Glycoconjugate Journal 15, 1079–1083 (1998)

Panosialins, inhibitors of an α1,3-fucosyltransferase Fuc-TVII, suppress the expression of selectin ligands on U937 cells

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Panosialins A and B were isolated as inhibitors of an α1,3-fucosyltransferase, Fuc-TVII, which is a key enzyme in the biosynthesis of selectin ligands, from culture broth of *Streptomyces* sp. Panosialins A and B inhibited the Fuc-TVII activity with IC_{50} values of 4.8 and 5.3 μg/ml, respectively. Panosialin A suppressed expression of selectin ligands on U937 cells, and inhibited the cell adhesion to immobilized E-selectin-immunoglobulin. Panosialins are the first reported Fuc-TVII inhibitors which can suppress the biosynthesis of selectin ligands and then inhibit selectin-mediated cell adhesion.

Keywords: **cell adhesion, Fuc-TVII, inhibitor, panosialin, selectin, sialyl Lewis x**

Abbreviations: **sLe^x, sialyl Lewis x (NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc); GDP-fucose, guanosine 5′-diphosphate-β-L-fucose; mAb, monoclonal antibody; FBS, fetal bovine serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Ig, immunoglobulin; HM-VEC, human microvessel endothelial cells; TNF-α, tumor necrosis factor alpha.**

Introduction

Selectin-mediated cell adhesion is a crucial step in the initial events involved in the recruitment of leukocytes to lymphoid tissues and the sites of inflammation [1–3]. The three known selectins (E-, P-, and L-selectin) recognize the sialyl Lewis x (sLe^x; NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc) and related oligosaccharides [4]. The biosynthesis of these oligosaccharides requires several steps of glycosylation catalyzed by glycosyltransferases. Among these glycosyltransferases, an α1,3-fucosyltransferase, which catalyzes the transfer of fucose from GDP-fucose to *N*-acetylglucosamine via an α1,3-linkage, plays important roles in the biosynthesis of these oligosaccharides.

Among five cloned α1,3-fucosyltransferases (Fuc-TIII, Fuc-TIV, Fuc-TV, Fuc-TVI, and Fuc-TVII), Fuc-TVII is a key enzyme in the biosynthesis of selectin ligands [5–10]. Fuc-TVII shows activity only toward α2,3-sialylated type 2 oligosaccharides *in vitro,* and generates the sLe^x epitopes that bind to E- and P-selectin *in vivo* [6,7]. Recent studies have also demonstrated its involvement in the biosynthesis of L-selectin ligands [8]. Furthermore, Fuc-TVII-deficient mice were shown to exhibit a leukocyte adhesion deficiency that is characterized by both the absence of leukocyte E- and P-selectin ligand activity, and a deficiency of L-selectin ligand activity in high endothelial venules [9]. Therefore, the inhibition of Fuc-TVII activity should suppress the expression of selectin ligands, and selective inhibitors of Fuc-TVII are expected to be potential therapeutics for the treatment of inflammatory diseases.

To date, only limited inhibitors of α1,3-fucosyltransferases, most of which are unreactive mimetics of the donor substrates, have been reported, but their effects on the expression of cell surface selectin ligands were not examined [11–14]. In the course of our screening program to discover selective inhibitors of Fuc-TVII, we found that *Streptomyces* sp. KY11789 produces two known compounds, panosialins A and B, with inhibitory activity toward Fuc-TVII. In this study, we describe the inhibitory effects of panosialins A and B on Fuc-TVII. Furthermore, we also show that panosialin A inhibits the expression of selectin ligands leading to a reduction in the selectin-dependent adhesion of U937 cells to immobilized E-selectin or cytokine-activated endothelial cells.

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Materials and methods

α1,3-Fucosyltransferase assay

Soluble recombinant Fuc-TVII and Fuc-TVI were prepared as described previously [10]. The standard α1,3-fucosyltransferase assay was performed in a total volume of 30 μl of 100 mM cacodylate buffer (pH 7.5), 25 mM $MnCl_2$, 0.05 mM GDP-fucose, 0.025 mM pyridylaminated α2,3-sialyl lacto-*N*-neotetraose (NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc), and one of the recombinant enzymes. To examine the effect of panosialins on the enzyme activity, they were added to the mixture. After incubation at 37 °C for 2 h, the reaction was stopped by boiling for 5 min. After centrifugation, the reaction mixture was subjected to HPLC analysis on an ODS column (YMC-pack ODS-AQ, 6 i.d. × 150 mm; YMC, Japan). The reaction product was eluted with 20 mM NH_4OAc (pH 4.0) at the flow rate of 1.0 ml/min and monitored with a fluorescence spectrometer.

