Angiogenesis (2010) 13:25–42
DOI 10.1007/s10456-010-9162-4

ORIGINAL PAPER

Transcriptional regulation of the vascular endothelial glycome by angiogenic and inflammatory signalling

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Received: 21 September 2009 / Accepted: 15 January 2010 / Published online: 17 February 2010

Abstract Vascular endothelial cells undergo many molecular changes during pathological processes such as inflammation and tumour development. Tumours such as malignant lymphomas affecting bone marrow are dependent on interactions with endothelial cells for (1) site-specific homing and (2) tumour-induced angiogenesis. Modifications in glycosylation are responsible for fine-tuning of distinct endothelial surface receptors. In order to gain a comprehensive insight into the regulation of the endothelial glycome, comprising genes encoding for sugar transporters (sugar s/t), glycosyltransferases (GT), glycan-degrading enzymes (GD) and lectins (GBP), we performed gene profiling analysis of the human bone marrow–derived microvascular endothelial cell line HBMEC-60 that resembles closely in its biological behaviour primary bone marrow endothelial cells. HBMEC were activated by either angiogenic VEGF or the inflammatory cytokine TNF. Approximately 48% (207 genes) of the 432 glycome genes tested were found to be expressed in HBMEC-60 cells. Inflammatory and angiogenic signals produce different profiles of up- or down-regulated glycome genes, most prominent changes were seen under TNF stimulation in terms of signal intensity and number of alterations. Stimulation by VEGF and TNF affected primarily genes encoding for glycosyltransferases and in particular those important for terminal modulation. For instance, an enhanced α2,6 sialylation was observed after TNF stimulation at the transcriptional and glycan expression level whereas transcription of ST3Gal1 sialylating in α2,3 position was enhanced after VEGF stimulation. Transcriptional analysis of the glycome gives insights into the differential regulation of glycosylation pathways and may help to understand the functional impact of endothelial glycosylation.

Keywords Gene expression profile (GEP) · Glycome · Glycosyltransferases · Transcriptional regulation · TNF · VEGF · Vascular endothelium

Introduction

Cellular glycans expressed on glycoproteins (GP), glycosphingolipids (GSL) and proteoglycans, defined as the glycome, play a crucial role in many biological events such as cell recognition, adhesion, cell–cell interaction and fine-tuning of receptor-mediated signal transduction [1–7]. Several observations underline the importance of endothelial glycosylation for various steps of angiogenesis and the homing of leucocytes to inflammatory tissue: during first stages of angiogenesis VEGF-mediated signalling is dependent on interactions between vascular endothelial growth factor (VEGF) and distinct heparan sulfate sequences on vascular endothelial cells [8]. The blood-histo oligosaccharide group Lewis^y (Le^y, CD174) seems to be necessary for first endothelial cell–cell contacts during angiogenesis [9]. The Notch ligand Jagged 1 is a proangiogenic regulator. The enhanced affinity of the Notch receptor for Jagged 1 over Notch ligand Delta-like 4 is

Electronic supplementary material The online version of this article (doi:10.1007/s10456-010-9162-4) contains supplementary material, which is available to authorized users.

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modified by glycosyltransferases of the Fringe family [10] and possibly also by O-fucosyltransferase which finally contributes to signalling leading to a coordinated endothelial sprouting [11]. Inflammatory signals may have an impact on both angiogenesis and homing of normal and malignant leucocytes to activated vascular endothelium. Activation of human endothelial cells by tumour necrosis factor (TNF) entails major changes in the expression of glycosylation-related genes [12]. First adhesive contacts of leucocytes to activated vascular endothelium during the rolling phase of homing are primarily mediated between selectin E and its ligands of the Lewis^x histo blood group on leucocytes [7]. Synthesis and modifications of oligo- and polysaccharide moieties of protein and lipid carriers are regulated by a complex network of glycosyltransferases (GT), glycan-degrading enzymes (GD) for trimming the growing carbohydrate chains, sugar transporters (sugar s/t), chaperons and those enzymes which add modifications to the oligo- and polysaccharide backbone, such as sulfotransferases (Sulfo-T). While the transcriptome of vascular endothelial cells has been analysed [13–15] few data are available with regard to the transcriptional regulation of genes involved in the endothelial glycosylation process. In a previous study transcription profiles of glycosyltransferases, trimming glycosidases and sulfotransferases responsible for the synthesis and modification of N- and O-linked oligosaccharides have been analysed for human umbilical cord vein endothelial cells (HUVEC) using RT-PCR technology [12]. No comprehensive analysis of glycome GEP (gene expression profiles) was yet available for microvascular endothelial cells under the influence of either angiogenic or inflammatory signalling. We therefore studied genes encoding for enzymes of the glycome and those for lectins (glycan-binding proteins, GBP) using a microvascular endothelial cell type derived from bone marrow [16]. The cells were stimulated in vitro by VEGF or TNF and the effects on glycome transcription were analysed by gene expression profiling. Since inflammatory activation of endothelial cells often accompanies the outgrowth of tumours, these findings may also have implications for tumour-induced angiogenesis. The importance of angiogenesis for the successful outgrowth of tumours and metastasis is not only recognized for solid tumours [17] but also recognized for haematological malignancies including those which affect the bone marrow [18, 19]. During the process of malignancy, both leukaemic blasts and bone marrow microenvironment contribute to neoangiogenesis by secretion of angiogenic factors [20]. Moreover, specific contacts to bone marrow endothelial cells facilitate homing of multiple myeloma cells to this organ [21]. In order to study reactions of endothelial cells to specific cytokine signalling which likely may occur during invasion of tumours to the bone marrow we chose for our study cell line HBMEC-60 which resembles in its biological behaviour primary endothelial cells of the bone marrow, as for instance by increased receptor expression after VEGF stimulation [16].

Materials and methods

Cell culture

HBMEC-60 (retrovirally immortalized human bone marrow endothelial cells) [9, 16] were cultured in endothelial-specific culture medium using endothelial cell basal medium (PromoCell, Heidelberg, Germany) supplemented with fetal calf serum (FCS) (Biochrom, Berlin, Germany) 20%; hydrocortisone, 1 μg/ml; hEGF (human epidermal growth factor), 0.1 ng/ml and hbFGF (fibroblast growth factor), 1 ng/ml as recommended by the manufacturer. Prior to stimulation cells were cultured for 12 h in starvation medium (endothelial cell basal medium supplemented with 5% FCS). For stimulation with recombinant TNF (PromoCell) 40 and 10 ng/ml VEGF (BD, Heidelberg, Germany) were used. HBMEC-60 with and without stimulation were harvested after 6, 12, 24 and 48 h. For specific experiments, also HUVEC (human umbilical cord vein endothelial cells), supplied by PromoCell, were used. HUVEC were cultivated under conditions as described for HBMEC-60.

Flow cytometry

Human endothelial cells (1×10^5 cells/sample) as indicated for the respective experiments were incubated with primary antibodies as listed below on ice for 30 min. Cells were washed once in phosphate-buffered saline (PBS) and incubated with goat anti-mouse conjugated to FITC (Dianova, Hamburg, Germany) for 30 min on ice. Flow cytometry was performed on an FACSCANTO II (BD). The following primary monoclonal antibodies or lectins, coupled to fluorescent dyes or non-coupled, were used in this study: CD31-FITC (Invitrogen, Karlsruhe, Germany); CD51-APC (BD); CD54-APC (BD); CD62E-PE (Biolegend, Uithoorn, The Netherlands); CD105-APC (Caltag Laboratories, Buckingham, UK); CD141 (BD); CD144-Biotin (R&D Systems (Minneapolis, MN). Anti-glycan antibody against CD75s (HH2) was kindly donated by Dr. S. Funderud, Radium Hospital, Oslo, Norway and CD15 antibody supplied by BD. Both monoclonal antibodies are of IgM isotype; a mouse IgM antibody coupled to FITC (Chemicon CBL, Schwalbach, Germany) was used as isotype control. Lectins MAA (Mackia amurensis agglutinin) and SNA (Sambucus nigra agglutinin) coupled to biotin were supplied by Linaris (Wertheim, Germany). The monoclonal antibody STG against human ST6Gal1 was

produced by the authors and described earlier [22]. Production of CD22Rg and the staining procedure for CD22Rg was performed as described [22]. As secondary reagents, goat anti-mouse IgG + IgM coupled to FITC and streptavidin coupled to FITC (both Dianova) were used.

For exclusion of dead cells, Viaprobe© (BD) was added to the cell preparations shortly before cytometric measurement according to the manufacturer's instructions.

