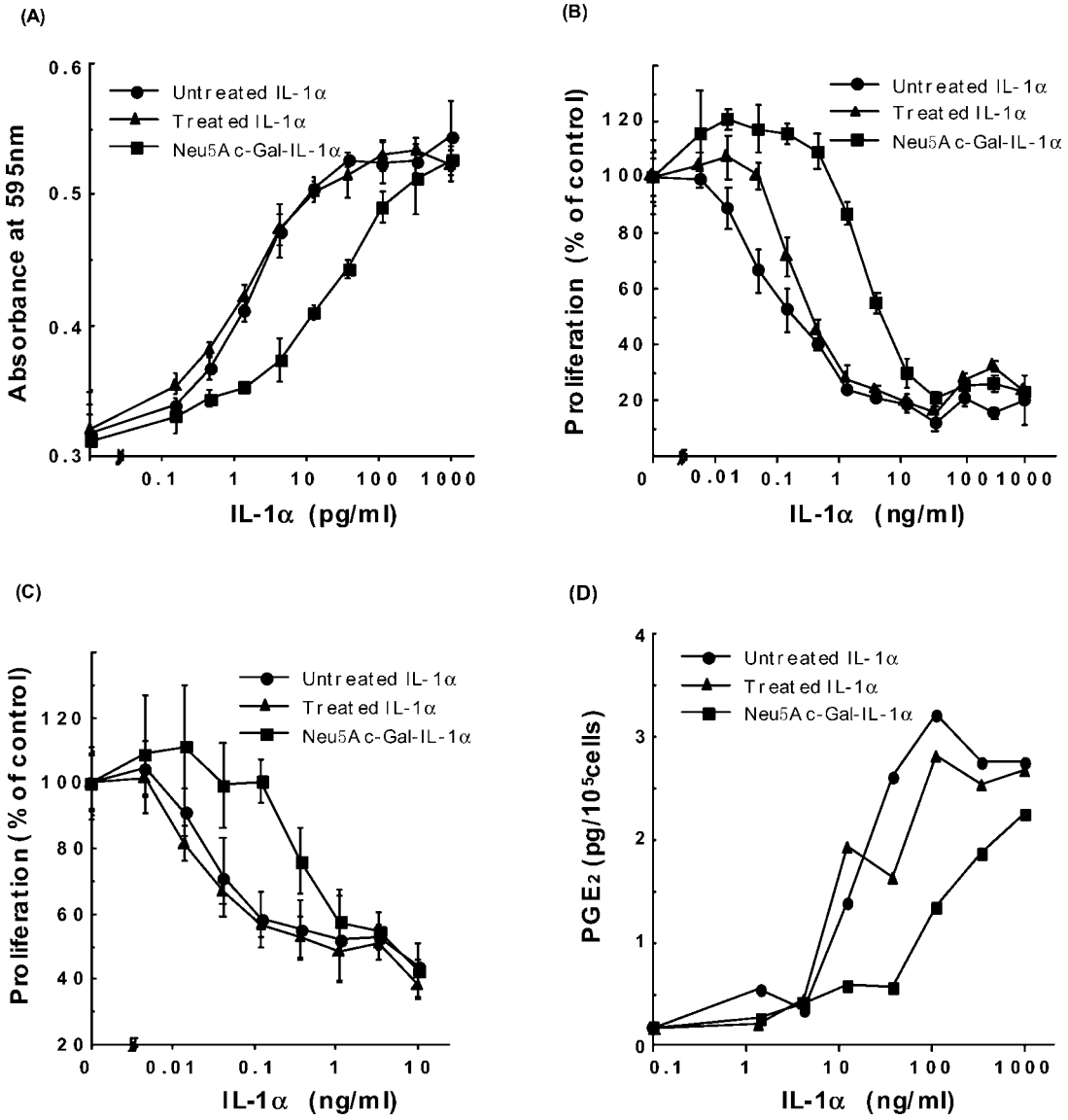

Figure 4. Biological activities of IL-1α *in vitro*. (A) Effects of IL-1s on proliferation of D10H.2 cells. Mouse T cell line D10H.2 cells were cultured at 37°C for 4 days with or without varying doses of IL-1αs. After culture, cell proliferation was determined by MTT method. (B, C) Antiproliferative effect of IL-1 on A375-6 cells (B), and M1 cells (C). Human melanoma cell line A375-6 cells were cultured at 37°C for 3 days with or without varying doses of IL-1αs. After culture, cells were stained with crystal violet. Mouse myeloid leukemic cell line M1-3b cells were cultured at 37°C for 4 days with or without varying doses of IL-1αs. After culture, cell proliferation was determined by MTT method. Representative data of three independent experiments are shown. (D) Effect of IL-1 on the PGE_2 production by TIG-1 cells. Human fibroblast cell line TIG-1 cells were cultured with or without varying doses of IL-1αs. After 24 h culture, the amount of PGE_2 in the supernatants was determined by ELISA. Mean of duplicate cultures are shown. Representative data of two independent experiments are shown.

Recovery of myelosuppression as a result of IL-1α treatment in 5-fluorouracil-treated mice

