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In vitro biological activities of glycosylated human interleukin-1α, neoglyco IL-1α, coupled with *N*-acetylneuraminic acid

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In the previous study, *N*-acetylneuraminic acid (NANA) with C9 spacer was chemically coupled to human recombinant (rh) IL-1α in order to study the effect of glycosylation on its biological activities, and to develop IL-1 with less deleterious effects. In this study we examined a variety of IL-1 activities *in vitro,* including proliferative effect on T cells, antiproliferative effect on myeloid leukemic cells and melanoma cells, stimulatory effects on IL-6 synthesis by melanoma cells and PGE_2 synthesis by fibroblast cells. NANA-introduced IL-1α (NANA-IL-1α) exhibited reduced activities about ten times compared with original IL-1α in all the activities performed *in vitro.* The competitive binding of ^{125}I-IL-1α to mouse T cells and pre-B cells with unlabeled IL-1αs suggests the decrease in binding affinities of NANA-IL-1α to both type I and type II IL-1 receptors. Therefore, reduced activities of NANA-IL-1α well correlated with the decrease in its receptor binding affinities.

Keywords: neoglycoprotein, interleukin 1, cytokine, sialic acid

Introduction

Most of the discovered natural cytokines are glycosylated. Although the recombinant cytokines generated by *E. coli* are devoid of carbohydrates, most of them exhibit the same biological activities *in vitro* as natural glycosylated counterparts. However, in several cytokines carbohydrates play an important role in its stability and biological activity. Recombinant interferons (IFN) generated by *E. coli* are unstable [1] and removal of carbohydrates from erythropoietin results in inactivation [2]. Carbohydrates in cytokine receptors are also important. In IFN and IL-1, carbohydrates in receptor molecule contribute to its ligand binding affinity [3,4]. It is also known that on the cell surface there are many kinds of lectins, through which cell to cell or cell to glycoporteins interactions and recognitions are initiated [5]. Therefore, it is possible that the mode of actions of recombinant cytokines *in vivo* are different from those of glycosylated natural counterparts. Also it is possible to manipulate the biological activity, tissue distribution and stability of cytokines by coupling of carbohydrates into recombinant cytokines.

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Human IL-1 is a nonglycosylated cytokine mainly produced by macrophages and monocytes, although murine IL-1 may be glycosylated. There are two types of IL-1, IL-1α and IL-1β and they exhibit almost the same activities *in vitro* and *in vivo* through binding to the same cell surface receptor. IL-1 exhibits pleiotropic effects on various cell types [6]. IL-1 is potentially useful for therapy because of its antitumor effect, protective effect on animals against microorganism infection, radiation, and chemotherapy. IL-1 also may be useful against hyperglycemia. However, because of its proinflammatory activity, IL-1 exhibits serious deleterious effects, including endogenous pyrogen activity, induction of other proinflammatory cytokines, tumor necrosis factor (TNF), IL-6, IL-8 and prostanoids, tissue injury and hypotension.

In the previous study, we chemically synthesized D-Manα(1-6)Man [Man_2α(1-6)] conjugated-human recombinant interleukin-1α (IL-1α), neoglyco IL-1α, to know the effect of glycosylation on its activities and to develop IL-1 with less deleterious effects [7–9]. Man_2α(1-6)-IL-1α exhibited impairment in both biological ativities in all the experiments *in vitro* and receptor-binding capacity compared to intact IL-1α [7]. However, Man_2α(1-6)-IL-1α exhibited selective activities *in vivo* [8]. Furthermore, the tissue distribution of Man_2α(1-6)-IL-1α in mice differed from that of intact IL-1α [9]. We also synthesized D-Gal conjugated

IL-1α [10]. It exhibited the similar decrease in its biological activities *in vitro* and receptor-binding capacity [11]. However, *in vivo* the magnitude of its decrease was less than that *in vitro* [12].

