Advanced Research in Gastroenterology & Hepatology
ISSN: 2472-6400

Research Article
Volume 21 Issue 2- December 2024
DOI: 10.19080/ARGH.2024.21.556056

Adv Res Gastroentero Hepatol

Glycosylated Lysozyme Mutants act as Potential Anti-Inflammatory and Anti-Metastatic Agents

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Submission: November 25, 2024 **Published:** December 10, 2024

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Abstract

The glycosylated human lysozyme mutant hLysII/IVT has been expressed and characterized in CHO cells expressing fucosyltransferases FUCIII, IV, V, VI, or VII. Digestion analysis with endo- and exoglycosidases showed that hLysII/IV-FUCTVI contains complex N-glycans with a variable number of lactosamine repeats; these were terminally sialylated and fucosylated, suggesting the presence of the sLe^x antigen further confirmed by immune-blotting. In contrast, hLysII/IVT-FUCTIII and IV lack terminal sialylation and hLysII/IVT-FUCV and VII lack terminal fucosylation. In a static adhesion assay, binding of U937 to activated HUVEC cells was efficiently blocked in the presence of medium supernatant containing hLysII/IV-FUCTVI and by affinity-purified hLysII/IVT-FUCTVI. The binding was not attenuated in the presence of hLysII/IVT-FUCTIII, IV or V secretions. Titration experiments showed that significant inhibition of adhesion is achieved at 0.07nM hLysII/IVT-FUCTVI, making it the most potent known inhibitors of adhesion described to date. As a proof of principle, cell adhesion was also efficiently blocked in a flow-adhesion assay, which has to be characterized in more detail in further studies.

Keywords: Fructosyltransferases; Glycosylation; Lysozyme; Oligosaccharide; Polylactosamines; sLe^x

Abbreviations: hLys: Human Lysozyme; FUCT: Fucosyl Transferase; sLe^x : Sialyl Lewis^x

Introduction

The evascularization of cells is a critical step in immune defense responses and the spread of metastases (reviewed in [1]). During inflammation, the endothelium of the affected tissue becomes activated and interacts with cells bearing cognate structures such as sialyl LewisX (sLe^x) substances. These substances bind to selectins, which are displayed in a regulated manner on the surface of the endothelium and platelets. sLe^x structures are located at the nonreducing termini of various glycoconjugates with extensions made of N-acetyllactosamine repeats. These repeats are suggested to serve as potential immune regulatory factors, thought to suppress excessive immune responses and play a significant role in the immune systems function [2]. Several studies have demonstrated that the synthesis of these repeats is promoted by the expression of the elongating β1-3 N-acetylglucosaminyltransferase [3-5] and branching β1-6 N-acetylglucosaminyltransferases [6], whereas it is inhibited by sialyltransferases, which terminate the elongation process [7]. N-acetylglucosaminyltransferase V, responsible for synthesizing the β1,6-linked branch in complex oligosaccharides, supports the formation of triantennary oligosaccharides, which are better substrates for elongation than their biantennary counterparts [6]. Increased branching of complex oligosaccharides and elongation of N-acetyllactosamine repeats are observed in neoplastic human cells, along with the synthesis of sLe^x substances by terminal fucosylation, which correlates with metastatic potential (reviewed in [8]).

In previous studies, we analysed glycosylation of lysozyme mutants at specific sites denoted as I (N22), II (N68), and IV (N49). By combining sites I, II, and IV, we generated glycosylated lysozyme mutants I/IV and II/IV. Stable transfection of cells with both singly and doubly glycosylated mutants enabled characterization of glycosylated lysozyme forms and their complex side chains. Notably, the double mutant hLysII/IV exhibited the highest abundance of lactosamine repeats, with oligosaccharides attached to residues N68 (site II) and N49 (site IV) [9].

Further analysis of this glycoprotein involved overexpressing it in wild type CHO cells, followed by purification and separation of the mono- and di-glycosylated forms [10,11]. Mass spectrometric analysis of glycopeptides derived from molecules glycosylated solely at site IV or at both sites simultaneously revealed that glycosylation at site II of the mutant lysozyme not only enhanced the synthesis of triantennary oligosaccharides at site IV but also promoted their elongation with N-acetyllactosamine repeats. This finding offers new insights into the interaction between glycosylation sites and the resulting oligosaccharide structures [10,11].

In this study, we aim to generate glycosylated lysozyme mutants with terminal sLe^x oligosaccharides for potential E-Selectin blockade applications. This can be achieved by α1-3 fucosylation of the existing terminal lactosamine repeats of hLysII/IVT. However, the CHO wild-type cells used previously lack α1-3-fucosyltransferases, and thus are unable to perform this fucosylation. To overcome this limitation, CHO cells that stably express fucosyltransferases III, IV, V, VI, and VII (referred to as CHO-FUCTIII-VII) will be transiently and stably transfected with the pCMI-hLysII/IVT-cDNA plasmid vector. The resulting hLysII/IVT-FUCTIII-VII protein will be purified, and the glycosylation patterns of the mutant proteins will be analyzed through glycosidase digestion. An adhesion experiment will then be conducted to identify the most promising candidate for inhibiting cell adhesion. This candidate will undergo further characterization by Western blot analysis, following by additional adhesion experiment under flow conditions to assess its effectiveness in such an environment.