We determined the ability of IL-1α to recover the peripheral white blood cell (WBC) count in 5-fluorouracil (5-FU) treated mice. Mice were injected with 5-FU (12 mg/mouse) as a single dose intraperitoneally on day 0. From day 1, IL-1s were administered intraperitoneally twice a day (0.2 μg/injection, 0.4 μg/day); at the dosage IL-1α has been shown to exhibit significant recovery effect on peripheral WBC count. Treatment with 5-FU resulted in a decrease in WBC in peripheral blood on day 2 (Figure 9). On day 4 the effect of IL-1, either IL-1α or NeuAc-Gal-IL-1α, was not apparent. On day 13 the level of WBC in 5-FU treated mice remained low. However, both IL-1α and Neu5Ac-Gal-IL-1α treated groups increased the level of WBC higher than control when they were injected into control

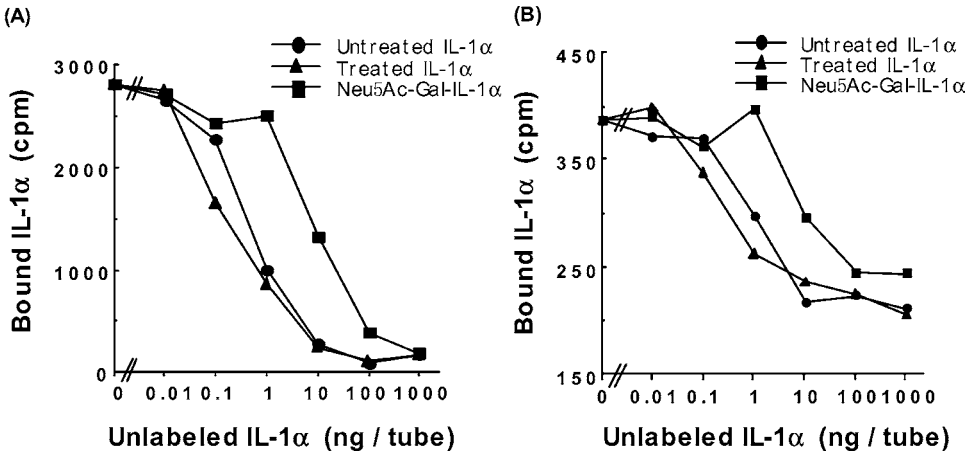

Figure 5. Inhibition of ^{125}I-IL-1α binding to EL-4 6.1 C10 cells (A), and 70Z/3.12 cells (B) by untreated IL-1α. Mouse T cell line EL-4 6.1 C10 cells and mouse pre-B cell line 70Z/3.12 cells were incubated with ^{125}I-IL-1α in the presence of varying doses of unlabeled IL-1αs for 1 h at 4°C. The free and bound radioactivity was separated by the binding oil column method.