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 [13]. 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 [14]. Indeed, sialic acid is present in most of the serum glycoproteins and its content amounts to about 20% in erythropoietin and α1-acid glycoprotein [15]. Furthermore, recent studies revealed that sialic acid is also important as a ligand for selectins [16] and sialoadhesion family of cell surface lectins. Sialoadhesion family are consist of Sialoadhesion, CD22, myelin associated glycoprotein (MAG) and CD33, present in macrophage subsets, B lymphocytes, oligodendrocytes/Schwann cells and myeloid cells, respectively [17]. Therefore, it is possible that coupling of sialic acid enables its conjugates to bind to a variety of cell types. As well, sialic acid on the cell surface of tumor cells is implicated in tumor invasion and metastatis through interaction with E-selectin in endothelial cells [18].

In the previous study, we coupled *N*-acetylneuraminic acid (NANA), a major constituent of sialic acid, to rhIL-1α. In this report we studied the *in vitro* biological activities of NANA-introduced IL-1α and its receptor binding ability.

Materials and methods

Reagents

RPMI 1640, bovine serum albumin (BSA), and polymixin B were 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)(2×10^7U/ml) was provided by Dr. M. Yamada of Dainippon Pharmaceutical Co. (Osaka, Japan). Human recombinant IL-6 (rhIL-6) was provided by Dr. Y. Akiyama of Ajinomoto Co. (Osaka, Japan).

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) [19] 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 MH60.BSF2 cells provided by Dr. T. Hirano (University of Osaka) were maintained in culture medium supplemented with 10% FBS and 1 U/ml rhIL-6 [20]. 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, A375 which originally given by Dr. R. Ruddon (NCI, Bethesda, MD). 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 were 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 microliters of cell suspension (2×10^5 cells/ml) was added to each flat-bottomed well of 96 well microtiter plate (Falcon, Lincoln, NJ). Fifty microliters 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-diphenyl tetrazolium bromide (MTT) assay [21].

Assay for M1-3b growth inhibition

Cultured M1-3b cells washed three times with culture medium. Fifty microliters of cell suspension (2×10^5 cells/ml) with 5μg/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 microliters 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 [21]. 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 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 microtiter plate. After 24h

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 [22]. After solubilization of the dye-staining the absorbance at 595 nm was determined using an ELISA autoreader (Bio-Rad Laboratories, Richmond, CA). The percentage of cell growth was calculated as the same method as M1-3b.

Assay for IL-6 activity produced by A375-6 cells

A375-6 cells were cultured at 1×10^5 cells/ml for 24 h with varying concentrations of IL-1s. IL-6 activity in the supernatant was determined using the proliferation of IL-6-dependent MH60.BSF2 cells as described [20]. IL-6 activity was expressed as the equivalent amount of rhIL-6.

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 (81.01 TBq/mmol) was purchased from Amersham Pharmacia biotech (Aylesbury, UK). EL-4 6.1 C10 cells (1×10^6) or 70 Z/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 1mg/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 was separated by the binding oil column method [23] and measured with a γ-counter (Aloka, Tokyo, Japan).

Results

IL-1 proliferative effect on T cells

Biological activities of NANA-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 [19]. The cells were cultured for 4 days with or without IL-1s, and then the cell proliferation was determined. As shown in Fig. 1, control IL-1 exhibited the same or more strength of activity than untreated IL-1. In contrast, NANA-IL-1 exhibited about 1/30 activity of untreated IL-1.

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

IL-1 inhibits the growth of mouse myeloid leukemic cells M1 and human melanoma cells A375-6 [24,25]. To determine the IL-1 antiproliferative activity, M1 cells and A375-6 cells were treated with or without IL-1s for 4 and 3 days, respectively, and then cell proliferation was determined. As shown in Fig. 2A, control IL-1 exhibited the same strength of activity as untreated IL-1. NANA-IL-1 exhibited the activity about 1/10 of untreated IL-1. Control IL-1 also exhibited the same strength of activity as untreated IL-1 in inhibition of A375-6 cell proliferation (Fig. 2B). The activity of Gal-IL-1 decreased to about 1/10 of untreated IL-1.