For measuring possible apoptotic effects of TNF treatment, HBMEC-60 cells were harvested at times indicated, washed two times with ice-cold PBS, and then 1×10^6 cells/sample resuspended in binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin V-PE (BD) (3.5 µl) together with 5 µl Viaprobe was added to each sample and incubated for 15 min in the dark. The cell suspension was supplemented with 200 µl binding buffer and analysed in flow cytometry as described earlier. As positive control for dead cells, samples were treated with 3% formaldehyde. Apoptotic cells were quantitatively assessed by measuring annexin V positive cells and dead cells by Viaprobe.

Microscopic immunofluorescence analysis

HBMEC-60 cells were incubated on chamber slides (Nalge-Nunc, Rochester, NY) which were precoated with fibronectin (10 µg/ml in PBS) for 1 h at 37°C, and treated with and without TNF as described earlier. Cells on slides were washed in PBS for 5 min at room temperature (RT), fixed with 4% paraformaldehyde (PFA) for 10 min at RT, washed again in PBS, treated with 0.2% saponin in PBS for 5 min at RT and washed again with PBS. Thereafter, cells were incubated with first antibody/lectin for 30 min at RT, followed by extensive washing, incubated with second reagent for 30 min at RT (goat anti-mouse IgG coupled to Cy3 or streptavidin coupled to Cy3) in the dark followed by washing in PBS. Cells were then incubated with Hoechst 33342 DNA dye (Invitrogen) for 10 min, washed and the slides finally covered with fluorescence mounting medium (Dako, Hamburg, Germany). Slides were analysed using a Keyence fluorescence microscope Biozero BZ (Neu-Isenburg, Germany).

Matrigel in vitro angiogenesis assay

Plates (48 wells) were coated with matrigel (150 µl/well; BD Biosciences) on ice as previously described and according to the manufacturer's recommendations. Before the onset of the experiment (48 h) HBMEC-60 cells (4×10^4 cells) were placed into complete ECGM containing 40 ng/ml TNF-α or 10 ng/ml VEGF. Subsequently cells were placed into matrigel coated wells in triplicates or quadruplicates. Incubation of cells on matrigel was performed for 17 h and then at least three representative images of the centre of each well were captured using an Axiomat 25 microscope (objectives 5×, and 10×) and an AxioCam MRC camera (Zeiss, Wetzlar, Germany). Each experiment was repeated at least three times.

RNA isolation

Cellular RNA was isolated using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland) according to the manufacturer's instruction. RNA was eluted in DEPC-treated water. The quality of total RNA was checked by gel analysis using the total RNA Nano chip assay on an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Berlin, Germany). Only samples with RNA index values greater than 8.5 were selected for expression profiling. RNA concentrations were determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

cDNA synthesis

One microgram of total RNA was Oligo(dT) primed and first-strand cDNA synthesis was performed according to the manufacturer's guidelines (Super Script™ First-Strand Synthesis System for RT-PCR, Invitrogen, Karlsruhe, Germany).

Real-time PCR

For quantification of glycosyltransferase mRNA expression, cDNA samples were analysed by real-time quantitative PCR. A total of 2.5 µl cDNA was amplified in 25 µl of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in the presence of 900 nmol of the specific primers for: ST3Gal1, ST6Gal1, B4GalT1, FUT4, FX and SELE as described (Garcia-Vallejo [12]) using the 7300 Real-Time PCR system (Applied Biosystems). Oligonucleotide specificity, synthesized by MWG Biotech, Ebersberg, Germany, was computer-tested (BLAST, NCBI) by homology search with the human genome. Samples were run in triplicate and experiments were repeated twice. The thermal profile for the reaction was 2 min at 50°C, followed by 10 min at 95°C and then 40 cycles of 15 s at 95°C and 1 min at 60°C. In order to exclude non-specific amplification dissociation curve analysis and agarose gel electrophoresis of the PCR products were performed at the end of the run. The endogenous reference gene GAPDH was chosen for normalization. Relative gene expression was calculated using the comparative C_t method [23].

Microarray analysis of using Illumina human Sentrix 6V2

cRNA labelling, hybridisation and scanning of the Illumina human Sentrix 6V2 chip (San Diego, CA) were performed in the Genomics and Proteomics Core Facility of the German Cancer Research Center according to Illumina's recommended protocols. The Sentrix 6V2 bead chip covers an expression level of 48,600 human transcripts, variants, and EST clusters.

Probe labelling and Illumina Sentrix BeadChip array hybridization

Biotin-labelled cRNA samples for hybridization on Illumina Human Sentrix 6V2 BeadChip arrays were prepared according to Illumina's recommended sample labelling procedure based on the modified Eberwine protocol [24]. In brief, 250 ng total RNA was used for complementary DNA (cDNA) synthesis, followed by an amplification/labelling step (in vitro transcription) to synthesize biotin-labelled cRNA according to the MessageAmp II aRNA Amplification kit (Ambion, Austin, TX). Biotin-16-UTP was purchased from Roche Applied Science, Penzberg, Germany. The cRNA was column purified by TotalPrep RNA Amplification Kit and eluted in 60 µl of water. Quality of cRNA was controlled using the RNA Nano Chip Assay on an Agilent 2100 Bioanalyzer and spectrophotometrically quantified (NanoDrop).

Hybridization was performed at 58°C, in GEX-HCB buffer (Illumina) at a concentration of 50 ng cRNA/µl, unsealed in a wet chamber for 20 h. Spike-in controls for low, medium and highly abundant RNAs were added, as well as mismatch control and biotinylation control oligonucleotides. Microarrays were washed twice in E1BC buffer (Illumina) at RT for 5 min. After blocking for 5 min in 4 ml of 1% (wt/vol) Blocker Casein in phosphate buffered saline Hammarsten grade (Pierce Biotechnology, Rockford, IL), array signals were developed by a 10 min incubation in 2 ml of 1 µg/ml Cy3-streptavidin (Amersham Biosciences, Buckinghamshire, UK) solution and 1% blocking solution. After a final wash in E1BC, the arrays were dried and scanned.

Scanning and data analysis

Microarray scanning was done using a Beadstation array scanner, setting adjusted to a scaling factor of 1 and PMT settings at 430 nm. Data extraction was done for all beads individually, and outliers were removed when >2.5 median absolute deviation (MAD). All remaining data points are used for the calculation of the mean average signal for a given probe and standard deviation for each probe was calculated. Genes were regarded as expressed with a mean average signal >20.

Data analysis was done by normalization of the signals using the cubic spline/quantile algorithm after background subtraction, and differentially regulated genes are defined by calculating the standard deviation differences of a given probe in a one-on-one comparison of samples or groups. Genes were considered arbitrarily as regulated if gene expression of stimulated cells exceeded the respective expression of non-treated cells by ±1.4.

Results

Effects of TNF and VEGF stimulation on expression of endothelial surface receptors and angiogenesis

In order to investigate whether stimulation of HBMEC-60 cells by either VEGF or TNF has effects on surface expression of endothelial marker glycoproteins, we performed flow cytometric analysis using specific monoclonal antibodies (Table 1). Significantly increased surface expression after TNF treatment was observed with ICAM-1 (CD54) and HLA class I, to a lesser extent with thrombomodulin (CD141). These data are in confirmation with described inflammatory effects of TNF on activated endothelial cells [25, 26]. After VEGF stimulation, only CD141 expression was increased. Flow cytometric data are shown for an activation time of 24 h. It is known that strong selectin E (CD62E) expression is a transient state most obvious after short periods of incubation, e.g. 4 h [27], with a decline in expression thereafter. This kinetic of CD62E expression was confirmed with HBMEC-60 cells.

Table 1 Expression of endothelial surface markers (flow cytometric analysis)

Antigen	CD-no.	Mean fluorescence intensity		
		Non-treated	VEGF-treated	TNF-treated
PECAM-1	CD31	260	278	254
ICAM-1	CD54	681	222	3,687
Selectin E[a]	CD62 E	11	33	157
Endoglin	CD105	7,807	5,333	4,696
Thrombomodulin	CD141	55	629	146
VE-cadherin[a]	CD144	69	45	223
	HLA class I	182	189	1,821

[a] Surface markers were analysed after 24 h of incubation with and without VEGF/TNF stimulation, as an exception selectin E was measured after 4 h and VE-cadherin after 13 h of incubation because after 24 h expression of these antigens was already significantly decreased

Also, VE-cadherin (CD144) showed a maximal expression after 13 h of incubation with TNF.