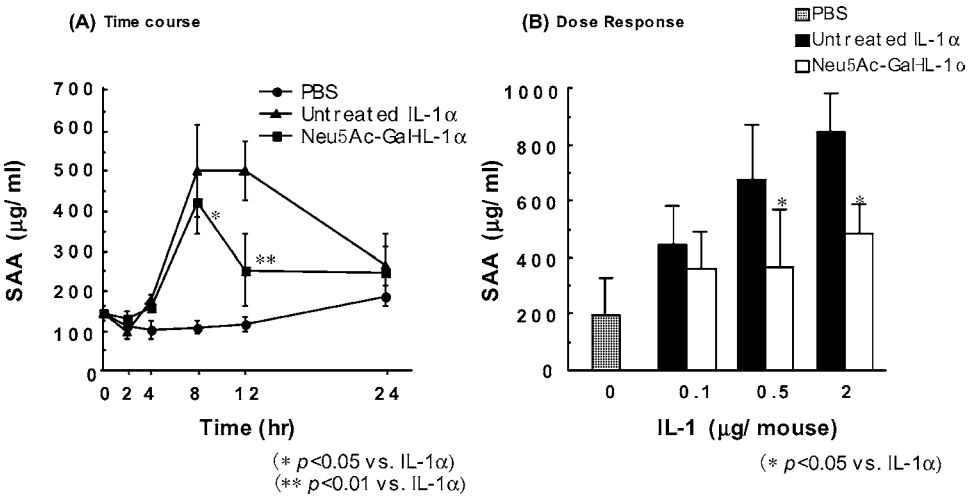

Figure 6. Effect of IL-1 on SAA level in mice. Neu5Ac-Gal-IL-1α, IL-1α or PBS (containing polymyxin B) was administered intraperitoneally and mice were bled at the time indicated (A) or 8 h later (B). SAA level was determined by ELISA. Each point represents the mean ± SD ($n = 5$).

mice. In addition, both IL-1α and Neu5Ac-Gal-IL-1α treatment caused the recovery in the level of WBC in 5-FU treated mice. There was no difference in the potency between IL-1α and Neu5Ac-Gal-IL-1α in this regard.

Tissue distribution of IL-1α

In order to determine whether conjugation of Neu5Ac-Gal affects the tissue distribution of IL-1α, ^{125}I labeled IL-1α and Neu5Ac-Gal-IL-1α were administered intraperitoneally into mice, their content in various tissues over time was determined and compared (Figure 10). At 2 h a relatively high amount of ^{125}I-IL-1α was found in the kidneys and lungs compared to other tissues. The amount of IL-1α in the kidney decreased at 4 h. At 20 h the level of IL-1α in all the tissues decreased to quite a low level. On the other hand, Neu5Ac-Gal-IL-1α distributed at higher levels than IL-1α in the kidney at 2 h, and the kidney, heart and blood at 4 h, and the liver at 20 h.

Discussion

In this study, we conjugated Neu5Ac-Gal to IL-1α. The conjugation was confirmed by oxidation with $NaIO_4$, which is specific to carbohydrate. Analysis with TOF-MAS revealed that about 2.5 moles of Neu5Ac-Gal were coupled per mole of IL-1α. The ratio was almost the same in the case of Neu5Ac, 2.7 moles of Neu5Ac was coupled per mole of IL-1α [20]. This is interesting because $Man_2\alpha$(1-4), $Man_2\alpha$(1-6) and Gal monomer coupled to per mole of IL-1α were averagely 4.7, 5.2 and 9.1 moles, respectively [14,17]. X-ray crystallographic