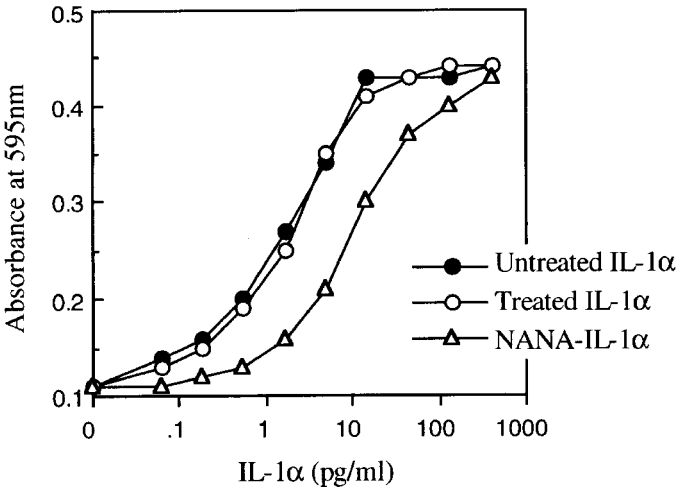

Figure 1. Effects of IL-1s on proliferation of D10H.2 cells. Mouse T cell line D10H.2 cells were cultured at 37°C for 4 days with or without varying doses of IL-1αs. After culture, cell proliferation was determined by MTT method.

IL-1 induction of IL-6 by melanoma cells

To evaluate the IL-6 inducing activity of IL-1 [26], A375-6 cells were cultured with or without IL-1s for 24h, and then the amount of IL-6 in the culture supernatants was determined. As shown in Fig. 3, control IL-1 exhibited the same potency as untreated IL-1. NANA-IL-1 exhibited the activity about 1/10 of untreated IL-1.

IL-1 stimulation of PGE_2 production by fibrolast cells

To evaluate the stimulating activity of PGE_2 production by fibroblast cells [27], human fibroblast cell line TIG-1 cells were cultured with or without IL-1s for 24 hr, and then the amount of PGE_2 in the culture supernatants was determined (Fig. 4). Control IL-1 activity was the same as that of untreated IL-1. NANA-IL-1 exhibited the activity about 1/10 of untreated IL-1.

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 [28,29]. To determine the ability of IL-1s to bind type I IL-1R the competitive

