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Glycosylated human interleukin-1α, neoglyco IL-1α, coupled with *N*-acetylneuraminic acid exhibits selective activities *in vivo* and altered tissue distribution

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In order to study the effect of glycosylation on its biological activities and to develop IL-1 with less deleterious effects, *N*-acetylneuraminic acid (NeuAc) with C9 spacer was chemically coupled to human recombinant IL-1α. NeuAc-coupled IL-1α (NeuAc-IL-1α) exhibited reduced activities *in vitro* and receptor-binding affinities by about ten times compared to IL-1α. In this study, we examined a variety of IL-1 activities *in vivo*. NeuAc-IL-1α exhibited a marked reduction in the activity to up-regulate serum IL-6, moderate reduction in the activities to up-regulate serum amyloid A and NOx. However, it exhibited comparable activities as IL-1α to down-regulate serum glucose and to improve the recovery of peripheral white blood cells from myelosuppression in 5-fluorouracil-treated mice. In addition, tissue level of NeuAc-IL-1α was high compared to IL-1α. These results indicate that coupling with NeuAc enabled us to develop neo-IL-1 with selective activities *in vivo* and enhanced tissue level.

Keywords: **neoglycoprotein, sialic acid, interleukin 1, cytokine**

Introduction

Glycoproteins are widely distributed in animals, plants and microorganisms. Neoglycoproteins, proteins chemically or enzymatically coupled with carbohydrates, are quite useful for investigating the functions of carbohydrates and the physicochemical properties of glycoproteins. The great advantage to synthesize neoglycoproteins is that both natural and chemically synthesized artificial carbohydrates can be coupled to proteins [1–4].

Sialic acid, usually present at the non-reducing position of oligosaccharide in glycoproteins and glycolipids, is important for the function, stability and tissue distribution of glycoproteins [5] and can also act as a ligand for viruses, including influenza viruses, paramyxoviruses, coronaviruses, polyomaviruses and retoviruses [6]. Sialic acid is especially important for the retension of glycoproteins in serum because asialoglycoproteins are rapidly cleared through galactose binding lectins present in the liver [7]. Indeed, sialic acid is present in most of the serum glycoproteins and is content amounts to about 20% in erythroprotein and α1-acid glycoprotein [8]. Furthermore, recent studies revealed that sialic acid is also important as a ligand for selectins [9] and sialoadhesin family of cell surface lectins. Sialoadhesin family consists of Sialoadhesin, CD22, myelin associated glycoprotein (MAG) and CD33, which are present in macrophage subsets, B lymphocytes, oligodendrocytes/Schwann cells and myeloid cells, respectively [10]. Therefore, it is possible that coupling of sialic acid enables its conjugates to bind to a variety of cell types and prolong its serum level as well.

Interleukin 1 (IL-1) is a cytokine involved in immune and inflammatory responses, hematopoiesis, and homeostatic reactions [11]. 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 leading to hypotension, fever, anorexia and diarrhea. Human IL-1 is nonglycosylated. To develop neoIL-1 with less deleterious effects but preserving beneficial effects, we have chemi-

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cally introduced carbohydrate moieties to recombinant human (rh) IL-1α [12–17]. D-Manα(1-6)Man[Man_2α(1-6)] conjugated rh IL-1α exhibited a decrease in biological activities *in vitro* and receptor binding affinity compared to IL-1α [12]. However, Man_2α(1-6)-IL-1α exhibited selective activities *in vivo* [13]. Furthermore, the tissue distribution of Man_2α(1-6)-IL-1α differed from that of IL-1α [14]. D-Gal conjugated IL-1α manifested a similar decrease in biological activities *in vitro* and receptor binding affinity [15,16], however, the magnitude of its decrease in activities *in vivo* was less than that *in vitro* [17]. To study the effect of sialic acid modification on IL-1α activity, we coupled *N*-acetylneuraminic acid (NeuAc), a major member of sialic acid, to rhIL-1α [18]. NeuAc-IL-1α exhibited a reduction in biological activities to about 1/10 of IL-1α in all the activities assayed *in vitro* and IL-1 receptor binding affinity [19]. In this study, we sought to examine the *in vivo* biological activities and tissue distribution of NeuAc-IL-1α.

Materials and Methods

Animals

ICR female mice (6 weeks old) were purchased from Charles River (Yokohama, Japan), housed in temperature- and light-controlled (12 h/day) rooms, and fed *ad lib*. Mice were used in experiments after one week of acclimation.