Panosialins A and B

Panosialins A and B were isolated from the fermentation broth of Streptomyces sp. KY11789 by the following procedure. A methanolic extract of a mycelial cake was subjected to HP-20 (Mitsubishi Chemical, Japan) column chromatography. The eluent with methanol was fractionated by ODS flash chromatography with increasing amounts of methanol in water. The active fraction eluted with 90% methanol was purified by HPLC on an ODS column (YMC-Pack ODS-AQ, 20 i.d. × 250 mm; YMC) with acetonitrile/50 mM phosphate buffer (pH 7.0) (1 : 1).

Cell lines

Human lymphoma line U937 was obtained from the American Type Culture Collection and cultured in RPMI 1640 medium containing 10% FBS. Human microvascular endothelial cells, HM-VEC, were purchased from KURABO (Osaka, Japan) and cultured in E-GM MV medium (KURABO).

Selectin chimeras and mAb

Human E- and P-selectin-IgG chimeras were purified from the conditioned medium of Namalwa KJM-1 cells transfected with the E- or P-selectin chimera expression vector according to the method of Yago *et al.* [15]. Mouse anti-sLe^x mAb KM93 (IgM) was prepared as described [16].

Flow cytometer analysis

U937 cells were incubated with or without panosialin A for various periods in RPMI 1640 medium supplemented with 10% FBS. The cells were then centrifuged and washed with PBS containing 1% BSA, and then incubated at 4 °C for 1 h with either E-selectin-IgG, P-selectin-IgG, or anti-sLe^{x}mAb KM93, which were preincubated with a fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (H + L), F$(ab')_2$ (Wako Pure Chemicals, Japan), at 4 °C for 1 h. The cells were then washed again, resuspended in PBS, and analyzed by cell flow cytometry (EPICS Elite; Coulter).

U937 cell adhesion to plates coated with E-selectin-IgG or HM-VEC

Cell adhesion assays on immobilized E-selectin-IgG were performed as follows. U937 cells were incubated with or without panosialin A for 72 h in RPMI 1640 medium supplemented with 10% FBS. Sumilon microwell plates (Sumitomo Bakelite, Japan) were coated overnight at 4 °C with 100 μl PBS containing E-selectin-IgG (0.75, 1.5, and 3.0 μg/ml). The E-selectin coated plates were washed with PBS and then blocked with PBS containing 1% BSA. After washing to remove unbound cells, the adherent cells were counted with a Cell counting kit (Dojin Kagaku, Japan). The cell adhesion to HM-VEC was observed as follows. HM-VEC were cultured confluently on 48-well collagen-coated plates (Sumitomo Bakelite, Japan) and then stimulated with 10 units/ml of TNF-α for 4 h. Panosialin A- and non-treated U937 cells were biotinylated according to the method of Pearce-Pratt *et al.* [17] and then added (1×10^6 cells) to the HM-VEC plates, followed by incubation at 37 °C for 30 min. Unbound cells were removed by aspiration and the bound cells were fixed with 0.25% glutalaldehyde at room temperature for 10 min. Each well was washed with PBS containing 0.02% Tween 20 and then 50 μl of biotinylated peroxidase (Vector Laboratories) diluted 4000 times with 1% BSA-PBS was added. After 30 min, each well was washed and then 100 μl of an ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium) solution was added. The optimal density of each well was measured using a microplate reader (Emax; Molecular Devices).

Results

Panosialins A and B, inhibitors of Fuc-TVII

The inhibitory effect on Fuc-TVII was measured using a protein A-fused soluble human Fuc-TVII enzyme. Bioassay-guided isolation yielded two active compounds from the fermentation broth of *Streptomyces* sp. KY11789. The spectroscopic data for these compounds were identical with those for panosilains A and B (Fig. 1) [18,19]. Both panosialin A and B inhibited the activity of Fuc-TVII with IC_{50} values of 4.8 and 5.3 μg/ml, respectively (Fig. 2). On the other hand, they inhibited Fuc-TVII more potently than Fuc-TVI another α1,3-fucosyltransferase. The IC_{50} values for panosialins A and B toward Fuc-TVI were 28.7 and 30.1 μg/ml, respectively (Fig. 2).