In a next step, we analysed the effects of TNF and VEGF on angiogenesis in a standardized in vitro matrigel tubing assay as described in "Materials and methods" (Fig. 1). Differences in numbers of nodal points, outlets per nodal points and overall length of differentially developed tubes were quantitatively assessed using a software program (S.Core lifescan, Garching, Germany). After TNF stimulation, the number of nodal points and length of well-developed tubes was reduced, and after VEGF treatment, the opposite effect was seen.

Since intracellular signalling induced by TNF stimulation can either lead to activation via the NF-κB pathway or lead to apoptosis via complex II formation [28], we also looked for possible induction of apoptosis after 24 h TNF stimulation in HBMEC-60 cells. The cells were monitored for apoptotic events in flow cytometry by measuring annexin V binding on apoptotic cells versus DNA intercalation of propidium iodide in dead cells. During this time of incubation, no significant death or apoptosis was observed in TNF-treated and non-treated HBMEC-60 cells (data not shown). Gene expression data showed an increased transcriptional level of NF-κB1A after TNF treatment (non-treated cells: 883, VEGF-treated cells: 2,157, TNF-treated cells: 4,639). In contrast, caspase 8 and 9 transcriptions were at the detection limit. Thus, we conclude that in our in vitro assays TNF had at least during 24 h incubation an activating rather than an apoptotic effect on HBMEC-60 cells.

Effects of TNF and VEGF stimulation on transcription of endothelial marker glycoproteins

In order to examine the significance of our gene expression data obtained by Illumina array technology, we also looked for transcription levels of typical endothelial markers (Table 2). As expected, transcription for CD54 and Endoglin (CD105) increased considerably after TNF treatment. PECAM-1 (CD31) transcription was reduced and that of VCAM-1 (CD106) was induced after TNF treatment. Transcription of CD144 was enhanced after stimulation with both TNF and VEGF. In contrast to the timely restricted surface expression of CD62E, its transcription was significantly enhanced after 12 h when surface expression had already decreased. Transcription of CD141 was increased after VEGF and decreased after TNF stimulation.

Dynamics of gene expression

First, we were interested in the assessment of an appropriate time point at which major gene regulation occurs after stimulation with VEGF and TNF and which can be used for further GEP analysis. For this purpose, we selected four genes covering different stages of glycan biosynthesis. Gene expression kinetics of FX (involved in fucose metabolism), B4GALT1 (involved in the initiation of neolacto-series and keratan sulfate as well as in elongation

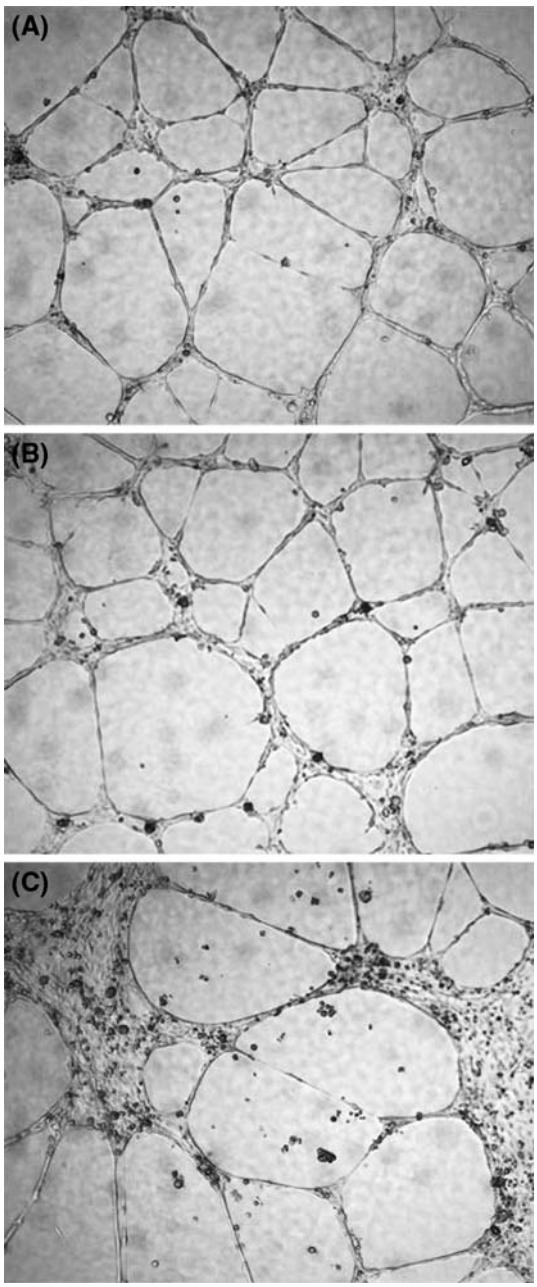

Fig. 1 Tube formation of HBMEC-60 in matrigel angiogenesis assay. **a** Non-treated cells, **b** VEGF-treated cells, **c** TNF-treated cells. Magnification ×50. Different parameters measured are expressed in numbers of pixel. Significant changes were quantitatively observed in the following parameters: branching (outlet) points **a**:315, **b**:342, **c**:216; nodal structures: **a**:237, **b**:251, **c**:162; total length of well-developed tubes: **a**:2066, **b**:2658, **c**:1462

Table 2 Transcription of endothelial surface glycoproteins in HBMEC-60 cells with and without TNF/VEGF treatment

Endothelial markers	Non-treated	VEGF-treated	TNF-treated
PECAM-1 (CD31)	1,478[a]	1,703	761
ICAM-1 (CD54)	50	120	2,199
Selectin E (CD62E)	–[b]	–[b]	1,330
Endoglin (CD105)	3,684	3,496	5,246
VCAM-1 (CD106)	–[b]	–[b]	177
Thrombomodulin (CD141)	176	377	–[b]
VE-cadherin (CD144)	2,837	4,201	4,109
Von Willebrand factor (VWF)	4,708	4,244	2,942

[a] Gene expression evaluated by microarray analysis

[b] Below detection limit

of N-linked glycans) and ST6Gal1 and ST3Gal1 (responsible for terminal sialylation of GP/GSL) were analysed by real-time PCR after 6, 12, 24 and 48 h of incubation (Fig. 2). Stimulation of 12 h with either cytokine turned out to be a representative time point at which major modulations of glycome gene expression occured. After VEGF stimulation, all four genes analysed displayed enhanced expression with B4GALT1 having a further expression peak after 48 h after VEGF stimulation. After TNF stimulation, major expression peaks were detected at 12 h incubation for ST6Gal1, B4GALT1 and FX, with the exception of ST3Gal1. Based on these results gene expression profiling using microarray technology was performed with RNA of HBMEC-60 harvested after 12 h of stimulation with both cytokines.

Glycan-specific gene expression of non-stimulated bone marrow endothelial cells

Microarray analysis was performed on Sentrix 6 BeadChip covering an expression level of 48,600 human transcripts, variants and EST clusters. Of these, a total of 10,560 genes were expressed in HBMEC-60. Here, we focus on a set of glycosylation relevant enzymes and proteins that were classified into different gene groups: glycosyltransferases (GT), Sulfo-T, glycan-degrading enzymes (GD), genes necessary for sugar synthesis and transport (sugar s/t) and glycan-binding proteins (GBP). The gene families of each enzyme group analysed are listed in Table 3 (see detailed list of glycome genes analysed and list of glycome gene groups in "Electronic supplementary material"). The classification and nomenclature was adapted from the Glycov4 chip of the consortium for functional glycomics (CFG) [29]. From the CFG selection of genes, we excluded those which are not directly associated to the glycosylation