Materials and Methods

Cells and culturing

Wild type CHO cells were obtained from the American Type Culture Collection (ATCC CRL1736) and cultivated in α-minimum essential medium (Thermo Fisher Scientific, Waltham, U.S.), supplemented with 10% (v/v) fetal bovine serum (Sigma Aldrich, Darmstadt, Germany), and Pen/Strep (1 x 10^5 u/lM; Gibco, Carlsbad, USA, Fisher Scientific, Schwerte, Germany). CHO dhfr- cells stably transfected with fructosyltransferases III, IV, V, VI, VII (CHO-FUCTIII-VII [12]), kindly provided by Prof. Dr. D. Vestweber (University of Muenster, Germany), were cultivated in α-MEM with 0,4mg/ml G418 (Caymen Chemicals, MI, US). Cells were passaged with trypsin-EDTA (Gibco, Carlsbad, USA, Fisher Scientific, Schwerte, Germany) when reaching 70% confluency.

Overexpression of hLysII/IVT(FUCTIII-VII)

Subconfluent cultures of CHO-FUCTIII-VII cells, grown in 6 well plates, were transfected with pMCI/hLysII/IV [11] by lipofection, following a modified protocol as per the Thermo Fisher Scientific, Waltham, U.S. manual, with 2.5μg circular DNA per 2.5x10^5 cells. Transient expression was assessed in the culture supernatants 48h post-transfection, while stable transfectants were isolated by puromycin (14μg/ml) (Sigma Aldrich, Darmstadt, Germany).

Quantification of lysozyme

The lysozyme activity assay relies on the digestion of *Micrococcus luteus* cell walls by lysozyme. A *Micrococcus luteus* suspension (4mg dissolved in 1ml 67mM sodium phosphate buffer) (Sigma Aldrich, Darmstadt, Germany) was mixed 1:1 with 10μl of culture medium. After 4h incubation at 37°C, the optical density at 630nm was measured using a spectrophotometer (LKB Novaspect II, Pharmacia, Freiburg). The decrease in optical density was compared to that obtained with a lysozyme standard (Roche Diagnostic, Mannheim, Germany).

Characterization of hLysII/IV (FUCTIII-VI)

CHO cells expressing fucosyltransferases FUCTIII-VII and glycosylated lysozyme were metabolically labeled with 4MBq of a [^{35}S]-methionine and cysteine mixture (Tran35S-label from ICN Biomedicals, Eschwege, specific activity 40GBq/mmol) in 3.4cm diameter dishes for 18h. The labeled lysozyme was then isolated by immunoprecipitation, separated by SDS-PAGE, and visualized by fluorography as previously described [7,13]. For glycosidase treatments, the metabolically labeled immunoprecipitated pellets were resuspended in 0.1 M Tris/HCl (pH 7.4) containing 0.02% (w/w) SDS (Sigma Aldrich, Darmstadt, Germany). Glycosidase digestions were conducted according to the manufacturer's instructions, with β-N-Acetylglucosaminidase and β-galactosidase from Sigma Aldrich (Darmstadt, Germany). Endo-β-galactosidase, endo-β-N-acetylglucosaminidase F2, and sialidase were sourced from Roche Diagnostic (Mannheim, Germany). Endo-β-N-acetylglucosaminidase F2 cleaves biantennery complex oligosaccharides, while sialidase treatment was used to detect terminal sialic acid residues. The presence of fucosylated lactosamine repeats was confirmed through combined digestion with sialidase, galactosidase and N-acetylglucosaminidase; fucosylation terminates further digestion. Fucosylation also prevented lactosamine digestion by endo-β-galactosidase.

Immunoaffinity purification of lysozyme II/IV (FUCTIII-VII)

Nonradioactive lysozyme was purified from the spent culture medium of a CHO-FUCTIII-VII cultures expressing the mutant II/IV. Lysozyme was bound to an immobilized rabbit polyclonal anti-human lysozyme antibody column (DAKO, Denmark). Antibodies were purified from antiserum using immobilized human lysozyme and attached to cyanogen bromide-activated Sepharose 4B-CL (Pharmacia, Freiburg). Bound proteins were eluted with 0.1M glycine/ Tris/HCl buffer (pH 2.65) and with 2M $MgCl_2$. Samples were further dialyzed and concentrated. The ability of purified lysozyme to block cell adhesion was tested using a static adhesion assay. All chemicals were obtained from Merck, Germany.

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Western blot

Conditioned media were collected in 15 mL tubes and centrifuged at 5000 rpm for 5 minutes to pellet debris. Sample buffer (10% glycerol, 0.4% sodium dodecyl sulfate (SDS), 0.3% bromophenol blue, and 0.2% pyronin Y in 1 x stacking buffer with Tris base at 0.5mol/L and 0.8% SDS, with 20% mercaptoethanol; all from Sigma-Aldrich) was added to the medium, and samples were heat-denatured at 95°C for 3 minutes. Protein (50µg) was loaded onto a SDS-PAGE with 12.5% - 15% polyacrylamide and fractionated by electrophoresis. Proteins were transferred onto Immobilon-P PVDF transfer membrane (Millipore, Bedford, MA, USA) and equal protein loading was confirmed by Ponceau S (Sigma-Aldrich) staining. Blots were probed with rabbit or mouse antibodies [against human lysozyme (EC 3.2.1.17, DAKO Cytomation, Denmark), human fucosyltransferase VI (ab197289, Abcam, Berlin, Germany) or sLex (1. sLex from FH6-cells; 2. Sialyl Lex from Abnova MAB3343; 3. Anti-Sialy Lex Clone KM93, MC-014 from Kamiya)]. Detection was achieved using a chemoluminescence system (Clarity Western ECL, Bio-Rad). Each experiment was repeated at least three times.