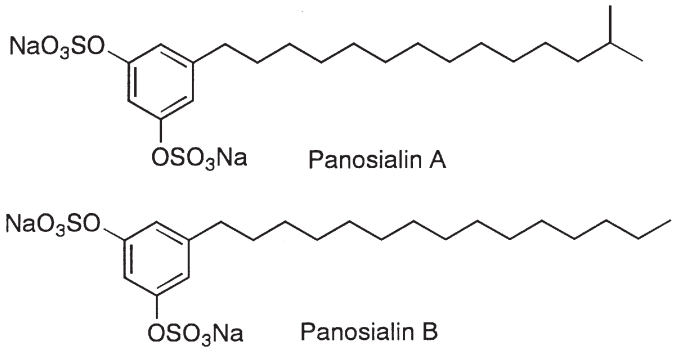

Figure 1. Structures of panosilalins A and B [18].

Suppression of selectin ligand expression on U937 cells

The inhibitory activity of panosialins toward Fuc-TVII suggested that they might suppress the cell surface expression of selectin ligands. Therefore, U937 cells were cultured in the presence of panosialin A (0–25 μg/ml), and then expression of selectin ligands on the cell surface was measured by flow cytometry analysis. Because of the limited amount of panosialin B, only panosialin A was used in the following experiment. After treating the cells with panosialin A for 72 h, the cell surface expression of the E-selectin ligand, P-selectin ligand, and sLe^x was measured using E-selectin-IgG, P-selectin-IgG, and anti-sLe^xmAb KM93, respectively. The expression of the E-selectin ligand was suppressed most potently, followed by that of sLe^x and the P-selectin ligand (Fig. 3). After treatment of the cells with 25 μg/ml of panosialin A, the expression of the E-selectin ligand decreased to approximately 30% compared with in untreated cells.

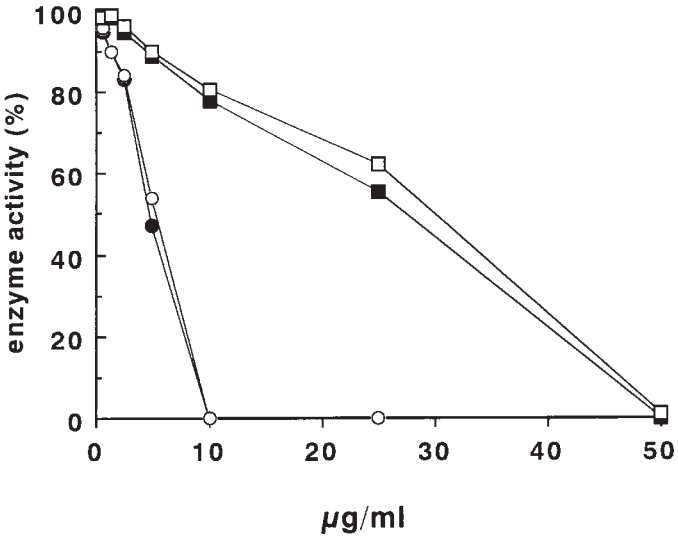

Figure 2. Effects of panosialins A and B on Fuc-TVII and Fuc-TVI activity. Fuc-TVII activity was assayed in the presence of panosialin A (●) or panosialin B (○). Fuc-TVI activity was also assayed in the presence of panosialin A (■) or panosialin B (□).