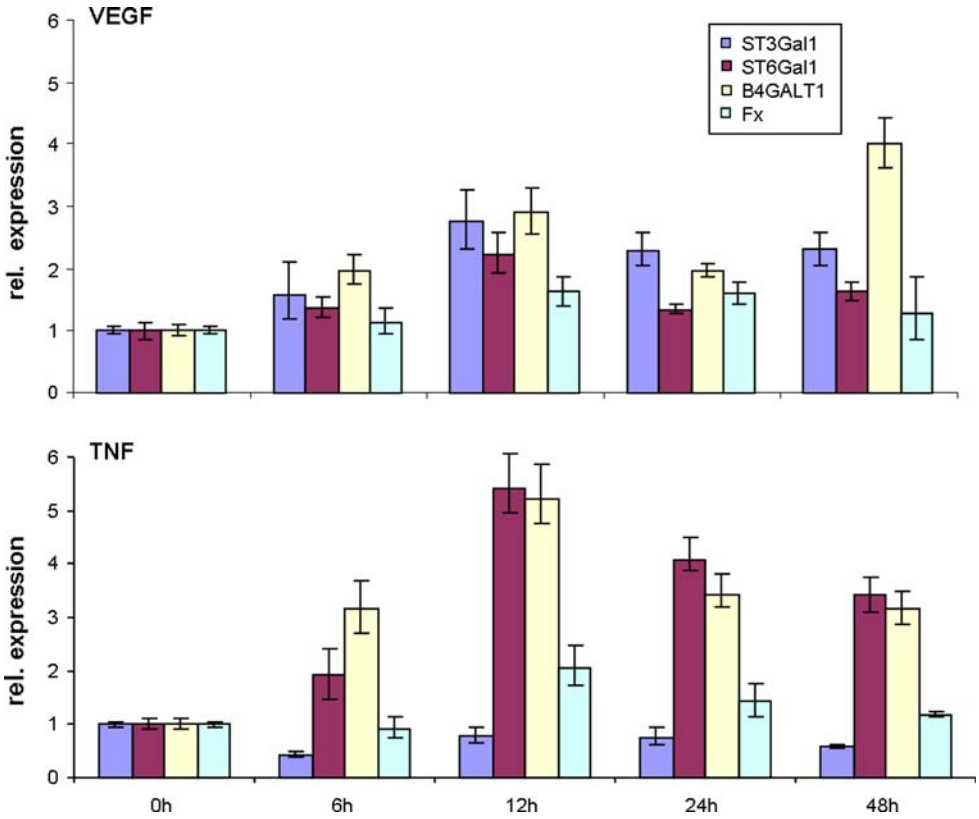

Fig. 2 Kinetic of gene expression of TNF and VEGF–stimulated HBMEC-60 (RT-PCR). HBMEC-60 were grown to confluency under standard conditions and were then incubated in the presence TNF or VEGF. Cells were harvested after different time points 0, 6, 12, 24 and 48 h and the mRNA expression levels for four genes FX, B4GALT1, ST6Gal1 and ST3Gal1, covering different stages of glycan biosynthesis were determined by real-time PCR as described in "Material and methods"

Table 3 Distribution of the analysed glycome relevant gene families

	Gene families	No. of analysed genes	No. of expressed genes
GT	Fucosyltransferases	13	3
	Sialyltransferases	21	10
	Sulfotransferases	36	12
	Galactosyltransferases	21	13
	GalNAc-transferases	25	5
	Glucosyltransferases	10	7
	GlucNAc-transferases	37	16
	GlucUA-transferases	10	2
	Mannosyltransferases	13	12
	HS GlcNAc/GlA transferase	2	2
	Hyaluronansynthases	4	1
	N-glycantransferases	4	4
	Xylosyltransferases	2	1
GD	Arylsulfatases	9	4
	Fucosidases	1	1
	Galactosidases		3
	Heparanases	3	1
	Hexosaminidases	2	2
	Hyaluronoglucosaminidases	6	4
	Iduronidases	1	1
	Lysosomal enzymes/proteins	19	14
	Mannosidases	10	7
	Sialidases	4	1
	Sulfatases	6	5
	Sulfohydrolases	1	1
GBP	C-type lectins	77	20
	I-type lectins	18	5
	Galectins	13	3
Sugar s/t	Nucleotide sugar transporters	23	16
	Nucleotide synthesis	34	28
Total		432	207

process and to glycan-binding proteins as for instance those which encode for cyto- or chemokines.

Glycan-specific gene expression of non-stimulated HBMEC-60 revealed that 207 of 432 genes analysed were detected as being present. Table 3 shows their distribution among the respective groups. Table 4 depicts glycome genes with an expression level >1,000.

Gene expression of non-stimulated HBMEC-60 was ranked and clustered according to their expression level in I. low, 10–40 (≤quantile P25), II. intermediate 59–441 and III. highly expressed 584–13313 (≥quantile P75). The corresponding levels of gene expression after VEGF and TNF stimulation are displayed in Fig. 3. Apparently the number of up- and down-regulated genes was much higher after TNF when compared to VEGF stimulation.

The proportional distribution pattern of the gene groups differed within the three expression levels. Genes of non-treated HBMEC-60 detected within the high expression cluster comprise genes involved in the synthesis of N-linked core sequences ubiquitously expressed in eukaryotic cells. For example, the glycosyltransferases DDOST, DAD1 and RPN1 detected in this group are members of the oligosaccharyltransferase complex (OST) which links high mannose oligosaccharides to asparagine residues. DPM3 functions as a stabilizer subunit of the dolichol–phosphate–mannosyltransferase complex essential for surface expression of GPI anchored proteins. Other highly expressed genes are involved in the first steps of chondroitin sulfate (XYLT2, ß4GALT7) and heparan sulfate (EXT1) biosynthesis. Enzymes involved in glycan degradation (GD), the second group of highly expressed enzymes of the glycome, comprise HexB (hexosaminidase B), AGAL (galactosaminidase). LAMP2 (CD107B; lysosomal associated membrane protein 2) also belongs to this group. In the group of GBP, PECAM-1, ICAM-2 and galectin-1 (LGALS1) displayed high expression levels (Table 4).

VEGF and TNF–induced shifts in glycan-specific gene regulation

Comparison of glycan-specific gene regulation induced by VEGF and TNF revealed differences with respect to the number of genes regulated by the respective cytokine and of an at least 1.4-fold down- or up-regulation of gene expression (Table 5). As can be seen, most changes occur in the GT group after both VEGF and TNF stimulation.

Distribution of glycosyltransferases involved in initiation, elongation and termination

GT distribution among the different expression levels (P75-P25) was further analysed according to their biosynthetic function, i.e. initiation (GT-I), elongation (GT-E) or termination (GT-T) of oligosaccharide chains (Fig. 4). The majority of GT ($n = 11$–12) within P75 in non-treated or stimulated HBMEC-60 belongs to GT involved in initiation events such as synthesis of N-linked core sequences. GT-extending carbohydrate structures represent a second group with 5–6 members and only 2–3 members of terminal transferases are represented in this expression cluster. Interestingly, no Sulfo-T were observed in P75. In the intermediate level, the quantitative distribution between non-treated versus treated HBMEC-60 did not display major differences according to quantities of GT-I, GT-E, GT-T and ST. The number of GT-I and GT-E varies from 13 to 15 genes, GT-T from 9 to 12 and ST from 7 to 9. For GT represented in the low expression level (P25), non-treated and VEGF/TNF-stimulated HBMEC-60 have an

Table 4 HBMEC-60 gene expression level >1,000

Category	Pathways	GEN	Non-treated	VEGF-treated	TNF-treated
Sugar s/t		SLC35A5	1204,26	1177,81	1161,48
		PAPSS2	1204,84	1193,50	1072,03
		GFPT2	1497,52	2088,04	3940,94
		GPI	1688,74	1969,92	1226,80
		NAGK	1707,32	1597,31	2207,94
		SLC35B1	2311,12	2378,30	2109,04
		GALK1	3242,10	3342,98	2066,08
		GNPDA1	2847,85	3020,51	2203,66
		GNB1	5600,60	6729,62	5947,30
		GNB2L1	13313,54	12829,66	15910,47
GT	GPI	PIGQ	1099,8	1397,2	990,3
	N-[a]	DPAGT1	1315,67	1356,50	1391,68
	N-	ALG3	1259,9	1486,8	1111,3
	N-	DAD1	7091,44	5376,08	5997,12
	N-	DPM3	2579,08	1980,54	1439,21
	N-	DDOST	3783,40	4133,10	3857,75
	N-	RPN1	2989,41	3844,91	3464,75
	N-	MGAT1	1622,19	2315,76	2217,91
	N-, KS, GSL	B4GALT3	1141,59	1527,99	1101,14
	HS	EXT1	1026,88	1724,37	1532,80
	HS, CS	B4GALT7	1408,99	1705,19	1552,75
GD	N-	MAN1B1	1203,21	1616,07	1419,80
	KS, GSL	HEXB	3878,11	3860,35	3613,54
	HS, KS	GNS	6352,58	6894,21	5488,21
		LAMP1	8661,98	11277,51	9118,58
		LAMP2	1683,97	1692,43	1852,04
		SLC17A5	1804,88	1878,15	973,89
		GLA	1817,81	1852,84	1445,49
		MGEA5	1293,44	1267,66	1264,99
GBP		PECAM1	1478,08	1702,52	761,10
		ICAM2	6541,39	6333,20	3304,82
		LGALS1	12727,93	12623,00	13318,31

[a] N-: N-linked oligosaccharides

identical distribution for GT-I, GT-E and Sulfo-T with the exception of GT-T which are only represented after TNF stimulus.