Reagents

RPMI 1640 was purchased from Sigma Chemical Co. (St. Louis, MO) and fetal bovine serum (FBS) from JRH Biosciences (Lenexa, KS). PBS was purchased from Nissui Seiyaku Co. (Tokyo, Japan). rhIL-1α (2×10^7 U/mg) was provided by Dr. Yamada (Dainippon Pharmaceutical Co., Osaka, Japan). Human recombinant IL-6 was provided by Dr. Y. Akiyama (Ajinomoto Co., Yokohama, Japan).

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 IL-1α

Acyl azide derivative of NeuAc with C9 spacer was synthesized and introduced to rhIL-1α as described previously [18]. The NeuAc-IL-1α was purified by anion-exchange chromatography, and the NeuAc-coupling was confirmed by oxidation with $NaIO_4$, the increase of its molecular weight on SDS-PAGE and time of flight mass spectroscopy (TOF-MS) analysis. Average number of carbohydrate molecules introduced was 2.9 molars per molecule of IL-1α. The levels of endotoxin in NeuAc-IL-1α and rhIL-1α were undetectable by the *Limulus amoebocyte* assay (sensitivity limit, 0.1 ng/ml).

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

NeuAc-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.

IL-6 activity in 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 serum was determined using a glucose B-test kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

The concentration of serum amyloid A (SAA) was measured by ELISA as described previously [21].

NOx levels in serum were measured according to the method described by Misko *et al.* [22]. 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 OD595 nm was measured using an ELISA autoreader (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 hemocytometer.

Radiolabeling of IL-1

rhIL-1α and NeuAc-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 NeuAc-IL-1α were 19.7×10^6 cpm/μg and 0.81×10^6 cpm/μg, respectively. The labeled IL-1αs were diluted with unlabeled IL-1α or NeuAc-IL-1α to achieve a radioactive concentration of 2×10^5 cpm/μg before intraperitoneally administration (4×10^5 cpm/2 μg/mouse) to mice to investigate their tissue distributions.

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

Ability of IL-1 to induce serum IL-6 in mice

Mice were injected intraperitoneally with IL-1α or NeuAc-IL-1α and serum IL-6 level was determined. Mice injected with IL-1α exhibited a sharp increase in IL-6 levels with a maximum level 2 h after IL-1 treatment (Figure 1A). The elevation of serum IL-6 at 2 h was observed as low as injection of 0.1 μg IL-1α/mouse (Figure 1B). A dose-response experiment at 2 h indicated that NeuAc-IL-1α exhibited remarkable decrease in its activity, less than 1/20 of IL-1α.

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 NeuAc-IL-1α, and then fasted. In control mice the serum glucose level decreased with duration of fasting up to 12 h, and then the level was sustained (Figure 2A). IL-1α caused a statistically significant reduction at 4 h after treatment and the decrease

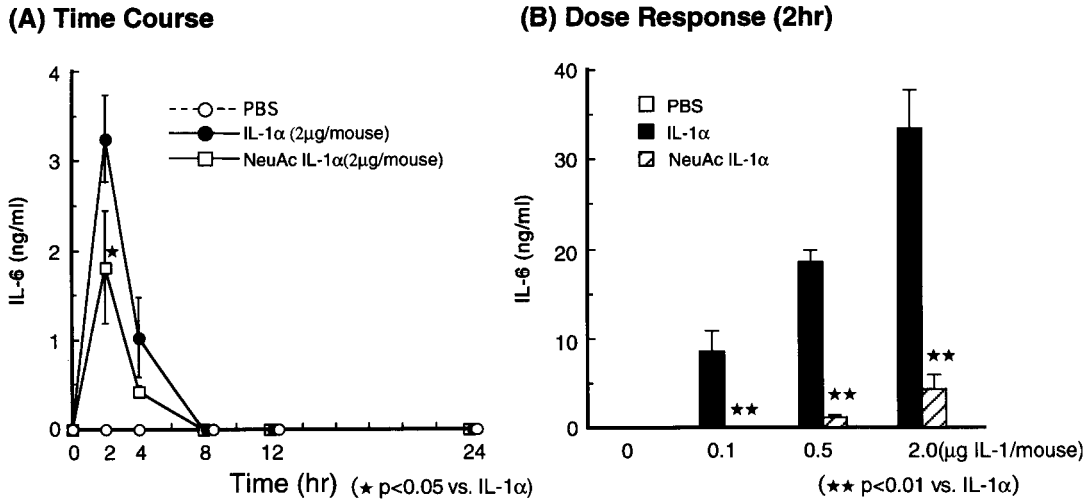

Figure 1. Effect of IL-1 on serum IL-6 level in mice. NeuAcIL-1α, IL-1α or PBS (containing polymyxin B) was administered intraperitoneally and mice were bled at the time indicated (A) or 2 h later (B). Serum IL-6 level was determined by MH60·BSF-2 bioassay. Each point represents the mean ± SD (n = 5).