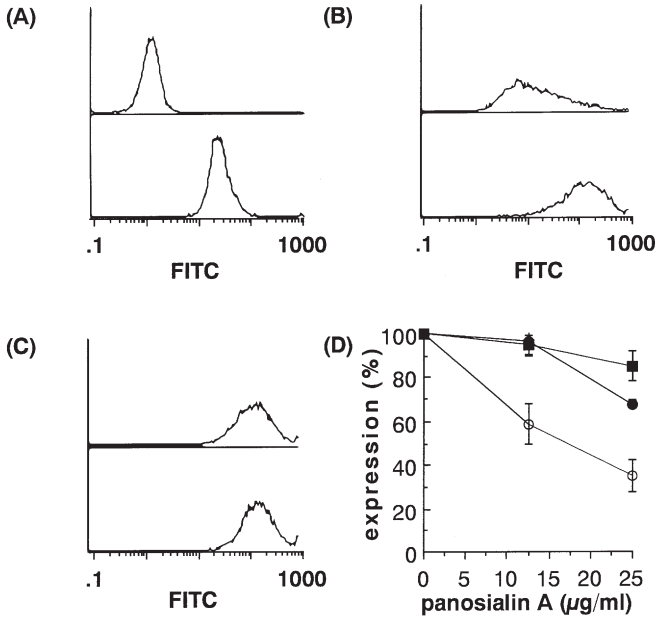

Figure 3. Flow cytometry analysis of panosialin A-treated U937 cells. The cells were treated with (*A*) anti-sLe^X mAb KM93, (*B*) E-selectin-IgG, and (*C*) P-selectin-IgG after incubation with (upper) or without (lower) panosialin A (25 μg/ml) for 72 h. (*D*) Panosialin A reduced the cell surface expression of sLe^X (●), the E-selectin ligand (○), and the P-selectin ligand (■) recognized by KM93, E-selectin-IgG, and P-selectin-IgG, respectively, after treatment of U937 cells for 72 h.

The effect of panosialin A on the expression of E-selectin ligands was monitored on incubation with panosialin A for various periods. The maximal effect was observed on 48–144 h of incubation with various concentrations of panosialin A, while the initial effect was detected after incubation for 24 h (60–70% of the maximal one, Fig. 4).

Inhibition of cell adhesion

The effect of panosialin A on E-selectin-dependent cell adhesion was assayed. After treatment of U937 cells with panosialin A for 72 h, the adhesion to plates coated with recombinant E-selectin-IgG was inhibited dose dependently (Fig. 5). The effect of panosialin A was most potent when the plates were coated with 75 ng of E-selectin-IgG (50% inhibition at 25 μg/ml of the compound). After treatment of U937 cells with panosialin A, the adhesion to TNF-α stimulated endothelial monolayers (HM-VEC) was also inhibited, although the inhibitory effect was less potent for the adhesion between U937 cells and HM-VEC than for that between U937 cells and immobilized E-selectin (data not shown).

Discussion

In the present study, we demonstrated that panosialins A and B inhibited Fuc-TVII. They are the first reported non-

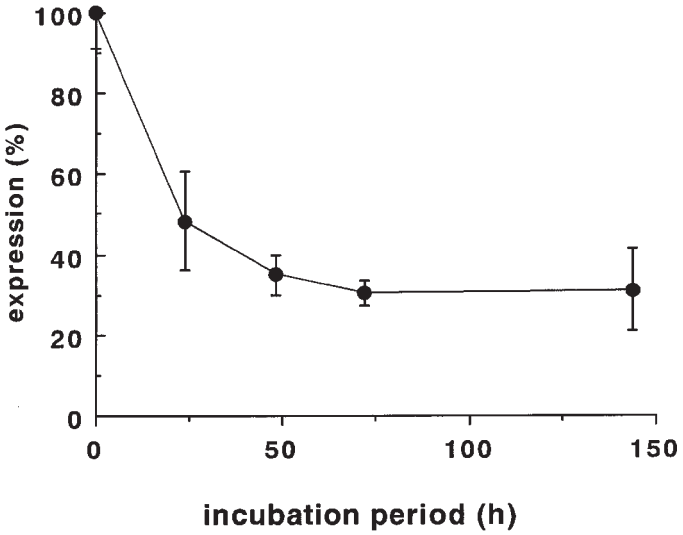

Figure 4. Effect of the incubation period with panosialin A on the expression of the E-selectin ligand on U937 cells.