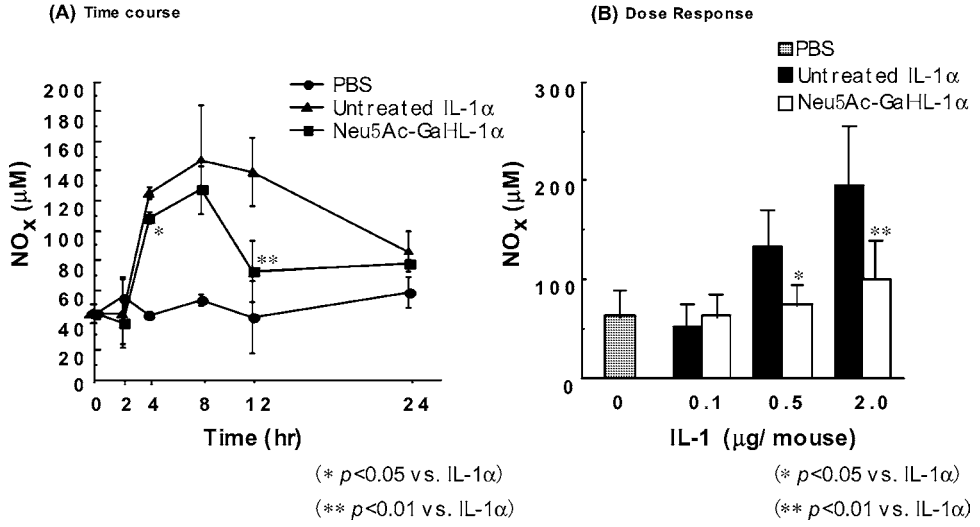

Figure 7. Effect of IL-1 on serum NO_x level in mice. Neu5Ac-Gal-IL-1α, IL-1α or PBS (containing polymyxin B) was administered intraperitoneally and mice were bled at the time indicated (A) or 8 h later (B). Serum NO_x level was determined as described in materials and methods. Each point represents the mean ± SD ($n = 5$).

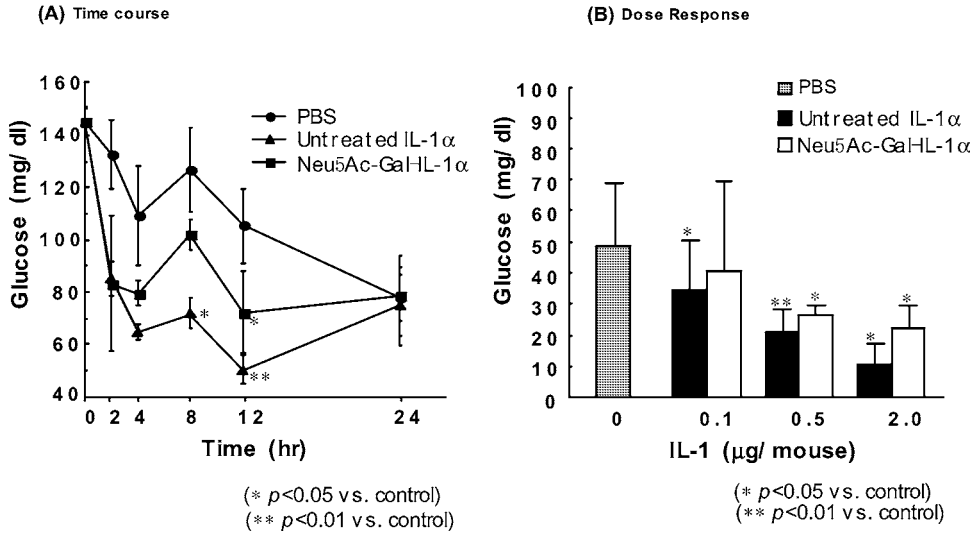

Figure 8. Effect of IL-1 on serum glucose level in mice. Neu5Ac-Gal-IL-1α, IL-1α or PBS (containing polymyxin B) was administered intraperitoneally and mice were bled at the time indicated (A) or 8 h later (B). Serum glucose level was determined using the glucose B-test (Wako). Each point represents the mean ± SD ($n = 5$).

analysis revealed that rhIL-1α contains 15 potentially reactive amino residues, 13 Lys, 1 Arg and N-terminal amino acid exposed on the surface of rhIL-1α [37]. Therefore, Gal monomer was estimated to have reacted to about 61% of the potential reactive amino residues. Probably the combination of the species and the size of carbohydrates will be important. Regarding to the number of carboydrates introduced, Gal monomer was mostly coupled, presumably because the size of the molecule was smallest in Gal, Man dimer (Man_2), Neu5Ac and Neu5Ac-Gal. In case of Neu5Ac and Neu5Ac-Gal, their negative charge will also contribute to the relatively small number of coupling.

Neu5Ac-Gal-IL-1α exhibited the reduction in all the biological activities examined *in vitro*. However, as control IL-1α exhibited the comparable activities to untreated IL-1α, the coupling condition did not affect IL-1 activity. This was the same in our previous studies employing Man_2, Gal monomer and Neu5Ac [14,18,21]. Biological activities of Neu5Ac-Gal-IL-1α was about 1/15 in all the assays performed *in vitro*, which is similar to Neu5Ac-IL-1α, about 1/10 activity of IL-1α.