Highly regulated glycome genes

Regarding the *x*-fold (non-treated HBMEC-60/stimulated HBMEC-60) gene expression, we observed that VEGF stimulation leads to regulation covering an *x*-fold change from −2.4 to 2.4×. Expression levels >500 were seen for 23% of upregulated and none of downregulated genes. After VEGF stimulus, no signs for induction or silencing effects were observed. TNF induced a much stronger effect on gene regulation ranging from −48× to 1,330×. Expression levels >500 were observed for 19% of the decreased and 22.7% of the enhanced genes. In particular, OLR1 (33.2×) galectin-9 (LGALS9) (35.9×), VCAM-1 (38.7×), ICAM-1 (43.8×) and selectin E (1330.4×) were genes with highest induction levels after TNF treatment.

The Sulfo-T GALNAC4S-6ST and HS3ST1 and the GBP THBD were downregulated to an expression level <10 (threshold of reliable detection of gene expression >20).

Predictive effects of VEGF and TNF stimulation on glycoconjugate structures

Glycan biosynthesis is a complex process comprising at least 11 different pathways. We compared gene expression of key enzymes with regard to their known function for

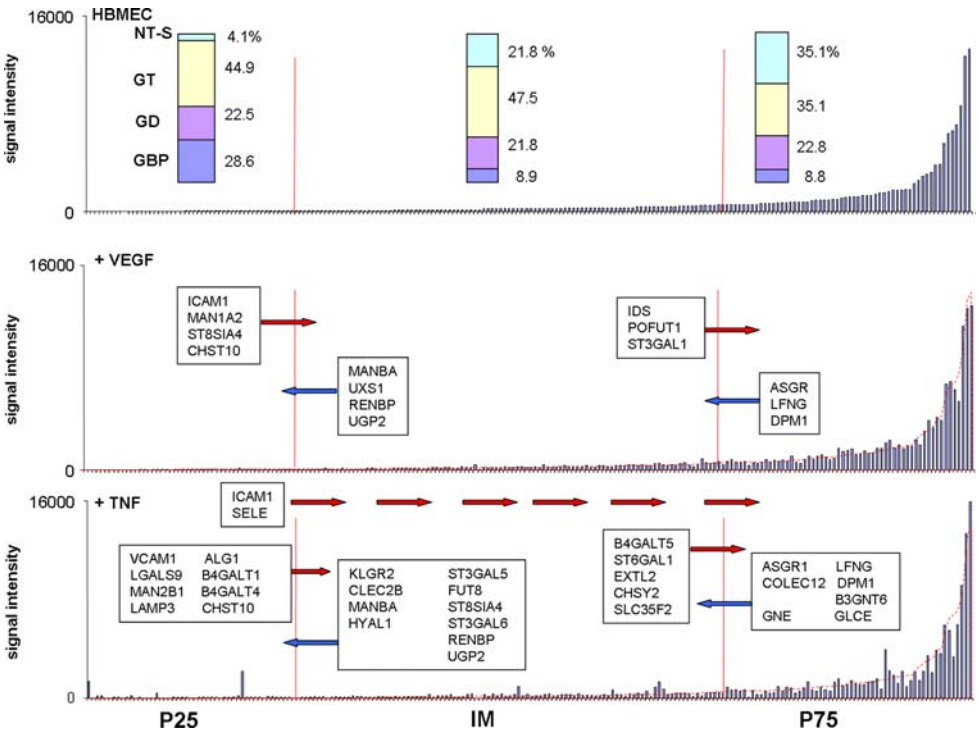

Fig. 3 Ranked expression levels of glycosylation relevant genes in VEGF/TNF-treated versus non-treated HBMEC-60 (GEP). Genes of non-treated HBMEC-60 were ranked in low (P25, 0–25%), intermediate (IM, 25–75%) and high (P75, 75–100%) expression levels. The proportional distribution pattern of the gene groups analysed is shown in *bars* within the profile of non-treated HBMEC-60. In comparison with non-treated HBMEC-60, the expression profiles of ranked genes after 12 h stimulation with either VEGF or TNF (*black arrows*) are shown. *Dotted lines* indicate the profile of non-treated cells in terms of signal intensities. Tendencies of prominent glycome genes (indicated in *boxes*) up- or down-regulated after VEGF or TNF treatment are marked with *arrows*

Table 5 Distribution of genes regulated after stimulation

	TNF ≥ 1.4	%	TNF ≤ −1.4	%	VEGF ≥ 1.4	%	VEGF ≤ −1.4	%
Glycome group								
Nucleotide sugars, sugars/T	8	18	7	17	2	9	2	12
GT[a]	19	44	16	38	14	64	8	47
GD	9	20	8	19	2	9	1	6
GBP	8	18	11	26	4	18	6	35
Total number	44	100	42	100	22	100	17	100

[a] Including Sulfo-T (classification according the Glycov4 chip of the consortium of functional glycomics)

synthesis of N-, O-linked glycans of GP, GSL, and of glycosaminoglycan (GAG) chains of non-treated versus VEGF/TNF-stimulated HBMEC-60. Although surface expression of glycans depends on many factors, the differential transcription levels of genes encoding for GT and GD may give insights into structural changes in the respective glycan species after stimulation. The glycan pathway description provided by the Kyoto Encyclopedia of Genes and Genomes (KEGG database) (www.genome.jp/kegg/pathway.html) [30] was used for evaluation of the gene expression and regulation along the different pathways. In our predictive analysis only genes with an *x*-fold change >1.4/<−1.4 were considered. The affiliation of glycome genes to the respective pathways and their variation in expression after VEGF/TNF stimulation is listed in Table 6.

For two oligosaccharide groups, the neolacto-series and the ganglio-series, the respective glycosyltransferases in relation to their possible GSL products are depicted in Figs. 5 and 6.

N-glycans

With regard to the initiation of N-glycan synthesis, we observed with both stimulation modes a decreased gene expression for enzymes of the first step of N-glycan synthesis. However, it has to be considered that the DPM genes were still highly expressed whereas the ALG14 expression was reduced to the detection limit. In contrast, glycan extension after VEGF treatment displayed transcriptional upregulation of the trimming mannosidase MAN2A1 and the N-acetylglucosamine transferases MGAT1, whereas after TNF stimulation all extension GT including B4GALT1 which completes the lactosaminyl

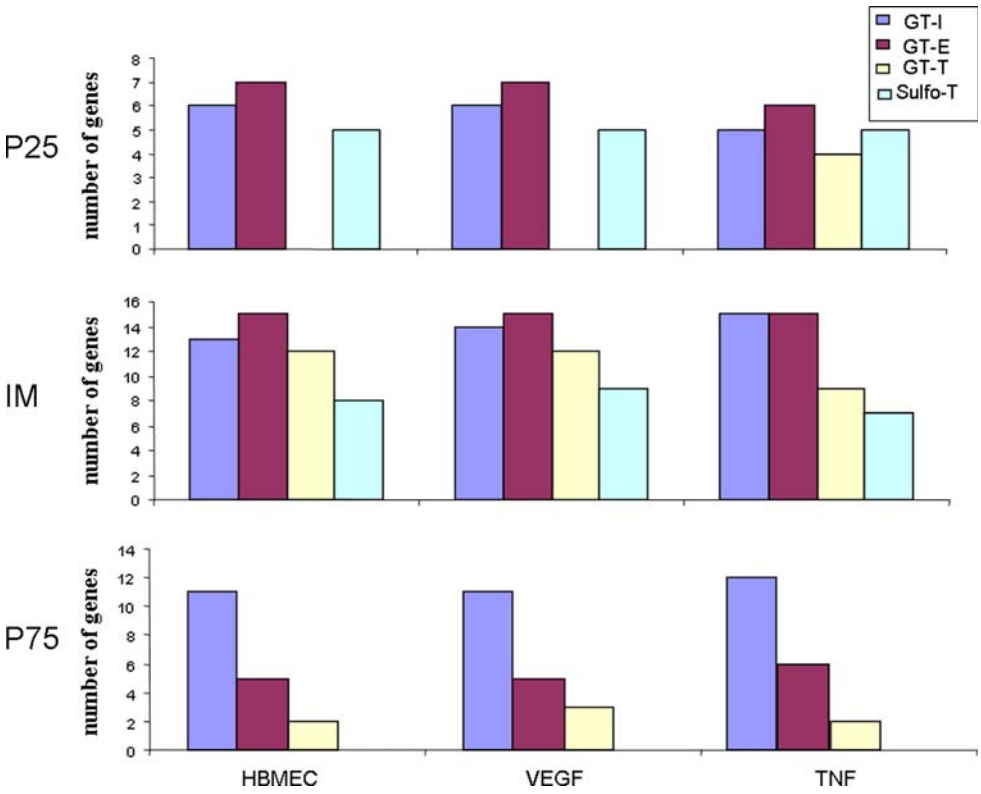

Fig. 4 Distribution of GT according to their biosynthetic function among the expression levels. Distribution of GT and Sulfo-T among the expression levels (P25–IM–P75, cf. Fig. 3) of non-treated and VEGF/TNF-treated HBMEC-60 was analysed after GEP. GT were grouped according to their enzymatic function during initiation (GT-I), elongation (GT-E) and termination (GT-T) of oligosaccharide chains

structures by addition of Gal to GlcNAc were upregulated to various extents. Strikingly, there seems to be a difference in terms of terminal sialylation between VEGF and TNF stimulation: after VEGF treatment, transcription of the gene encoding for α2,3 sialylation is much higher than ST6Gal1 responsible for α2,6 sialylation whereas after TNF treatment the reverse situation is observed.