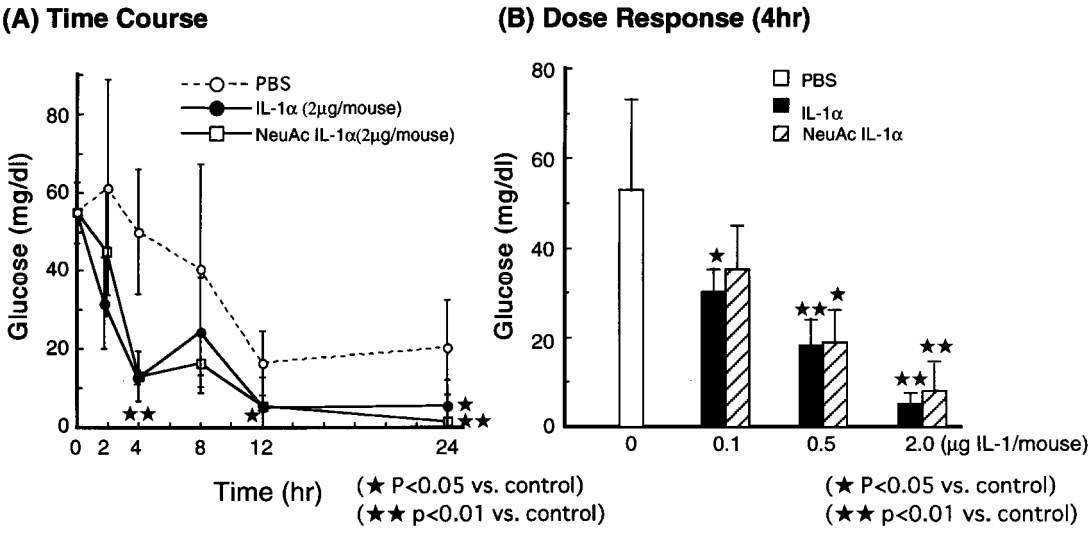

Figure 2. Effect of IL-1 on serum glucose level in mice. NeuAcIL-1α, IL-1α or PBS (containing polymyxin B) was administered intraperitoneally and mice were bled at the time indicated (A) or 4 h later (B). Serum glucose level was determined using the glucose B-test (Wako). Each point represents the mean ± SD of determinations from ten (A) and five (B) mice.

continued to 24 h. NeuAc-IL-1α also caused the reduction for up to 24 h after treatment. A dose-response experiment at 4 h indicated that NeuAc-IL-1α exhibited comparable activity as IL-1α (Figure 2B).

Ability of IL-1 to induce SAA in mice

IL-1α and NeuAc-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, was examined. IL-1α increased SAA level after 4 h treatment, which exhibited a peak at 8 h and tended to decrease at 12 h. After 24 h, SAA was no longer detected. In contrast, NeuAc-IL-1α increased SAA level in a time-dependent manner up to 12 h, and after 24 h it returned to an undetectable level. (Figure 3A). A dose-response experiment at 8 h indicated that NeuAc-IL-1α exhibited about 1/5 activity of IL-1α (Figure 3B).

Ability of IL-1 to induce serum NOx in mice

IL-1 induces NO synthesis in a variety of cell types. Nitric oxide (NOx) reacts with molecular oxygen and water to generate nitrite and nitrate that accumulate in biological fluids [23]. To measure the level of NOx, serum nitrate was converted to nitrite by nitrate reductase, and then the amount of total nitrite was determined. The serum NOx level of IL-1α-injected mice began to increase at 4 h, with a peak level at 24 h and decreased but still remained higher compared to control at 24 h (Figure 4A). NeuAc-IL-1α induced the similar time-dependent increase of serum NOx level. At this dosage (2 μg/mouse) there was no significant difference between IL-1α and NeuAc-IL-1α. However, a dose-response experiment at 8 h showed that NeuAc-IL-1α exhibited about 1/5 activity of IL-1α (Figure 4B).