IL-1 signal is mediated through its specific receptor on the cell surface. There are two types of IL-1 receptor. Type I IL-1R with molecular weight (MW) of 80 kDa is mainly expressed on

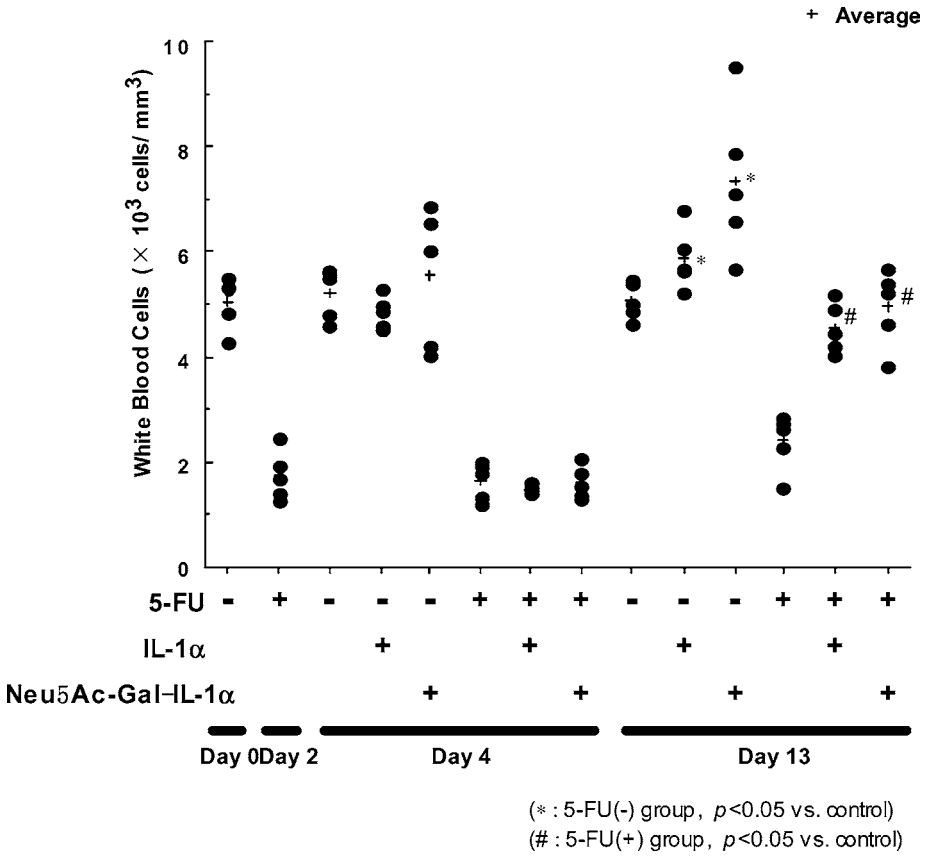

Figure 9. Recovery of myelosuppression as a result of IL-1α treatment in 5-FU-treated mice. Mice were administered 5-FU (12 mg/mouse) as a single dose intravenously on day 0. Neu5Ac-Gal-L-1α, IL-1α or PBS were administered intraperitoneally twice (200 ng each) daily from day 1 to day 12. Each group consisted of 5 mice. On day 4 and 13, mice were bled through the orbital vein and blood was suspended in Turk solution for WBC counting.