Static cell adhesion assay

Adhesion assays were conducted as previously described [14,15]. Briefly, U937 cells were labeled with BCECF (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein) (Thermo Fisher Scientific, Waltham, U.S.) by resuspending the cells (10^7 cells/ml) in HBSS/BSA/HEPES buffer with BCECF-AM at a final concentration of 2µM. After 20min incubation at room temperature, samples were spun at 400 x g for 5min and resuspended in the HBSS/BSA/HEPES buffer. Human umbilical vein endothelial cells (HUVEC), passage 1-3, were cultured in 6-well plates (RPMI 1640 medium Gibco, Carlsbad, USA) and plated to be confluent for the assay. HUVECs were activated with 50U/ml TNF-α at 37°C. Saturating amounts of E-Selectin antibodies (10µg/ml) (Murine IgG1, Ancell, Bayport, MN) or hLysII/IVT were added during the pre-incubation. After washing with RPMI + 1% FBS at 37°C, BCEDE-labeled U937 cells were added to each well. Triplicate 100µl aliquots were retained to determine average fluorescence per cell. After washing to remove of unbound U937 cells (by rinsing with 2ml PBS), HUVECs were transferred into tubes, centrifuged, and resuspended in 2ml 0,1% Triton/PBS; fluorescence was determined using a luminescence spectrometer.

Cell adhesion assay (flow conditions for HUVEC-cells)

The IBIDI pump system was set up as directed. 250.000 primary HUVEC cells were seeded onto a 0.6µm Slide (µ- Slide $I^{0.6}$ Luer) and incubated for 48 h at 37°C and 5% CO_2 under continuous flow (22ml/min) to form a monolayer. HUVECs were activated with TNFα 50U/ml (Sigma Aldrich, Darmstadt, Germany) for 30min and pretreated with either VCAM (20g/ml) (Cymbus Biotechnology LTD, NF, US) or hLysII/IVT (300ng/ml). Cells were washed with PBS and incubated for 30min with 150.000 BCECF-labeled U937 cells. Triplicate aliquots were retained to measure average cell fluorescence with a luminescence spectrometer.

Results

Overexpression of hLysII/IVT in CHO-FUCTIII-VI and characterization of oligosaccharide structures in hLysII/IVT-FUCTIII-VII.

To study oligosaccharide structures, spent media from cultured, stably transfected hLysII/IVT in CHO-FUCTIII, IV, V, VI and VII cells - metabolically labeled with radioactive amino acids - was collected. The synthesized lysozyme variants were then separated using SDS-PAGE (Figure 1). The fastest migrating band (labeled "L") resembled non-glycosylated lysozyme. A faint band ("mL"), which migrated just behind the L form, has previously been identified as bearing a mannose-rich oligosaccharide [16]. Singly-glycosylated molecules appeared as a group of slower bands ("sL"), while the slowest group ("dL") represented double-glycosylated molecules carrying complex bi- and/or Tri antennary oligosaccharides, with or without N-acetyllactosamine repeats [10]. Endo-β-N-acetylglucosaminidase F2 digestion revealed that the oligosaccharide structures of hLysII/IVT-FUCIII and IV predominantly carry biantennary complex N-glycans (Figure 2A).

Sensitivity to sialidase treatment indicated the presence of terminal sialic acid residues in hLysII/IVT-FUCTV, VI and VII. In contrast, the mobility of lysozyme from hLysII/IVT-FUCTIII and IV cells remained unchanged, suggesting the absence of sialylation in these cases (Figure 2B). Sequential digestion with sialidase, β-galactosidase and β-N-acetylglucosaminidase indicated indirectly that the terminal N-acetylglucosamine of the lactosamine repeats in hLysII/IV-FUCTIII, IV and VI were fucosylated, preventing complete digestion (Figure 3B). Digestion experiments with endo-ß-galactosidase similarly confirmed the presence of fucosylation on proximal lactosamine repeats in hLysII/IV-FUCTIII and IV (Figure 3A). Due to the lack of terminal sialic acid, hLysII/IV-FUCTIII and IV cells could only produce Le_x-antigens. However, in hLysII/IV-FUCTVI cells, terminal sialylation and fucosylation of the lactosamine repeats made the production of sLe_x-antigens feasible (Figure 4). Terminal fucosylation was absent in hLysII/IV-FUCTV and in VII cells. A combination of proximal fucosylation with terminal sialylation likely contributed to the formation of the VIM2-antigen [17] in hLysII/IV-FUCTV and VII cells.

Identification of hLysII/IVT-FUCTVI as most potent inhibitor of cell adhesion in a static adhesion-assay using hLysII/IVT-FUCTVIII-VII

The potential of hLysII/IV-FUVTIII-VII to block cell adhesion was evaluated in a static assay using U937 and TNF-α-activated HUVEC cells. To assess baseline effects, saturating concentrations of anti-E-selectin (10µg/ml) and anti-VCAM (10µg/ml) antibodies were applied, which reduced U937 cell adhesion by 29% and

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40%, respectively. Next, the supernatant from CHO-FUCTIII-VII cells, transiently transfected with hLysII/IV, was mixed with lysozyme to a final concentration of 370ng/ml (26 nM) and applied to the HUVEC cells. At this concentration, hLysII/IV-FUCTVI reduced U937 cell adhesion by 31% (p=0.009), indicating significant inhibition. No comparable effects were observed when conditioned media from hLysII/IV-FUCTIII, IV, V, and VII cultures were used (Figure 5A). To confirm that the inhibitory effect was due specifically to hLysII/IV, the adhesion assay was repeated using lysozyme purified from each conditioned medium.