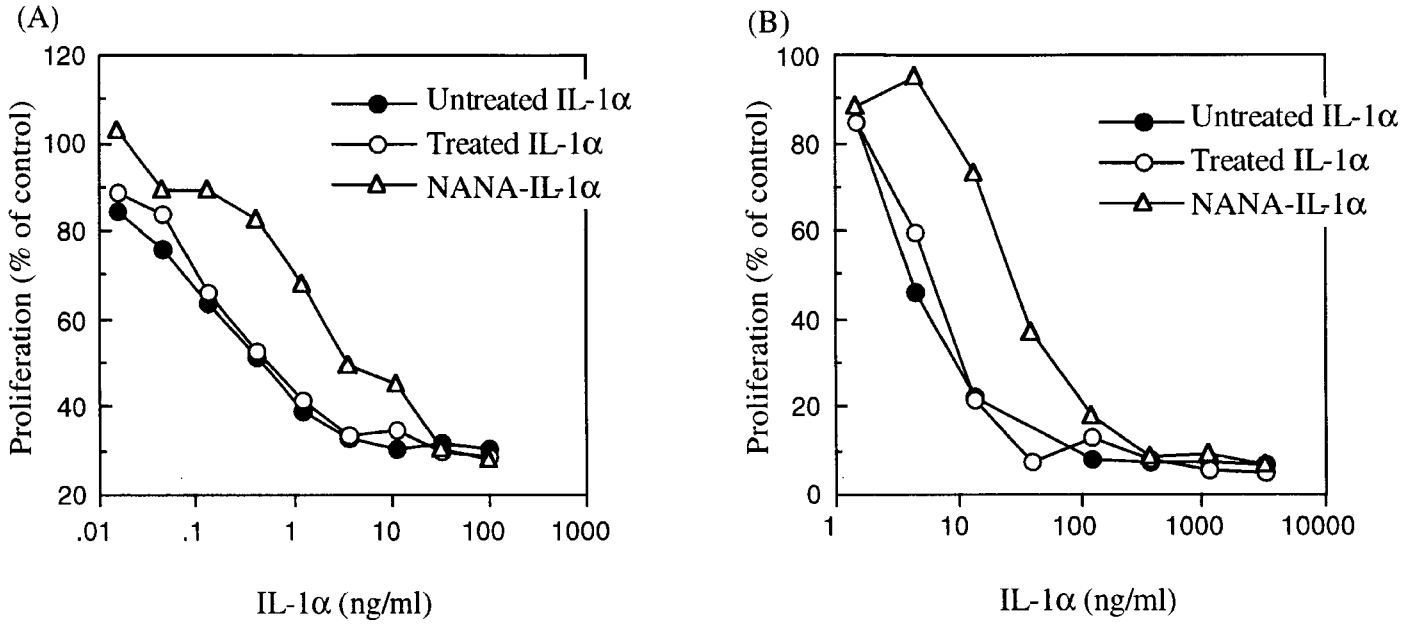

Figure 2. Antiproliferative effect of IL-1 on M1 cells(A), and A375-6 cells(B). (A) Mouse myeloid leukemic cell line M1-3b cells were cultured at 37°C for 4 days with or without varying doses of IL-1αs. After culture, cell proliferation was determined by MTT method. (B) Human melanoma cell line A375-6 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.

binding of ^{125}I-IL-1α with unlabeled IL-1s to mouse T cell line cells (EL-4) was examined. As shown in Fig. 5A, control IL-1 exhibited binding activity at the same level of untreated IL-1. The binding activity of NANA-IL-1 was about 1/10. Using mouse preB cell line 70/3.12 cells, the binding activity of IL-1 to Type II IL-1R was examined. As shown in Fig.5B, the binding activity of control IL-1 to Type II IL-1R was again at the same level as untreated IL-1. In contrast, the activity of NANA-IL-1 was about 1/10 of untreated IL-1.

Discussion

In this study, NANA-IL-1 exhibited the reduction in all the biological activities examined *in vitro.* However, as control IL-1 exhibited the same strength of the activities as un-

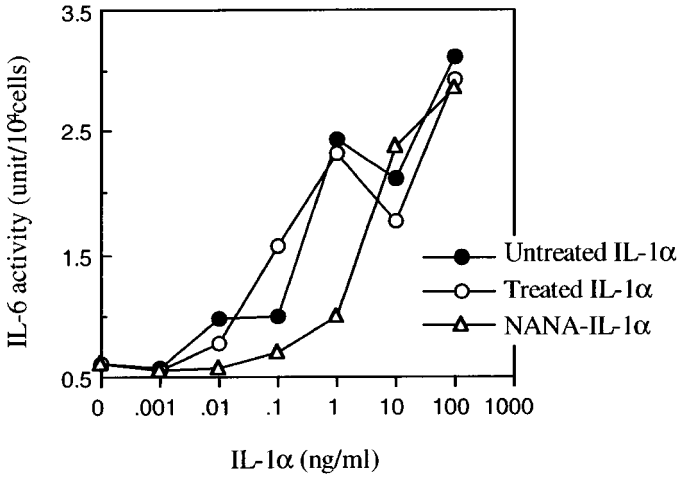

Figure 3. Effect of IL-1 on the IL-6 production by A375-6 cells. A375-6 cells were cultured with or without varying doses of IL-1αs. After 24h culture, IL-6 activity in the supernatants was determined by the proliferation of IL-6-dependent MH60-BSF2 cells.