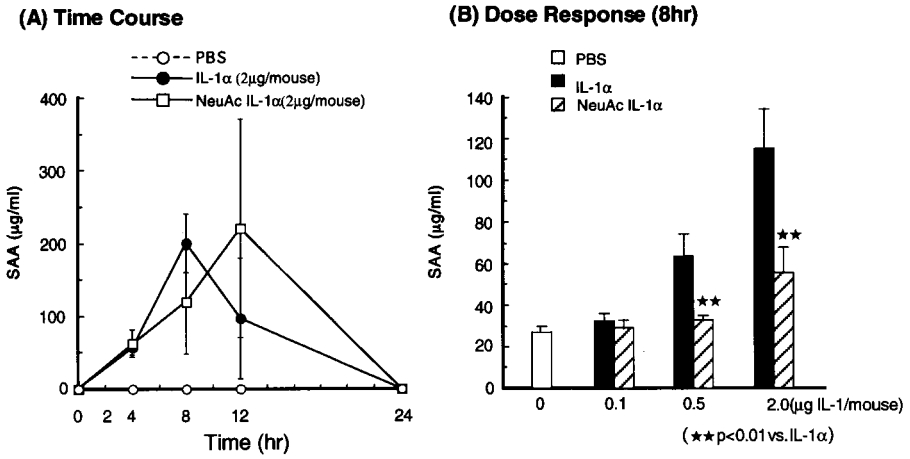

Figure 3. Effect of IL-1 on SAA level in mice. NeuAcIL-1α, IL-1α or PBS (containing polymyxin B) was administered intraperitoneally and mice were bled at the time indicated (A) or 8 h later (B). SAA level was determined by ELISA. Each point represents the mean ± SD (n = 5).

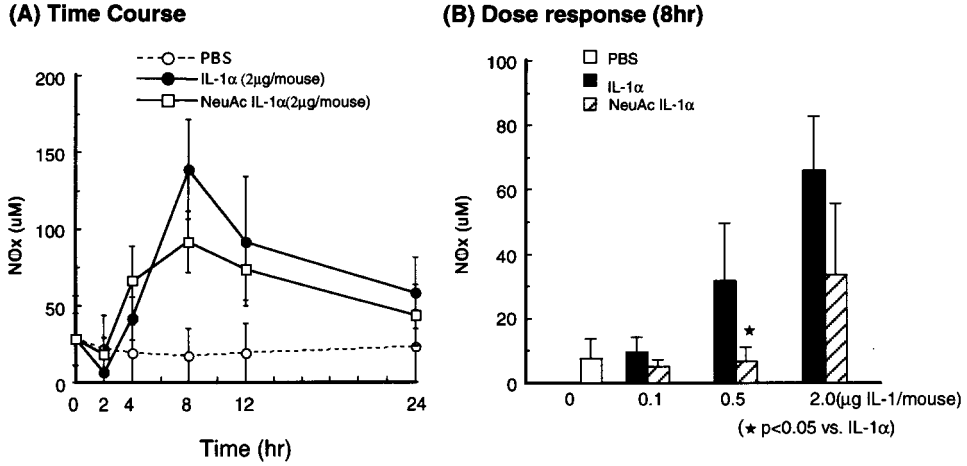

Figure 4. Effect of IL-1 on serum NOx level in mice. NeuAcIL-1α, IL-1α or PBS (containing polymyxin B) was administered intraperitoneally and mice were bled at the time indicated (A) or 8 h later (B). Serum NOx level was determined as described in materials and methods. Each point represents the mean ± SD (n = 5).

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-1αs 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. The number of WBC in control mice on day 0, 4 and 13 were $4.39 \pm 0.88 \times 10^3$ cells/mm^3, $4.94 \pm 0.88 \times 10^3$ cells/mm^3 and $3.99 \pm 0.55 \times 10^3$ cells/mm^3, respectively. Treatment with 5-FU resulted in a decrease in WBC in peripheral blood on day 2 with the average being $(1.66 \pm 0.48) \times 10^3$ cells/mm^3 (Figure 5). On day 4 the effect of IL-1, either IL-1α or NeuAc-IL-1α, was not apparent (data not shown). On day 13 the level of WBC in 5-FU treated mice remained low, $1.74 \pm 0.25 \times 10^3$ cells/mm^3. However, on day 13 both IL-1α and NeuAc-IL-1α treated groups increased the level of WBC higher than control when they were injected into control mice. Both IL-1α and NeuAc-IL-1α treatment caused the recovery in the level of WBC in 5-FU treated mice, and there was no difference in the potency between IL-1α and NeuAc-IL-1α in this regard.