substrate derived inhibitors of α1,3-fucosyltransferases. Their inhibitory effect on Fuc-TVII was more potent than that on Fuc-TVI, indicating that they may be selective for Fuc-TVII. Furthermore, panosialin A reduced the cell surface expression of selectin ligands on U937 cells. Panosialin A also reduced the cell surface expression of selectin ligands on HL-60 and THP-1 cells (data not shown). In contrast, panosialin A did not significantly alter the expression of other cell surface molecules on U937 cells; i.e.,9 gangliosides, sLe^a, GM3, GM2, GM1, fGM1, GQ1b, GD1b, GD3, and GD2, recognized by mAbs, 7 oligosaccharides recognized by lectins (LEA, MAA, PHA, SNA, ABA, Lotus, and *Helix pomatia*), and 2 glycoprotein antigens, CD43 and CD45 (data not shown). From these results it is considered that the inhibitory effect of panosialin A on Fuc-TVII causes a reduction in the cell surface expression of selectin ligands on U937 cells, since Fuc-TVII probably participates in their biosynthesis selectively. The initial effect of panosialin A was detected after incubation for 24 h and the maximal effect was observed after incubation for 48–144 h, suggesting that the turnover of selectin ligands on the cells might occur within 48 h. The suppression of cell surface selectin ligands by panosialin A reduced the adhesion of the cells to immobilized E-selectin or HM-VEC stimulated with TNF-α. The inhibitory effect of panosialin A was more potent when the treated cells were attached to recombinant E-selectin-IgG than when they were attached to HM-VEC stimulated with TNF-α. Other adhesion molecules, such as integrins and ICAM-1, may involved in the cell adhesion of U937 cells to HM-VEC.

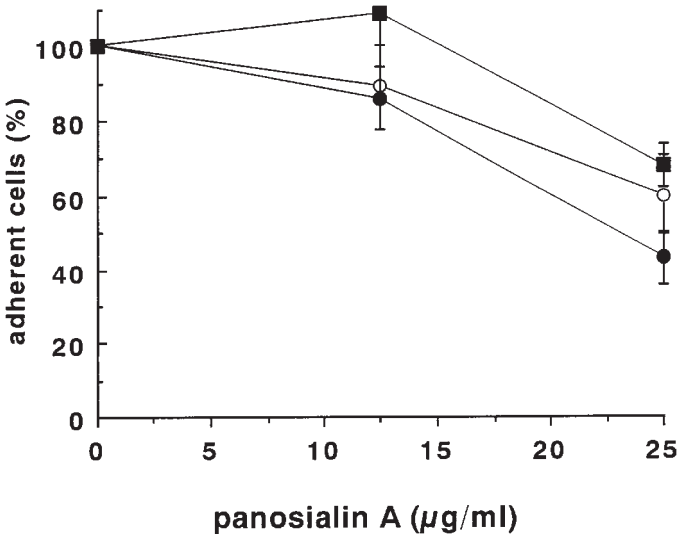

Figure 5. Adhesion of U937 cells to immobilized E-selectin. Binding of U937 cells to recombinant E-selectin (75 ng/well (●), 150 ng/well (○), and 300 ng/well (■)) after treatment of the cells with panosialin A for 72 h.

Panosialins were first isolated as inhibitors of viral sialidase, acid phosphatase, and polygalacturonase, and were recently reported to be inhibitors of glycosidases [18–20], although the selective effect of panosialin A on the cell surface expression of selectin ligands is considered to be due to its inhibitory effect on Fuc-TVII, as mentioned above. Some of these activities of panosialin A may cause its growth inhibitory effect on U937 cells (IC_{50} value of 50 μg/ml, data not shown). Since Fuc-TVII-deficient mice were reported to grow normally, the inhibition of Fuc-TVII activity is not considered to cause growth inhibition of the cells. The manner in which panosialins inhibit Fuc-TVII activity is not clear at present. Although its molecule has both hydrophobic and hydrophilic portions, in the molecule like those of some detergents, the activity of Fuc-TVII was not affected by the presence of Triton X-100 or Tween 20 up to a concentration of 5% (v/v, data not shown). Further investigation and research are required to obtain specific inhibitors of Fuc-TVII, which will inhibit selectin-mediated cell adhesion without any other effects on the cells.

In summary, we isolated panosialins A and B as the first non-substrate derived inhibitors of Fuc-TVII. Using panosialin A we showed that the inhibition of the Fuc-TVII activity could suppress the biosynthesis of selectin ligands, and then reduce selectin-mediated cell adhesion. Panosialin B is also considered to suppress the expression of selectin ligands, since both panosialin A and B inhibited Fuc-TVII to similar extents. These results suggested the importance of Fuc-TVII as a target molecule for the screening of anti-inflammatory drugs.

Acknowledgments

We are grateful to Kazumi Hasegawa and Aya Uehara for their technical assistance.

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Received 3 August 1998, revised 1 October 1998, accepted 9 October 1998