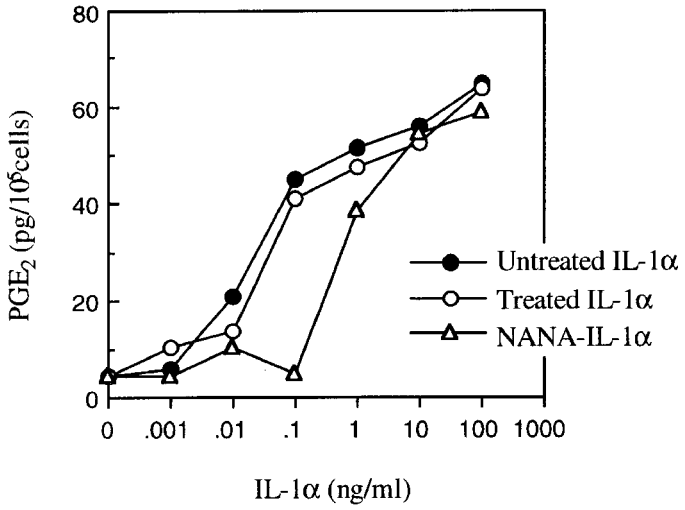

Figure 4. Effect of IL-1 on the PGE_2 production by TIG-1 cells. Human fibroblast cell line TIG-1 cells were cultured with or without varying doses of IL-1αs. After 24h culture, the amount of PGE_2 in the supernatants was determined by ELISA.

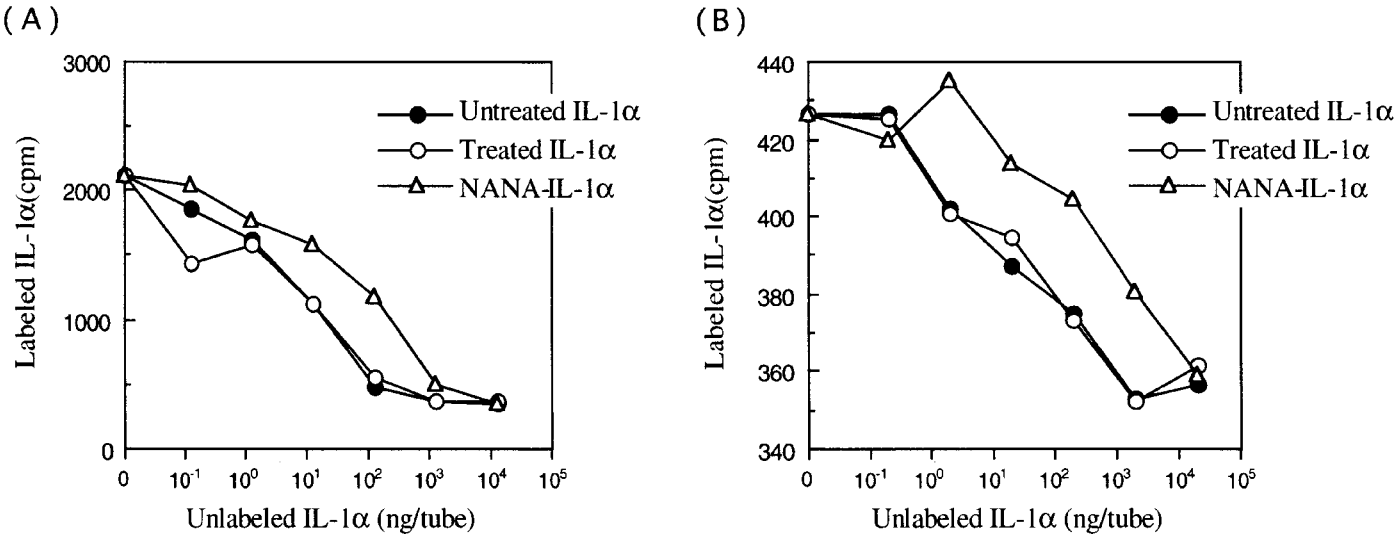

Figure 5. Inhibition of ^{125}I-IL-1α binding to EL-4 6.1 C10 cells (A), and 70Z/3.12 cells (B) by untreated IL-1α. Mouse T cell line EL-4 6.1 C10 cells and mouse pre-B cell line 70Z/3.12 cells were incubated with ^{125}I-IL-1α in the presence of varying doses of unlabeled IL-1αs for 1 h at 4°C. The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