O-glycans

Transcription of GALNT2 and GALNT6, involved in the first steps of O-glycan synthesis, were upregulated by VEGF treatment. GCNT1, responsible for core 2 and 6 structures, was downregulated after VEGF treatment. This decreased gene expression may also influence the keratan sulfate biosynthesis for which core 2 structures serve as initial substrates. B3GNT6, responsible for core 3 structures, was downregulated by TNF treatment. B4GALT1 and 5, involved in the synthesis for type 2 chains in N- and O-linked glycans, increased 3.9× and 2×, respectively, after TNF treatment. Genes for B4GALT5 also transfers Gal to core 6 structures necessary to synthesize F1α of mucins. TNF caused an increase in ST3Gal2 transcription and at the same time a decrease of ST3Gal1. The gene for Gal3ST4, involved in transferring a sulfate group to the 3′ position of galactose in Gal-ß1,3-GalNAc, i.e. to core 1 structures, was overexpressed after TNF treatment.

GAG, chondroitin sulfate (CS)

Both stimulations enhanced CSS3 and CSGlcA-T gene expression, involved in the synthesis of the repeating units CS (GalNACβ1–4–GlcAβ1–3). Sulfo-T GalNAc4S-6ST and CHST11 displayed decreased gene expression after VEGF treatment. After TNF stimulation, transcription of enzymes for sulfation, CHST12 was 2.9× downregulated and GalNAc4S-6ST switched off. The gene for SART2, responsible for dermatan sulfate synthesis by epimerizing GlcA to α-IdoA, was upregulated.

GAG, heparan sulfate (HS)

After VEGF stimulation, EXTL2, an enzyme responsible for HS elongation, was upregulated in its transcription, TNF stimulation entailed enhanced gene expression of both EXTL2 and EXT1. Downstream gene expression of NDST2, which completes and modifies the HS sugar backbone by deacetylation and N-sulfation was upregulated by VEGF and the respective isoenzyme NDST1 upregulated by TNF. In contrast to the VEGF regulation, TNF reduced transcription of HS3ST1, responsible for 3–0 sulfation.

Table 6 Expression of glycome genes along the pathways

Pathway	Enzyme	HBMEC gene expression	+VEGF *x*-fold change	+TNF *x*-fold change	Function	Glycosidic linkage	Structures	Involved in other pathways
N-linked glycosylation								
Ko00510	ALG13	541	−1.3	−1.4	Initiation	GlcNAc β1–4 GlcNAc	PP-Dol anchor	
	ALG14	58	−1.4	−2.0				
	ALG1	50.4	1.2	1.6		Man β1–4 GlcNAc		
	DPM1	768	−1.6	−1.5		Man β1-P-Dol		
	DPM3	2,579	−1.3	−1.8				
	MGAT1	1,622	1.4	1.4	Extension	GlcNAc β1–2 Man	Oligo- > complex glycans	
	MAN2A1	756	1.4	1.1	Trimming	Man α1–6 Man	High- > complex	
	MGAT2	142	−1.2	1.5		GlcNAc β1–2 Man	Complex/hybrid glycans	
	FUT 8	86	1.2	−1.9	Terminal	Fuc α1–6 GlcNAc	Core fucosylation	Keratan sulfate
	B4GALT1	49	1.0	3.9	Extension	Gal β1–4 GlcNAc	LacNAc	Keratan sulfate, O-GSL
	ST6Gal1	352	1.4	2.5	Terminal	Neu5Ac α2–6 Gal	sLacNAc	
	ST3Gal1	477	1.9	−2.0		Neu5Ac α2–3 Gal		O-, GSL, KS
	MGAT4A	81.6	1.0	1.4	Extension	GlcNAc β1–4 Man	Tri/multiantennary	
	MGAT4B	12.2	−1.3	3.45				
O-linked glycosylation								
Ko00512	GALNT2	24.5	1.4	−1.3	Initiation	GalNAc α1-Ser/Thr	Tn Antigen	
	GALNT6	43	1.5	1.4				
	GCNT1	135	−1.6	−1	Elongation	GlcNAc β1–6 GalNAc	core2, core6	Keratan sulfate, N
	ST3Gal1	477	1.9	−2.0	Terminal	Neu5Ac α2–3 Gal	sTF-antigen	GSL (Ganglio-)
	ST3Gal2	225	1.2	1.4				
	B3GNT6	612	−1.1	−2.3		GlcNAc β1–3 GalNAc	core3	
	B4GALT1	49	1.0	3.9	Elongation termination	Gal β1–4 GlcNAc	Type2 chain	Keratan sulfate, N-, GSL
	B4GALT5	366	1.2	2.0			FLα	N-linked
	Gal3ST4	76	−1.1	1.6	Sulfation	S-3 Gal	Gal-3-O-sulfation	
GAG								
Chondroitin sulfate								
Ko00532	CSS3	204	1.4	4.6	Elongation	GlcA β1–3 GalNAc GalNAc β1–4 GlcA	CS repeating units	
	CSGlcA-T	220	1.8	1.4		GlcA β1–3 GalNAc	CS repeating units	
	GalNAc4S-6ST	48.2	−2.0	−48.2	Sulfation	S-6 GalNAc	CS-E	
	CHST11	27	−1.4	1.1		S-4 GalNAc	CS-A,E,B,D	Dermatan sulfate
	CHST12	347	1.0	−2.9				
Dermatan sulfate								
	SART2	327	1.1	2.5	Epimerization of GlcA to α-IdoAL	Chondroitin-glucuronate 5-epimerase	DS	

Table 6 continued

Pathway	Enzyme	HBMEC gene expression	+VEGF *x*-fold change	+TNF *x*-fold change	Function	Glycosidic linkage	Structures	Involved in other pathways
Heparan sulfate								
Ko00534	EXTL2	362	1.3	3.5	Elongation	GlcNAc α1–4 GlcA	HS repeating units	
	EXT1	1,026	1.7	1.5		GlcA β1–4 GlcNAc		
	NDST1	68	1.2	2.3	Sulfation	S-2 GlcN (N-deacetylase/ N-sulfotransferase)	Sulfated HS repeating units	
	NDST2	26	1.8	1.0				
	HS3ST1	37	1.6	−5.9		S-3 GlcN	HS sulfation	
Keratan sulfate								
Ko00533	B4GALT1	49	1.0	3.9	Elongation	Gal β1–4 GlcNAc	KSII repeating units	O-, GSL
	B4GALT4	55	1.1	2.0		GlcNAc β1–3 Gal	KSI, KSII repeating units KSI, KSII repeating units	GSL
	B3GNT1/2	11.4	1.1	2.8				
	ST3Gal1	477	1.9	−2.0		Neu5Ac α2–3 Gal	sKSII	O-, N-, GSL
	ST3Gal2	225	1.2	1.4				
	FUT8	86	1.2	−1.9	Fucosylation	Fuc α1–6 GlcNAc	KSI, core fucosylation	N-
GSL								
Ganglio-series								
Ko00604	B3GALT4	57	−1.4	−1.0	Elongation	Gal β1–3 GalNAc	sialoGM1,GM1a, GD1b, GT1c	
	ST3Gal1	477	1.9	−2.0	Terminal	Neu5Ac α2–3 Gal	GT1b	O, N-, KSII
	ST3Gal2	225	1.2	1.4				
	ST3Gal5	77.2	1.4	−2.5			GM3	O-, N-
Neolacto-series								
Ko00602	B4GALT1	49	1.0	3.9	Elongation	GlcNAc β1–3 Gal	Poly LacNAc, i-Antigen	KSI, KSII, O-, N-
	B4GALT4	55	1.1	2.0				KSI, KSII
	B3GNT1/2	11.4	1.1	2.8				
	FUT4	87.8	1.1	2.3		Fuc α1–3 GlcNAc	Le^x, VIM-2 antigen	
	ST3Gal6	139	1.3	−2.1		Neu5Ac α2–3 Gal	Sialyl Le^x	