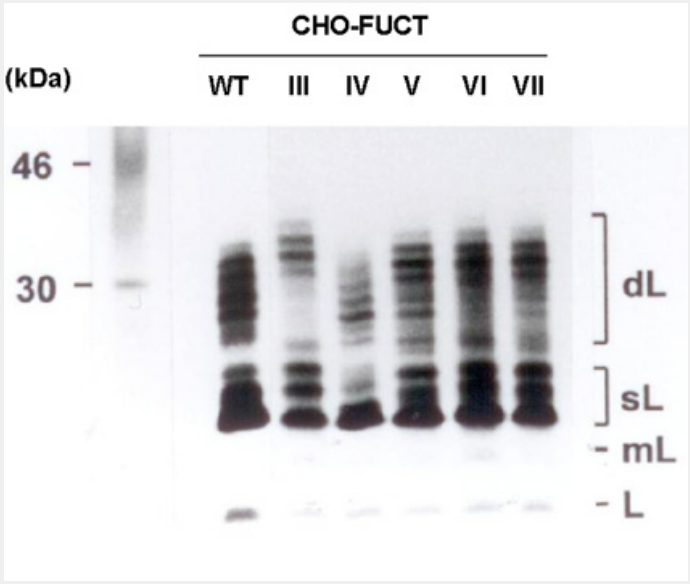

Figure 1: SDS-PAGE Analysis of Lysozyme Forms Overexpressed in CHO-FUCTIII, IV, V, VI, and VII Cells. Spent media were collected, and lysozyme variants were separated by SDS-PAGE. The fastest migrating band (L) corresponds to non-glycosylated lysozyme, while the faint band (mL) represents a mannose-rich glycosylated form. The "slower" bands (sL) represent singly glycosylated lysozyme species, and the slowest bands (dL) represent doubly glycosylated lysozyme variants with bi- and Tri antennary oligosaccharides.

The purified lysozyme species were used at final concentrations of 47ng/ml (3.4 nM). hLysII/IV-FUCTVI and hLysII/IV-FUCTVII significantly inhibited U937 cell adhesion, causing 33% (p=0.0003) and 20% (p=0.002) reductions, respectively. No such effects were observed with hLysII/IV-FUCTIII, IV or V (Figure 5B). Given the significant inhibition of adhesion observed with both conditioned media containing hLysII/IV-FUCTVI and the affinity-purified lysozyme, the inhibitory effect of this glycoprotein was tested at several concentrations. U937 cell adhesion was significantly inhibited by hLysII/IV-FUCTVI at concentrations of 1ng/ml (0.07 nM) or higher. Non-linear regression analysis produced the following model for the inhibition induced by this enzyme: y=27*EXP((-1)*2,5*x)+7, where y represents adhesion inhibiton [%] and x represents lysozyme concentration [ng/ml]) (Figure 6A & 6B). Using this expression, the maximal calculated adhesion inhibition by hLysII/IV-FUCTVI was 27%, with an IC_{50} of approximately 0.3ng/ml. No significant inhibition was observed at lysozyme concentrations of 0.1, 0.2, or 0.5ng/ml.

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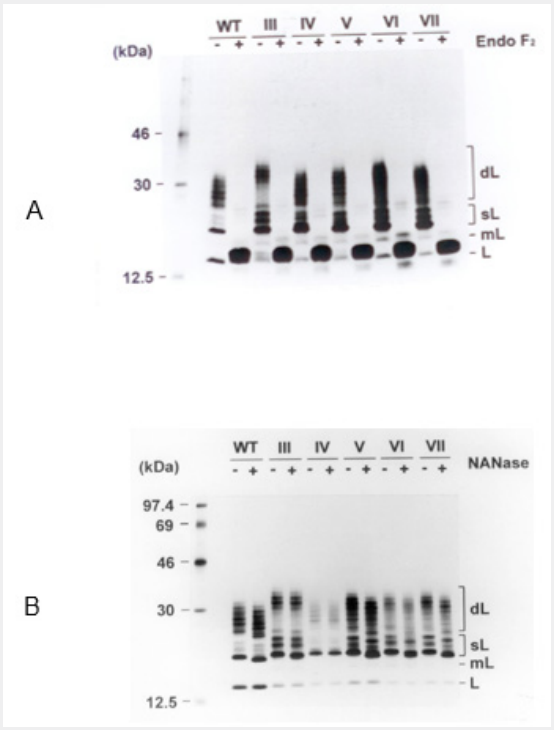

Figure 2: Analysis of glycosylation patterns of hLysII/IV-FUCTIII-VII. (A) Digestion with endo-β-N-acetylglucosaminidase F2 demonstrated that the oligosaccharide structures of hLysII/IVT-FUCIII-VII consist primarily of biantennary complex N-glycans. (B) Sensitivity to sialidase indicated the presence of terminal sialic acid residues in hLysII/IVT-FUCTV, VI and VII. The mobility of lysozyme from hLysII/IVT-FUCTIII and IV cells was not affected by this treatment indicating that no sialylation is present in these cases.

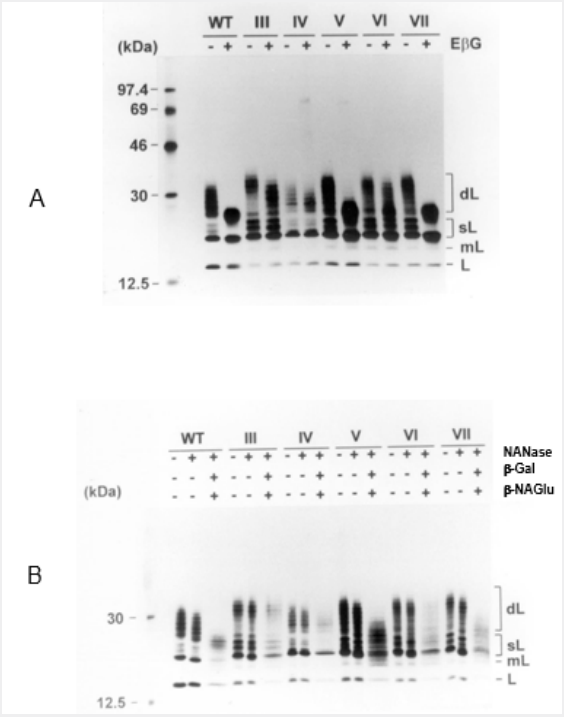

Figure 3: Analysis of glycosylation patterns of hLysII/IV-FUCTIII-VII. (A) Digestion experiments using endo-ß-galactosidase indicated the presence of fucosylation of the proximal lactosamine repeats in hLysII/IV-FUCTIII and IV (B) Digestion with sialidase, β-galactosidase and β-N-acetylglucosaminidase indirectly demonstrated that the terminal N-acetylglucosamine of the lactosamine repeats in hLysII/IV-FUCTIII, IV and VI were fucosylated, preventing their complete digestion.