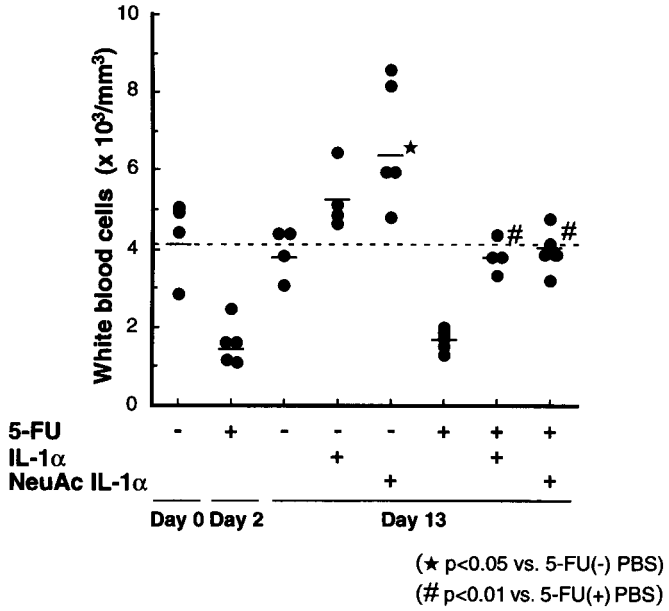

Figure 5. Recovery of myelosuppression as a result of IL-1α treatment in 5-FU-treated mice. Mice were administered 5-FU (12 mg/mouse) as a single dose intravenously on day 0. NeuAcIL-1α, IL-1α or PBS was administered intraperitoneally twice (200 ng each) a daily from day 1 to day 12. Each group consisted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

Tissue distribution of IL-1α

In order to determine whether glycosylation affects the tissue distribution of IL-1, ^{125}I labeled IL-1α and NeuAc-IL-1α were administered intraperitoneally into mice, their contents in various tissues over time was determined and compared. At 2 h a relative high amount of ^{125}I-IL-1α was found in the kidney, spleen and lung compared to the liver. The amount of IL-1α in the kidney, spleen, lung and blood slightly decreased at 4 h, and further decreased to quite low level at 20 h. In the liver, the amount of IL-1α was higher at 4 h than that at 2 h, and it also decreased to quite low level at 20 h. On the other hand, NeuAc-IL-1α distributed at higher levels than IL-1α in all the tissues except for the liver at 2 h. In particular, very high amount of NeuAc-IL-1α (approximately fivefold that of IL-1α) was found in the kidney. In other tissues approximately twofold NeuAc-IL-1α was found. Similar to IL-1α, the content of NeuAc-IL-1α in the liver was lowest among the tissues. The