treated IL-1, the coupling condition did not affect the IL-1 activity. This was the same in the cases of Man dimer (Man_2) or Gal monomer introduction into rhIL-1α [7,11]. It is of note, however, that the magnitude of the reduction of biological activities of NANA-IL-1 was the same in all the assays performed, which was in contrast to our earlier studies of Man_2-IL-1 or Gal-IL-1 [7,11]. In those IL-1s, the magnitude of the reduction varied depending on assays. In Man_2-IL-1, the least reduction was 1/10 to 1/30 in T cell proliferation activity, and most marked reduction was IL-6 induction by melanoma cells (Table 1). In case of Gal-IL-1, least reduction was 1/10 in IL-6 induction by melanoma cells, and most pronounced reduction was < 1/10000 in PGE_2 induction by fibroblast cells. Of particular interest is the stimulation of IL-6 production by melanoma cells. In the case of Man_2-IL-1, the reduction of IL-6 inducing activity was maximal, while it was least in Gal-IL-1α. The reduction rate was not correlated with the number of introduced carbohydrates because Man_2α(1-4), Man_2α(1-6), Gal and NANA coupled to per mole of IL-1 were averagely 4.7, 5.2, 9.1 and 2.9 moles, respectively. X-ray crystallographic 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 [30]. Therefore, Gal monomer was estimated to have reacted to about 61% of the potential reactive amino residues. Probably the combination of the region, species and the size of the introduced carbohydrates will be important. Regarding to the number of carbohydrates introduced, Gal monomer was mostly incorporated, presumably because the size of the molecule was smaller than those of Man_2 and NANA. In case of NANA, its negative charge may also contribute to the relative small number of coupling.

IL-1 action is mediated through its specific receptor on

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

Coupled carbohydrate	*Man_2α(1,4)*	*Man_2α(1,6)*	*Gal*	*NANA*
Molecules of carbohydrates introduced into molar of IL-1α	4.7	5.2	9.1	2.9
T cell proliferative activity	1/10	1/30	1/30	1/10
Antiproliferative effect on M1	1/20	1/100	1/100	1/10
on A375	1/150	1/300	1/100	1/10
IL-6 induction	<1/1000	<1/1000	1/10	1/10
PGE_2 induction	1/100	1/700	<1/10000	1/10
Affinity to type I IL-1R	1/400	1/700	1/500	1/10
type II IL-1R	ND	1/600	1/100	1/10

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

cell surface. There are two types of IL-1 receptor (IL-1R) cloned from many cell types. Type 1 IL-1R with molecular weight (MW) of 80 kDa is mainly expressed on T cells and fiblobrast cells [28], and type II IL-1R with MW of 60 kDa is mainly expressed on macrophages, bone marrow cells, and B cells [29]. Only type I IL-1R can deliver IL-1 signals into cells. Type II IL-1R is unable to transduce IL-1 signal, but works as a decoy receptor [31]. It is reported that even in macrophages, bone marrow cells and B cells small number of type I IL-1R is expressed that transduces IL-1 signals [32].

The most likely explanation of the reduced activity is due to the reduction in binding affinity to IL-1R. NANA-IL-1 exhibited the reduced binding affinity to both type I and type II IL-1Rs at the comparable level, which was also different from Gal-IL-1. It is possible that carbohydrate introduction causes conformational change that leads to the decrease in binding affinity, or carbohydrate interferes IL-1 binding to IL-1R. In case of NANA-IL-1, additionally negative charge will contribute to the altered binding affinity. Recent studies revealed that IL-1 binds to IL-1RI, then secondary chain of IL-IR, IL-IR associated protein (IL-1RAcP), is recruited to the IL-1/IL-1RI complex [33]. It is reported that binding affinity of IL-1 to IL-1RI is affected by both IL-1RAcP and carbohydrate moiety of IL-1RI molecule [4,34]. In cases of Man_2-IL-1 and Gal-IL-1, however, the reduction in binding affinity alone can not explain the variety of decreased biological activities depending on cell types. Probably other factors contribute to the varieties, such as multiple pathways of IL-1 signaling. In contrast, in case of NANA-IL-1, the magnitude of the IL-1RI binding affinity well correlated with those of its biological activities. Therefore, the decreased biological activities of NANA-IL-1 can be attributed to its decreased binding affinity to IL-1RI.

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