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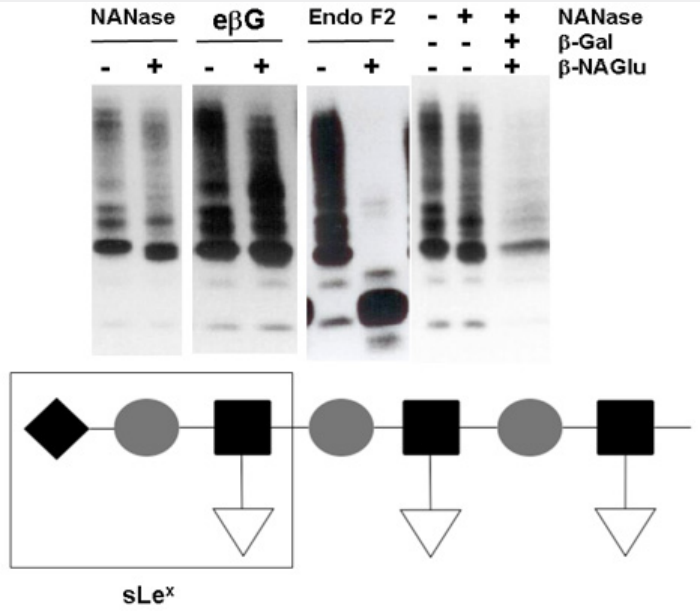

Figure 4: Analysis of glycosylation patterns of hLysII/IV-FUCTVII. In hLysII/IV-FUCTVI cells, terminal sialylation and fucosylation of the lactosamine repeats made the production of sLex-antigens feasible

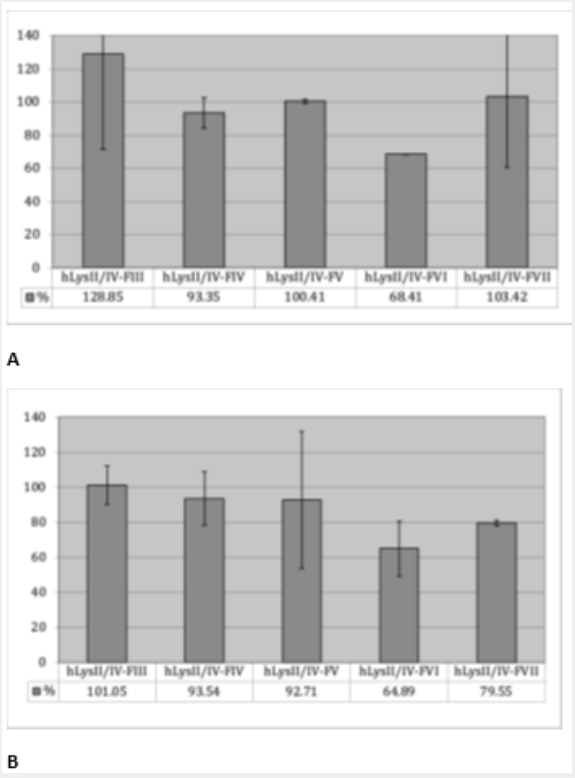

Figure 5: Adhesion inhibition of U937 cells to HUVEC cells by affinity-purified hLysII/IV-FUCTIII-VII in a static adhesion assay. (A) Supernatant of the medium transiently transfected with hLysII/IV at a concentration of 370 ng/ml (26 nM) and applied to the HUVEC-cells. U937 cell adhesion was reduced by 31% (p=0.009) at this concentration of hLysII/IV-FUCTVI. No effect was seen when the same procedure was performed using media that had been used to culture hLysII/IV-FUCTIII, IV, V, and VII (B) Purified lysozyme species were used at a final concentration of 47 ng/ml (3.4 nM). hLysII/IV-FUCTVI significantly inhibited U937 cell adhesion by 33% (p = 0.0003), while hLysII/IV-FUCT-VII caused a 20% reduction (p = 0.002). No significant adhesion inhibition was observed with hLysII/IV-FUCTIII, IV, or V..

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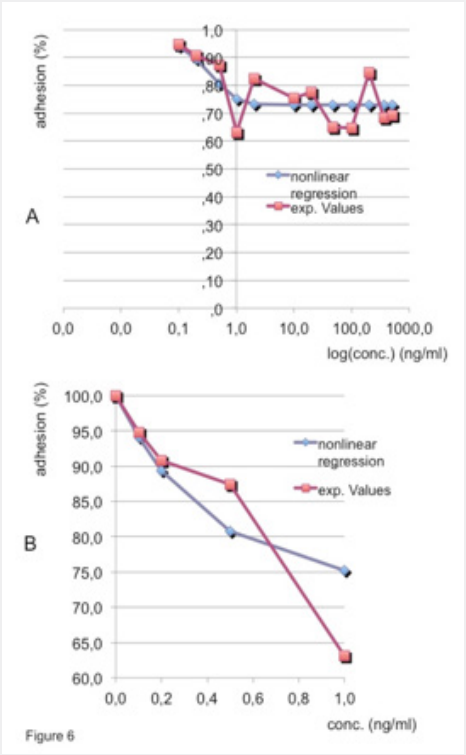