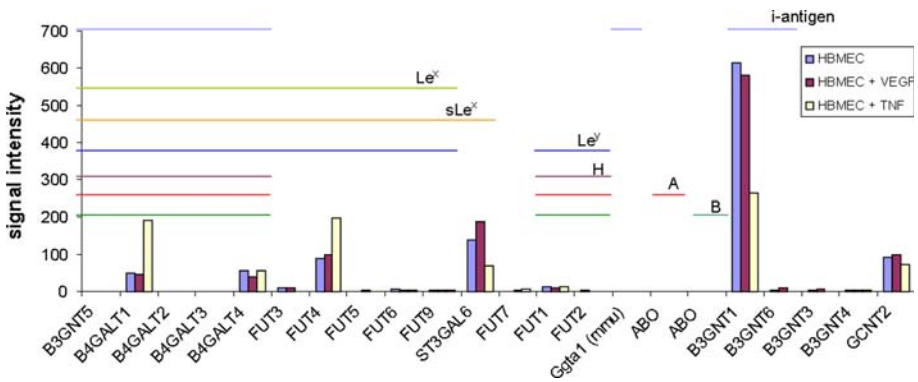

Fig. 5 Gene expression along the neolacto-series pathway. Gene expression along the neolacto-series pathway (according to the KEGG nomenclature) of non-treated and VEGF/TNF-treated HBMEC-60. *Lines* indicated the GT genes necessary for the synthesis of the respective carbohydrate structures

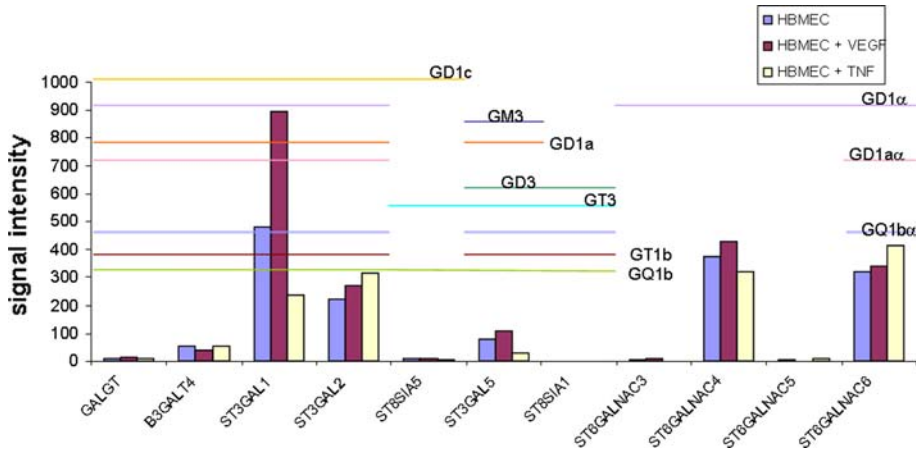

Fig. 6 Gene expression along the ganglio-series pathway. Gene expression along the ganglio-series pathway (according to the KEGG nomenclature) of non-treated and VEGF/TNF-treated HBMEC-60. *Lines* indicated the GT genes necessary to for the synthesis of the respective carbohydrate structures

Table 7 Transcription of representative glycome genes with and without TNF or VEGF treatment (RT-PCR). Comparison between HBMEC-60 cells and HUVEC

Genes	Non-treated HBMEC-60/HUVEC	HBMEC-60 + VEGF	HBMEC-60 +TNF	HUVEC +VEGF	HUVEC +TNF
ST6Gal1	1.0[a]	1.4	4.6	1.4	3.4
ST3Gal1	1.0	2.3	−1.7	1.5	0.7
B4GALT1	1.0	1.1	4.7	1.3	1.9
FUT4	1.0	1.3	2.1	1.4	3.9
SELE[b]	1.0	1.9	1,680	2.0	2,352

[a] Results were shown as *x*-fold gene expression of non-treated HBMEC-60 or HUVEC, respectively

[b] SELE = selectin E (CD62 E)

Keratan sulfate (KS)

Initial substrates for KS synthesis are provided from either N- or O-glycan synthesis, resulting in KSI or KSII structures, respectively.

No sign of apparent gene regulation for KS structures was observed after VEGF treatment.

The set of genes enhanced after TNF stimulation (B3GNT2, B4GALT1, ST3Gal1/2, belongs to the biosynthesis pathway of KSII structures. FUT8, fucosylating KSI structures, was downregulated by TNF.

GSL

We focussed on two of the GSL biosynthesis pathways, the ganglo-series and the neolacto-series. In both pathways, fucosyltransferases and sialyltransferases modify terminal glycan structures.

Ganglio-series

VEGF enhanced and TNF stimulation decreased ST3Gal5 (GM3 synthase) gene expression. HEXB, the GM2-degrading enzyme displaying high gene expression levels was not subjected to regulation. Two sialyltransferases with similar specificity were transcribed after TNF treatment in opposite ways: ST3Gal1 was down- and ST3Gal2 was up-regulated.

Neolacto-series

No regulation pattern for key enzymes of this pathway were observed after VEGF treatment. In contrast, TNF stimulation lead to upregulation of FUT4 transcription, essential for expression of Le^x. ST3Gal6 for synthesis of sialyl Le^x surprisingly was downregulated in its transcription. B3GNT1/2 expression, genes important for the synthesis of poly N-lactosaminyl structures were enhanced after TNF treatment. Together with B4GALT1, these gene products are responsible for synthesis of the I-, and i-antigen.

Comparison of glycome gene expression between HBMEC-60 cells and HUVEC

In order to see whether responses of HBMEC-60 to VEGF and TNF are comparable to those of native endothelial cells with regard to alterations in glycome genes we measured by RT-PCR in a different set of experiments mRNA

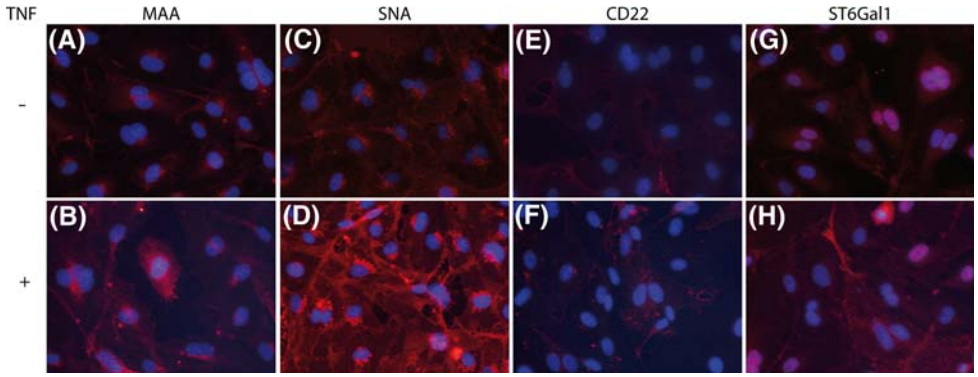

Fig. 7 Expression of sialoglycans and ST6Gal1 in TNF-stimulated HBMEC-60 cells. HBMEC-60 cells were cultivated on fibronectin-coated chamber slides, treated with or without TNF as described in "Materials and methods", and after PFA fixation and saponin treatment were incubated with lectins MAA (specific for α2,3 sialoglycans), SNA (specific for α2,6 sialoglycans), human CD22Rg (specific for α2,6 sialylated lactosamine structures) or a monoclonal antibody against the sialyltransferase ST6Gal1. Subsequently cells were stained by second antibody coupled to Cy3 (*red colour*). Nuclei were counterstained with Hoechst DNA dye (*blue colour*). Magnification ×40