T cells and fibroblast cells [33], and Type II IL-1R with MW of 60 kDa is mainly expressed on macrophages, bone marrow cells, and B cells [34]. Only Type I IL-1R can deliver IL-1 signals into cells. Type II IL-1R is unable to transduce IL-1 signals, but works as a decoy receptor, subsequently the ratio of IL-1RI/IL-RII on the cell surface influences the IL-1 activity. It is reported that even in macrophages, bone marrow cells and B cells a small number of Type I IL-1R is expressed that transduce IL-1 signals [38]. Neu5Ac-Gal-IL1α exhibited the reduced binding affinity to both Type I and Type II IL-1Rs about 1/10 and 1/15, respectively, which were the same levels as Neu5Ac-IL-1α whose binding affinity decreased to 1/10 for both Type I and II IL-1R. The magnitude of the IL-1RI binding affinity of Neu5Ac IL-1α [21] and Neu5Ac-Gal-IL-1α correlated well with those of its biological activities *in vitro*. Therefore, the decreased biological activities of Neu5Ac-Gal-IL-1α can be attributed to its decreased binding affinity to IL-1RI. It is possible that carbohydrate introduction causes a conformational change that leads to the decrease in binding affinity to IL-1R, or carbohydrate interferes IL-1 binding to IL-R. In case of Neu5Ac-IL-1α and Neu5Ac-Gal-IL-1α, the negative charge of sialic acid may also contribute to the altered binding affinity by exclusive interaction with cell surface sialic acid. In contrast to Neu5Ac-IL-1α and Neu5Ac-Gal-IL-1α, *in vitro* activities of $Man_2\alpha$(1-6)-IL-1α and Gal-IL-1α did not well correlate to their IL-1R binding affinities (Table 1). Probably other molecules on the cell surface also contribute to exerting their biological activities, which may be carbohydrate and cell-type specific because the potency of their activities differed depending on cell types.

Similar to the biological activities *in vitro*, Neu5Ac-Gal-IL-1α exhibited a reduction in activities *in vivo* at about 1/5–1/20, including up-regulation of serum level of SAA and NO_x, and down-regulation of serum glucose level. However, Neu5Ac-Gal-IL-1α exhibited a comparable activity as IL-1α to improve the recovery of peripheral WBC from myelosuppression in 5-fluorouracil-treated mice.

IL-1 alone or in synergy with IL-6 or glucocorticoid induces the synthesis of acute phase proteins by hepatocytes. SAA is the representative produced by hepatocytes in response to IL-1 [35]. NO_x is an important effector molecule in neurotransmission, vasodilatation and host defense against microorganism and tumor cells. IL-1 alone or in synergy with TNFα and interferon augments the production of NO_x from many cell types, including macrophages, hepatocytes, vascular endothelial cells and