Figure 6: Nonlinear Regression Analysis of U937 Cell Adhesion to HUVEC Cells as a Function of hLysII/IV-FUCTVI Concentration. (A) Plot showing the percentage of adhesion as a function of hLysII/IV-FUCTVI concentration on a logarithmic scale. The nonlinear regression and experimental values demonstrate a decreasing trend in adhesion with increasing hLysII/IV-FUCTVI concentration. The maximal calculated adhesion inhibition by hLysII/IV-FUCTVI was 27%, with an IC50 of approximately 0.3ng/ml. (B) Detailed analysis showing the concentration-dependent inhibition of U937 cell adhesion with hLysII/IV-FUCTVI. No significant inhibition was observed at lysozyme concentrations of 0.1, 0.2, or 0.5ng/ml Significant inhibition is observed at concentrations of 1 ng/ml and above.

Characterization of hlysII/VT-FUCTVI by Western-blotting

The highest performance in the static adhesion assay was achieved with hLysII/IVT-FucTVI. To further characterize this glycoprotein, we conducted a Western blot analysis. The overexpression of FUCTVI in CHO-FUCTVI cells was confirmed using a FUCTVI-specific antibody. For comparison, CHO-lec11 cells, known to overexpress Fructosyltransferase 6 as well [18], served as a control (Figure 7). Subsequently, the blot was stained with both lysozyme and sLe^x antibody FH6. A consistently weak band at approximately 25kDa was observed (Figure 8A). To enhance glycosylation and improve signal strength, cells were treated with butyrate, known to promote N-glycosylation site occupancy [19] (Figure 8B); 5-Aza-2′-deoxycytidine, which increases sLe^x expression [20]; and N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid, a specific endogenous sialidase inhibitor (Figure 8C). Following these treatments, a slight improvement in band intensity was observed compared to the untreated control.

Inhibition of cell adhesion using flow culturing conditions in HUVEC-cells

The ibid pump system was used to culture HUVEC cells under flow conditions, simulating blood vessel environment. An adhesion assay was then conducted with VCAM and hLysII/IV-FUCTVI at concentrations of 20µg/ml and 300ng/ml, respectively. Adhesion of U937 cells was inhibited by 68.5% with VCAM and 83% with hLysII/VI-FUCTVI (Figure 9). Notably, inhibition was significantly more effective under flow-culture conditions compared to static-culture conditions HUVEC-cells. Further experiments will explore varying concentrations of hLysIII/IV to determine IC_{50} under flow conditions.

Discussion

Overexpression of hLysII/IVT in CHO cells (CHO-FUCTIII-VII) and glycosylation characterization by glycosidases

In this study, hLysII/IVT was successfully transfected, overexpressed, and purified in CHO-FUCTIII, IV, V, VI and VII. In static adhesion assays, hLysII/IVT-FUCTVI emerged as the lysozyme variant most effective in inhibiting cell adhesion. Glycosidase analysis of the overexpressed hLysII/IVT-FUCTVI indicated a potential presence of terminal sLe_x structures. The six cloned human fucosyltransferases (FUCTIII, FUCTIV, FUCTV, FUCTVI, FUCTVII, and FUCTIX) form a family of type II transmembrane glycoproteins that contain highly similar domain structures and exhibit substantial amino acid sequence homology.

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All enzymes utilize GDP-fucose as a donor substrate but differ in oligosaccharide acceptor specificity. For instance, FUCTIII, known as Lewis enzyme, is involved in synthesizing all Lewis antigens (Le^a, sLe^a, Le^b, Le^x, sLe^x and Le^y [21]). In our experiments, Le^x was the predominant structure in hLysII/IV-FUCTIII. Aberrant Le^x expression due to FUCTIII overexpression has also been observed in conditions like gastritis, intestinal metaplasia and gastric carcinoma [22].

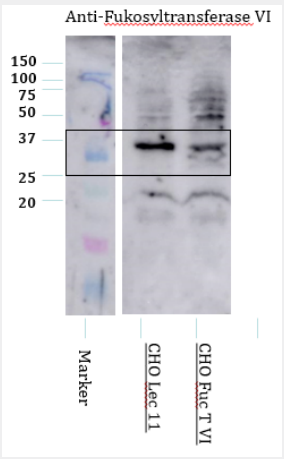

Figure 7: Confirmation of Continued Overexpression of FUCTVI in CHO-FUCTVI Cells by Western Blotting. A Western Blot with the FUCTVI antibody was performed to confirm the continued overexpression of FUCTVI in CHO-FUCTVI cells .The CHO-lec11 cell line, which also overexpresses fucosytransferase VI, was used as control.