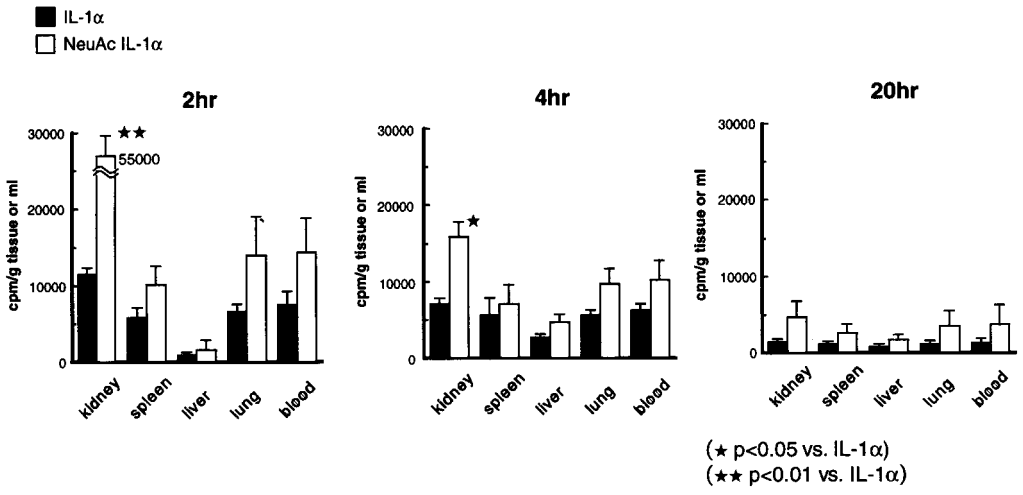

Figure 6. Tissue distribution of ^{125}I-IL-1α and ^{125}I-NeuAc-IL-1α. Radio-labeled NeuAc-IL-1α or IL-1α was intraperitoneally administered into mice. After taking blood through the inferior vena, tissues were removed immediately at the time indicated. After tissue wet weights were measured, radioactivity was determined using a γ-counter. Data were expressed as cpm per gram tissue or per ml blood (n = 5).

level of NeuAc-IL-1α remained higher than IL-1α in all the tissues (including the liver) at 4 h and 20 h after administration.

Discussion

In the present study, we demonstrated that NeuAc-IL-1α exhibits selective activities *in vivo*. As previously reported, in all the assays performed *in vitro*, including the proliferative effect on T cells, the antiproliferative effect on human melanoma cells and mouse myeloid leukemic cells, the stimulatory effect on IL-6 synthesis by melanoma cells, and the stimulatory effect on prostaglandin E_2 production by fibroblast cells, NeuAc-IL-1α exhibited reduced activities by about tenfold compared to IL-1α [19]. There are two types of IL-1R. Type I receptor (IL-1RI) transduces IL-1 signal, whereas type II receptor (IL-1RII) regulates IL-1 binding to IL-1RI as a decoy receptor. Receptor binding affinity of NeuAc-IL-1α to Type I and Type II IL-1R is also decreased by about tenfold [19], showing that the reduction in activities of NeuAc-IL-1 *in vitro* results from the decrease in receptor binding affinity. However, the decrease in receptor binding affinity can not explain the differential reduction of *in vivo* activities of NeuAc-IL-1α. Although NeuAc-IL-1α exhibited a marked reduction in activities to up-regulate serum level of IL-6, SAA, and NOx, it exhibited comparable activities as IL-1α to down-regulate serum glucose level and to improve the recovery of peripheral WBC from myelosuppression in 5-fluorouracil-treated mice. It is not known why the activities of NeuAc-IL-1α differ between *in vitro* and *in vivo* situations. *In vivo* system is more complex than that *in vitro* and the potency of IL-1 activity may not be determined solely by its affinity to IL-1RI. In accordance with our results in the present study, Man_2α(1,6)-IL-1α also exhibits comparable activities as IL-1α to decrease serum glucose level and to improve the recovery of WBC in 5-FU treated mice, although its binding affinity to IL-1RI was markedly decreased [12,13]. There are many factors affecting IL-1 binding to IL-1RI and the process for exerting its function. It is reported that urinary glycoprotein, uromodulin, binds IL-1 through its oligosaccharide, subsequently inhibits IL-1 function [23,24]. The ratio of IL-1RI/IL-1RII also influences the IL-1 binding to IL-1RI [25]. In the case of NeuAc-IL-1α, the negative charge of NeuAc may potentially influence its access to IL-1RI because cell surfaces are negatively charged with NeuAc-conjugated glycolipids or glycoproteins. Furthermore, lectins specific to NeuAc on cell surfaces and carbohydrate binding proteins in serum may also influence the IL-1 binding to IL-1RI as well as the post receptor signaling processes. Once IL-1 binds to IL-1RI, secondary subunit of IL-1RI, IL-1R accessory protein (IL-1RAcP), is recruited to the complex. Subsequently, IL-1R associated kinase is activated and transduces IL-1 signal [26]. Therefore, glycosylation of IL-1 may also influence the recruitment of IL-RAcP. It is also important whether IL-1 can access to target tissue cells, which is also likely to be influenced by glycosylation. Collectively, it is possible that these factors differently modulate IL-1 binding to IL-1RI, its distribution, retention, and the subsequent signaling process depending on cell types. IL-6 is produced by many cell types and is induced by IL-1 alone and in synergy with TNF. Among the activities studied *in vivo*, reduction in IL-6 inducing activity was the most remarkable. Although IL-6 is induced by many cell types *in vitro*, we do not know which cell types are the major IL-6 producer *in vivo*. May be these cells are relatively refractory to NeuAc-modified IL-1.