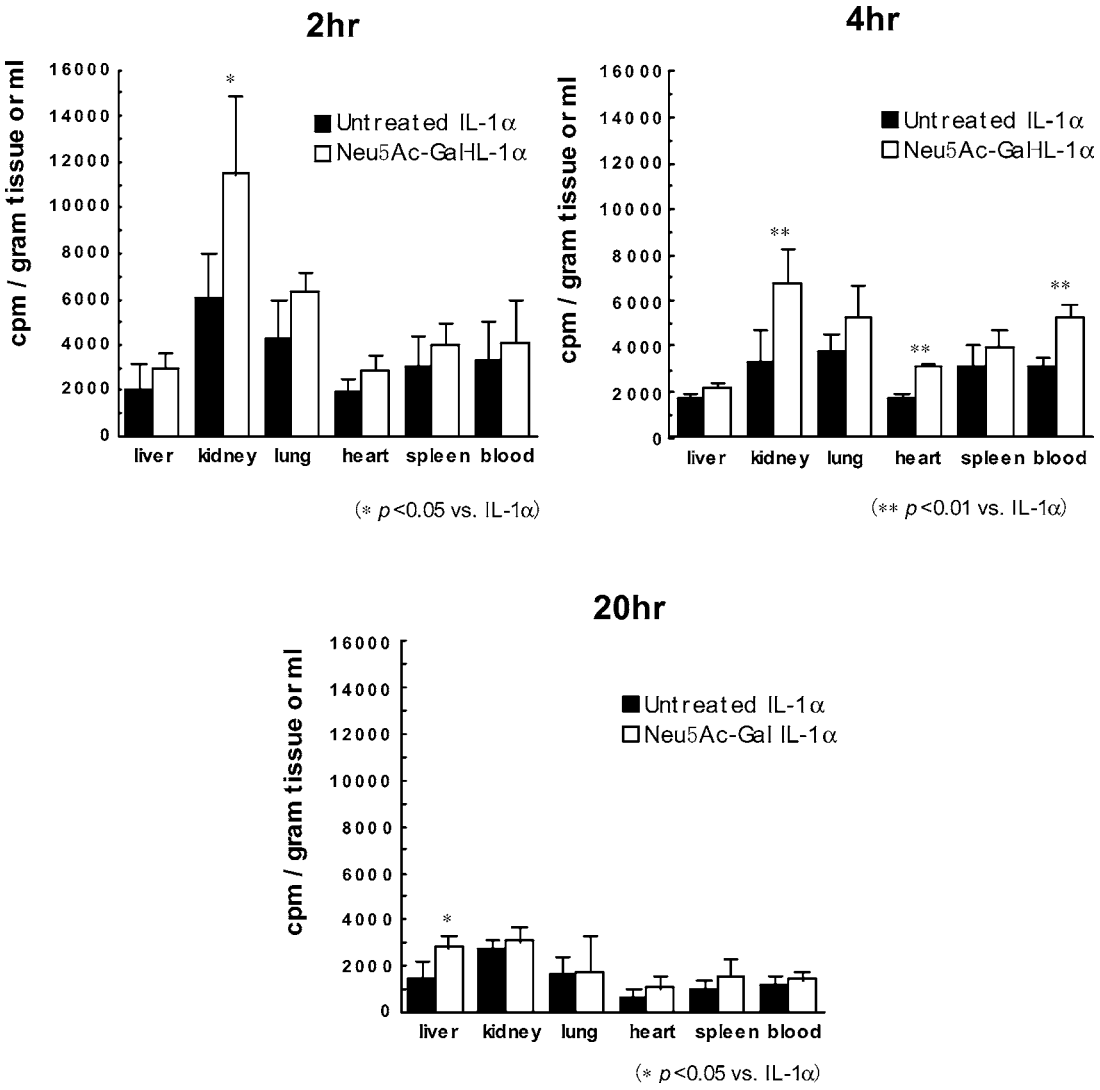

Figure 10. Tissue distribution of ^{125}I-IL-1α and ^{125}I-Neu5Ac-Gal-IL-1α. Radiolabeled Neu5Ac-Gal-IL-1α or IL-1α was intraperitoneally administered into mice. After taking blood through the inferior vena, tissues were removed immediately at the time indicated. After tissue wet weights were measured, radioactivity was determined using a γ-counter. Data were expressed as cpm per gram tissue or per ml blood ($n = 5$).

smooth muscle cells [36]. NO_x produced by smooth muscle cells, in conjunction with PGI_2 produced by endothelial cells, is implicated in IL-1-induced hypotension, which is the most serious deleterious effect in application of IL-1 to patients [13]. In induction of NO_x and SAA, Neu5Ac-Gal-IL-1α exhibited comparable time-dependent up-regulatory activities to IL-1α up to 8 h, however the up-regulatory activities of Neu5Ac-Gal-IL-1α decreased at 12 h. This is in contrast to Neu5Ac-IL-1α, which exhibited almost the same kinetics as IL-1α at 2 μg/mouse [22].

A similar difference was also observed in the activity to decrease a serum glucose level. Neu5Ac-Gal-IL-1α exhibited the reduced activity compared to IL-1α. However, the activity of NeuAc-IL-1α was indistinguishable from that of IL-1α [22]. Similar to NeuAc-IL-1α, $Man_2\alpha$(1-6)-IL-1α possessed the comparable activity to IL-1α [15]. In contrast, the activity was decreased in Gal-IL-1α [19]. Although the reason is not known, it may be due to the different distribution of these glycosylated IL-1s in the liver. Modification with galactose will enhance the uptake of Gal-IL-1α by hepatocytes, thus its degradation may be enhanced, whereas Neu5Ac-IL-1α is resistant to the uptake through Gal-specific lectin by hepatocytes [9]. $Man_2\alpha$(1-6)-IL-1α is also resistsant to the uptake through interaction with Man-specific lectin present in hepatocytes and macrophages [39].