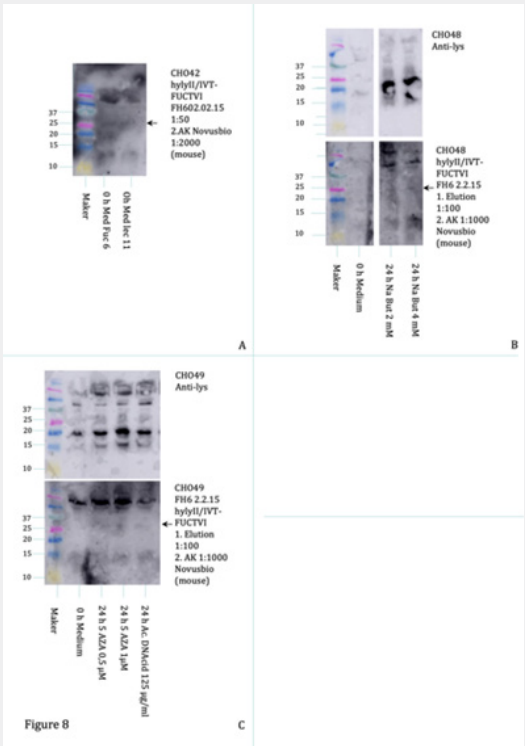

Figure 8: Western Blot Analysis of hLysII/IV-FUCTVI Glycosylation Enhancement by Chemical Treatments. (A) Medium control showing baseline expression of hLysII/IV-FUCTVI. (B) Treatment with butyrate resulted in improved glycosylation and stronger sLe^x expression. (C) Treatment with 5-Aza-2 deoxycytidine enhanced glycosylation at glycosylation sites, further increasing sLe^x expression. N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid also slightly improved sLe^x expression.

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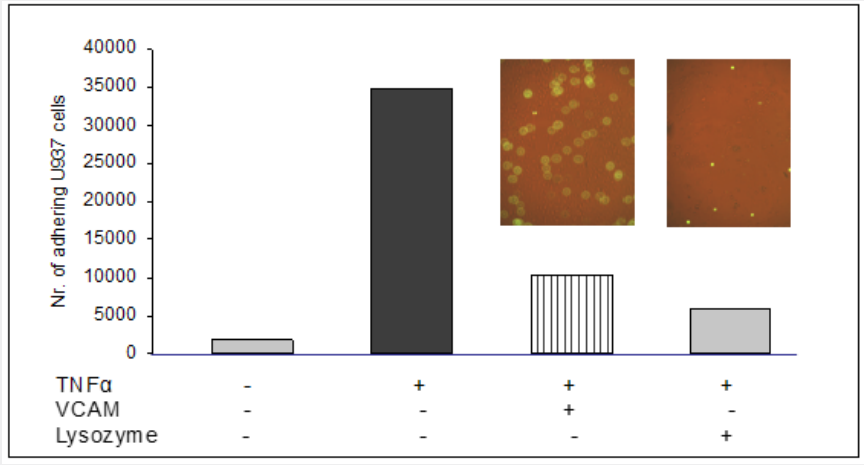

Figure 9: Inhibition of U937 Cell Adhesion to HUVEC Cells Under Flow Conditions Using hLysII/IV-FUCTVI and VCAM. U937 cells were incubated with TNF-α-activated HUVEC cells under flow conditions. hLysII/IV-FUCTVI (300 ng/ml) and VCAM (20 µg/ml) were used as inhibitors. hLysII/IV-FUCTVI significantly reduced U937 cell adhesion by 83%, while VCAM inhibited adhesion by 68.5%. These results demonstrate the superior efficiency of hLysII/IV-FUCTVI in inhibiting cell adhesion under physiological flow conditions compared to static conditions, highlighting its potential as a strong inhibitor of cell adhesion.

FUCTIV supports the expression of the Le^x, sLe^x, and difucosyl sLe^x epitopes [12]. It transfers fucose effectively to N-acetyllactosamine (LN) units in neutral polylactosamines and to the "inner" LN units of α2,3-sialylated acceptors, but is ineffective at transferring fucose to the distal α2,3-sialylated LN unit in α2,3-sialylated acceptors [23]. Our findings align with previous reports showing that FUCTIV-expressing CHO cells synthesize Le^x but not sLe^x determinants [24,25]. Furthermore, CHO-cells expressing FUCTV direct fucosylation of internal GlcNAc residues within lactosamine repeats, producing the VIM2 determinant structure. In contrast, FUCTVI preferentially fucosylates distal GlcNAc residues, leading to the synthesis of sialyl-Le^x determinants [26]. Unlike prior studies suggesting that FUCTVII effectively fucosylates only the distal α2,3-sialylated LN unit in α2,3-sialylated acceptors [23], we found limited evidence of distal fucosylation of the extended lactosamine chains of hLysII/IV-FUCTVII. Instead, either the proximal LN units are fucosylated, producing the VIM2 antigen, or distal α2,3-sialylated LN units are exclusively fucosylated in shorter lactosamine repeats.

Inhibition of U937 cell adhesion to HUVEC cells by hLysII/IV-FUCTVI in a static adhesion assay

Our study demonstrated that hLysII/IV-FUCTVI significantly reduces U937 cell adhesion to activated HUVEC cells at concentration of 1ng/ml (0.07 nM) and above. This result aligns with glycosylation studies, indicating that the lactosamine repeats of hLysII/IV-FUCVI are terminally sialylated and fucosylated, facilitating the formation of sLe^x-antigens. Previous research has shown that the maximum achievable reduction in E-selectin blocking in static adhesion assays using saturating concentrations of a monoclonal E-Selectin antibody is approximately 30% [14], comparable to the maximum reduction of 27 % (± 5%) observed in our experiments. In other studies, similar or higher concentrations of E-Selectin inhibitors were necessary to effectively block adhesion. For instance, a lipid-linked sLe^x achieved 30 ± 6% inhibition only at concentrations of 3nM or higher.