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 [27]. Although it was not significant, the peak of serum level of SAA tended to be delayed in mice administered with NeuAc-IL-1α. This was probably due to the prolonged serum level of NeuAc-IL-1α.

NOx is an important effector molecule in neurotransmission, vasodilatation and host defense against microorganism and tumor cells. IL-1α alone or in synergy with TNF and interferon augments the production of NOx from many cell types, including macrophages, hepatocytes, vascular endothelial cells and smooth muscle cells [28]. NOx produced by smooth muscle cells, in conjuction 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 [29]. NeuAc-IL-1α appeared to be weak in induction of serum NOx level, thus indicating an advantageous aspect for therapeutic use of NeuAc-IL-1α. A disadvantage aspect is that NeuAc-IL-1α may be weak in host defense against infection of microorganisms and tumors.

In response to IL-1, serum level of glucose decreases. Whether insulin is involved in the IL-1-dependent serum glucose reduction is controversial [30]. Several hypotheses are postulated. (I) IL-1 induces an accumulation of platelets in the liver, which in turn release serotonin, causing a decrease of serum glucose level [31]. (II) IL-1 decreases intracellular steroid receptors in hepatocytes, resulting in decreased induction of phosphoenolpyruvate carboxykinase and a subsequent reduction of glucogenesis and plasma glucose [32]. NeuAc-IL-1α exhibited a comparable activity as IL-1α. Similar to NeuAc-IL-1α, Man_2α(1-6)-IL-1α possessed the same potency as untreated IL-1α [14]. In contrast, the activity was decreased in GaI-IL-1α [17]. Although the reason is not known, it may be due to the different distribution of these neoIL-1s in the liver. Modification with galactose will enhance the uptake of GaI-IL-1α by hepatocytes, thus its degradation may be enhanced, whereas NeuAc-IL-1α is resistant to the uptake through GaI-specific lectin by hepatocytes [33]. Man_2α(1-6)-IL-1α is also resistant to the uptake through interaction with Man-specific lectin present in hepatocytes and macrophages [34].

One of the beneficial effects of IL-1 is the enhancement of recovery of peripheral WBC in chemotherapeutic drug-treated animals [35]. 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, such as granulocyte- and granulocyte-macrophage colony stimulating factors, IL-3, and IL-6 [11,36]. IL-1 also enhances the sensitivity of progenitor cells to the growth factors through up-regulation of their receptors [37]. NeuAc-IL-1α exhibited a comparable WBC recovering effect as IL-1α. At the dosage we used NeuAc-IL-1α exhibited no severe acute reactions, including down-regulation of serum level of glucose, up-regulation of serum level of IL-6, SAA and NOx. In addition, there were no toxic effects, such as anorexia, diarrhea, somnolence and body weight loss. This is important for therapeutic application of IL-1. It is of note that $Man_2\alpha$-(1-6)-IL-1α, but not GaI-IL-1α, also exhibited a comparable WBC recovering effects as IL-1α [14,17]. Probably GaI-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 NeuAc-IL-1α is its prolonged existence in serum and sustained *in vivo* effect. The level of radiolabeled NeuAc-IL-1α remained high compared to IL-1α in most of the tissues at either 2, 4 or 24 h. Especially at 2 h the level of NeuAc-IL-1α in kidney was extremely 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 [38]. The major route of clearance is the kidney, which is in accordance with our results. In contrast to NeuAc-IL-1α, $Man_2\alpha$(1-6)-IL-1α is distributed predominantly in the liver [15]. Therefore, the tissue distribution of IL-1 can be manipulated by changing carbohydrates introduced. We also observed the delayed peak of serum SAA in NeuAc-IL-1α injected mice, however, we did not observe the prolongation of other biological activities. The host may become refractory to successive stimuli of IL-1 through feedback mechanisms, such as down-regulation of IL-1RI expression, release of IL-1RII, induction of suppressive factors; glucocorticoid, IL-1 receptor antagonist and α-melanocyte stimulating hormone [11].

In conclusion, we have established a method to synthesize NeuAcIL-1α that exhibits reduced activities to increase serum IL-6, SAA, and NOx while maintaining activities to decrease serum glucose and to improve WBC recovery after myelosuppression *in vivo* in comparison to IL-1α. NeuAcIL-1α also has a prolonged half-life *in vivo*. NeuAcIL-1α, thus, may be useful for therapeutic application.

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