One of the beneficial effects of IL-1 is an enhancement of recovery of peripheral WBC in chemotherapeutic-drug-treated animals [40]. IL-1 is known to increase the survival of early progenitor cells and to enhance multipotential colony formation through the induction of several hematopoietic growth factors,

Table 1. Comparison of biological activities of carbohydrate-introduced IL-1αs

Coupled carbohydrate	*$Man_2\alpha(1,6)$*	*Gal*	*Neu5Ac*	*Neu5Ac-Gal*
Molecules of carbohydrates coupled to molar of IL-1α	5.2	9.1	2.7	2.5
T cell proliferative activity	1/30	1/30	1/10	1/15
Antiproliferative effect on M1	1/100	1/100	1/10	1/15
on A375	1/300	1/100	1/10	1/15
PGE_2 induction	1/700	$<$1/10000	1/10	1/15
Affinity to Type I IL-1R	1/700	1/500	1/10	1/15
Type II IL-1R	1/600	1/100	1/10	1/10
Induction of α1-acid glycoprotein* or SAA#	$>$1/20*	1/10–1/20*	1/5#	1/20#
Reduction of serum Glc level	1/1	1/20	1/1	1/5
Induction of serum NO_x	ND	1/20	1/5	1/5–1/10
Recovery of WBC	1/1	1/10	$\geq$1/1	1/1
Enhancement of tissue distribution	+	ND	++	+

Activities were compared to untreated IL-1α. ND: not determined.

such as G-CSF, GM-CSF, IL-3, and IL-6 [41]. IL-1 also enhances the sensitivity of progenitor cells to these growth factors through up-regulation of their receptors [42]. Neu5Ac-Gal-IL-1α exhibited the comparable WBC recovering activity to IL-1α. At the dosage we used Neu5Ac-Gal-IL-1α exhibited no severe acute reactions. In addition, there were no toxic effects, such as anorexia, diarrhea, somnolence and body weight loss (data not shown). This is important in consideration of the therapeutic application of IL-1. It has to be noted that $Man_2\alpha$(1-6)-IL-1α and Neu5Ac-IL-1α, but not Gal-IL-1α, also exhibited the comparable WBC recovering effect to IL-1α [15,19,22]. Probably Gal-IL-1α is more efficiently trapped in the liver than IL-1α, subsequently delivery of IL-1 into bone marrow will be reduced.

Another potential advantage for Neu5Ac-Gal-IL-1α is its prolonged existence in serum and sustained *in vivo* effects. The level of radiolabeled Neu5Ac-Gal-IL-1α remained high compared to IL-1α in the kidney at both 2 and 4 h, the heart and the blood at 4 h, and the liver at 20 h. Especially at 2 h the level of Neu5Ac-Gal-IL-1α in the kidney was high. It is reported that intraperitoneally injected human IL-1β into mice gives an initial peak in plasma after 10 min, and then the level declined with the duration of time [41]. The major route of clearance is the kidney, which is in accordance with our results. Similar augmented distribution of Neu5Ac-IL-1α was also observed [22]. However, the magnitude was different. Neu5Ac-Gal-IL-1α distributed in the kidney by about twofold, while that of Neu5Ac-IL-1α was by about fivefold. In addition Neu5Ac-IL-1α levels were high even after 20 h in all the tissues. Therefore, although the Neu5Ac-Gal-IL-1α level was high in some tissues, it was still low compard to that of Neu5Ac-IL-1α. Theses differences probably explain the potent biological activities of Neu5Ac-IL-1α relative to Neu5Ac-Gal-IL-1α. As *N*-acetylneuraminic acid (α 1-6) galactose (Neu5Ac-Gal) is present in natural oligosaccharides, the terminal Neu5Ac may be degraded by neuraminidase, subsequently desialylated IL-1α may be readily metabolized.

Acknowledgments

We thank Miss S. Kato for the ^{1}H NMR and ^{13}C NMR spectral measurements, Miss T. Naito for the microanalyses, Dr. R. Taguchi for TOF-MS analysis, and Dr. T. Matsumura for technical assistance. We also acknowledge NGK Insulators, Ltd. for providing sialic acid. This work was supported in part by Grant-in Aid for Scientific Research (B) from Japan Society for the Promotion of Science.

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Received 9 October 2003; revised 14 November 2003;
accepted 25 November 2003