Similarly, GM1292, a compound mimicking sLe^x inhibition of selectin-mediated adhesion, reduced eosinophil adhesion by 23 ± 5% and neutrophil adhesion by 20 ± 6% at a 1mM concentration [28]. Efomycine M, another selectin inhibitor, selectively reduced leukocyte-endothelial adhesion *in vitro* with an IC_{50} of 3µM and significantly diminished leukocyte rolling in mouse ear venules *in vivo*, as observed through intravital microscopy [27]. Molecular modelling studies suggests that efomycines can adopt conformations similar to natural selectin ligands [29]. In 1999, Rittershaus et al. engineered a form of soluble complement receptor 1 (sCR1, CD35) known as sCR1-sLe^x, which expressed sLe^x moieties on certain N-linked oligosaccharides through FT-VI-expressing LEC11 CHO cell line secretions [29]. Later, in 2004, Thomas et al. described an *in vitro* enzymatic remodeling of sCR1 using recombinant glycosyltransferases, which doubled the number of sLe^x moieties and increased apparent binding affinity for E-selectin by nearly tenfold (IC50 of ~5nM for sCR1-sLe^x versus ~0.4nM for sCR1-S/F) [30]. This enhancement likely arises from a multivalent binding reaction where the widely distributed sLe^x on sCR1-S/F engage multiple E-selectin molecules.

The inhibition of adhesion observed with hLysII/IV-FUCTVI appears to be comparable or even more potent than with sCR1-S/F [29]. Unlike sCR1, glycosylated lysozyme has a smaller protein moiety and fewer oligosaccharide side chains, differing by roughly an order of magnitude. The isoelectric points also vary

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significantly; with theoretical values of 9.28 for human lysozyme and 6.57 for sCR-1. The strong adhesion inhibition by modified sCR-1 has been attributed to its polyvalent structure, and it will be of interest to further investigate which structural properties of lysozyme contribute to its potent inhibition of cell adhesion in its glycosylated form.

Further analysis of the hLysII/IVT-FUCTVI mutant by western blotting

Western blot analysis of hLysII/IV-FUCTVI using the (sLe^x-) FH6 antibody revealed a reproducible but faint band at 25kD. Visualization was slightly enhanced by promoting glycosylation with 5-Aza-2′-deoxycytidine, butyrate and N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid (Figure 8B & 8C). With two possible glycosylation sites involved in the synthesis of terminal sLe^x antigens on glycosylated lysozyme mutants, these antigens may occur on both singly and doubly glycosylated mutants. The 25kDa band suggests that singly glycosylated forms are unlikely, as they would have a lower molecular weight. However, due to the faint band intensity, we cannot completely rule out a weaker band corresponding to singly glycosylated forms. A molecular weight of 25kDa is characteristic of doubly glycosylated forms. In a study from 2000, Melcher et al. demonstrated via MALDI-TOF that, in hLysII/IVT from CHO wild-type cells, glycosylation site IV is predominantly used in doubly glycosylated forms. Through interaction with the adjacent glycosylation site II, these forms can produce triantennary complex structures with a variable number of lactosamine repeats [10].

Inhibition of U937 adhesion to HUVEC cells by hLysII/IV-FUCTVI in a flow adhesion assay

In the flow adhesion assay, U937 cells adhesion to HUVEC cells was inhibited by 68.5% with VCAM and by 83% with hLysII/IV-FUCTVI. Notably, inhibition was significantly more effective with flow-cultured HUVEC cells than with static-cultured cells. Since the experiment using the ibidi flow system was conducted only once, these findings should be interpreted with caution and confirmed through further replications. If consistent results are obtained, the IC_{50} (half maximal inhibitory concentration) should also be determined by testing various concentrations of hLysII/IVT-FUCTVI. Establishing the IC_{50} will help define the effective dose for achieving the desired inhibitory effect. Multiple trials and thorough validation are essential to ensure the reliability and robustness of these findings.

Future Directions

If the effectiveness of hLysII/IVT-FucTVI in blocking adhesion is confirmed in subsequent studies, several applications could be explored. Firstly, hLysII/IVT-FucTVI could be administered parenterally in mouse models as a potential therapeutic for inflammatory conditions (e.g., chronic inflammatory bowel diseases) or as a prophylactic treatment to prevent tumor metastasis. Given the previously reported anti-inflammatory and anti-tumor effects of lysozyme (reviewed in [31]), these properties could provide additional benefits in the treatment of inflammation or tumors.

Another potential application involves incorporating lysozyme into liposomes. Previous studies have demonstrated the use of conventional lysozyme in liposomes, which successfully targeted the bacterial cell wall (the enzymatic target of lysozyme) and released gentamicin from within the liposomes. In another study, sLe^x molecules were incorporated into the surface of liposomes, enabling targeted delivery to tumor vasculature and the release of tumor-toxic substances [32]. Therefore, it is conceivable to combine these strategies by incorporating sLe^x-modified hLysII/IVT-FUCVI into liposome surfaces, which would allow for targeting delivery to tumors or inflammatory sites, followed by the release of tumor-toxic or anti-inflammatory agents at those locations. Further research is required to thoroughly investigate these potential applications.

Conclusion

In summary, hLysII/IV was successfully overexpressed in CHO-FUCTIII-VII cells, resulting in the production of hLysII/IV-FUCTIII-VII. Digestion analysis confirmed the terminal sialylation and fucosylation of lactosamine repeats in hLysII/IV-FUCTVI, facilitating the expression of sLe^x-antigens. In a static adhesion assay, hLysII/IV-FUCTVI efficiently blocked U937 cells adhesion to activated HUVEC cells. The observed inhibition was comparable to or exceeded the most potent inhibitors reported in previous studies (IC_{50} 10pM). Western-Blotting confirmed the expression of sLe^x, likely located on the terminal lactosamin repeats of tetraantennery complex glycoconjugates attached to glycosylation site IV of hLysII/IVT-FUCTVI. While binding inhibition was limited by 30% in the static adhesion assay, it was significantly enhanced to 83% in a proof-of-principle experiment using flow-cultured HUVEC cells (IBIDI pump system).

Acknowledgments

We thank Professor Vestweber, University of Münster, for kindly providing the CHO-FucTIII-VIII cell lines.

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