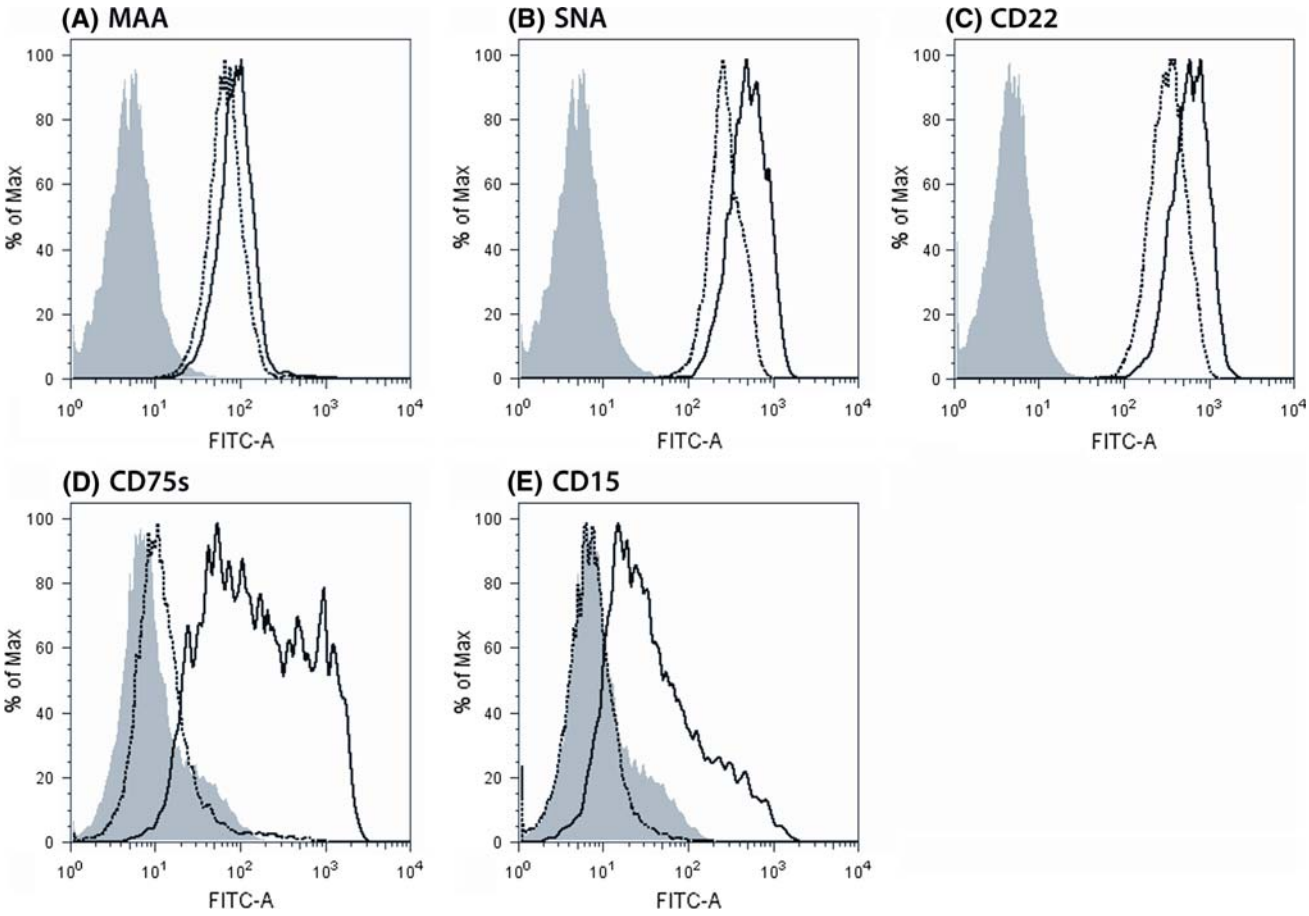

Fig. 8 Surface expression of sialoglycans and Lewis^x (CD15) on TNF-stimulated HBMEC-60 cells. HBMEC-60 cells were cultivated with (*bold line*) or without TNF (*dotted line*) and stained for flow cytometric analysis with MAA (**a**), SNA (**b**), CD22Rg (**c**), CD75s monoclonal antibody HH2 (**d**) and a CD15 specific monoclonal antibody (**e**). The *shaded curve* represents staining with an irrelevant IgM isotype control antibody

expression levels of representative glycosyltransferases and selectin E of HBMEC-60 and HUVEC after 12 h TNF or VEGF stimulation. Indeed, HUVEC displayed the same tendencies of up or downregulated glycome genes as HBMEC-60 (Table 7). In particular, both cell types showed upregulation of ST6Gal1 and downregulation of

ST3Gal1 after TNF stimulation. Also for HBMEC-60 itself, the same tendencies of up- and down-regulated glycome genes as observed in the preceding experiments could be reproduced (cf. Fig. 2; Table 6). An extreme upregulation of the selectin E gene was observed after TNF treatment (cf. Table 1).

Upregulation of HBMEC-60 sialoglycans under TNF treatment

Next, we analysed the effects of TNF treatment on the expression of sialoglycans in HBMEC-60 cells. According to the results of mRNA measurement, we expected an increase in α2,6 sialoglycans. To this end, we stained the cells with lectins differentiating between α2,3 (MAA) and α2,6 (SNA) sialylation. As could be seen in immunofluorescence microscopy, SNA staining of TNF-stimulated cells was much stronger when compared to that of non-treated cells. MAA also showed a small increase in staining after TNF treatment (Fig. 7). Binding of CD22Rg which specifically recognizes the subgroup of α2,6 sialylated lactosamines was also enhanced after TNF. In the same line, the ST6Gal1 responsible for α2,6 sialylation of lactosamine glycans, was also enhanced in its expression after TNF stimulation.

The increase in α2,6 sialoglycans after TNF treatment became even more obvious when we analysed their surface expression by flow cytometry. Staining of all reagents binding to α2,6 sialoglycans was much stronger after TNF stimulation (Fig. 8). Interestingly, an antibody against CD75s, which reacts with an even smaller subgroup of α2,6 sialolactosamines than CD22Rg [31], showed weak binding before and very strong binding after TNF treatment. It may be that its surface expression undergoes different dynamics after TNF stimulation than other sialoglycans as detected by SNA or CD22. In gene expression profiles, an increase of FUT4, the key enzyme for Lewis^x, was observed after TNF treatment (cf. Table 6). Whereas in non-treated HBMEC 60 cells surface expression of Lewis^x (CD15) was not detectable, it was significantly expressed after TNF treatment.

Discussion

The functional influence of interactions mediated by particular components of the endothelial glycome and lectins (GBP) during endothelium-mediated processes such as homing and angiogenesis is well documented [6–9]. HBMEC-60 cells were used here as a model cell system to obtain first insights into changes in endothelial glycome genes occurring during inflammatory processes or tumour invasion in bone marrow. Changes in surface receptors and synthesis of angiogenic factors have been described during multiple myeloma-induced angiogenesis. For this tumour entity, angiogenic signalling is a diagnostic factor, and on the other hand, anti-angiogenic therapeutic strategies have been successfully applied for multiple myeloma treatment [18–20]. Cell line HBMEC-60 displays typical surface markers of endothelial cells and is responsive to cytokine signalling as shown earlier [16]. HBMEC-60 cells regulate their expression after stimulation as previously described for other microvascular cell lines [25–27]. Also as shown in this study, alterations in typical endothelial surface receptors and in glycome genes of HBMEC-60 are similar to those observed with native HUVEC. In particular, major surface receptor like CD54 and CD62E were significantly enhanced after TNF stimulation. After VEGF stimulation, changes were less obvious, CD141 displayed increased expression as previously reported [32]. Most of these changes in surface expression correlated with their respective trends of up- or down-modulation at the transcriptional level. Therefore, cell line HBMEC-60 shows features characteristic for microvascular endothelial cells as confirmed by changes visible at the cell surface as well at the transcriptional level after stimulation with either VEGF or TNF. Apparently, initial virus transformation of this cell line does not seem to change its behaviour with regard to expression of surface receptors and changes in glycome genes when compared to native endothelial cells. TNF is considered as a major mediator of inflammation with great impact on tumourigenesis, survival, proliferation, invasion and angiogenesis [33]. TNF acts on endothelial cells by enhancing the extravasation of leucocytes from vasculature to the inflammatory tissue. Since TNF treatment of cells may either lead to apoptosis or lead to a NF-κB-mediated anti-apoptotic regulation [28], we also looked at survival rates of HBMEC-60 cells and transcriptional expression of those genes responsible for either induction of apoptosis or survival. Knowledge about the differential mechanisms induced by TNF are important with regard to glycome regulation. For instance, apoptosis of endothelial cells genes can promote the sulfation of proteoheparan sulfates as evidenced by RT-PCR and gene array analysis of the respective genes [34, 35]. We could show that during a time of 24 h incubation the rate of dead cells and cells positive for annexin V binding was minimal; moreover, NFκB1 was upregulated and BCL2L1 remained at unchanged levels whereas caspase 9 transcription was lowered after TNF treatment. Therefore, we presume that after TNF treatment of HBMEC-60 cells no obvious apoptosis signalling was active.

With regard to the enzymatic regulation of the glycome, several observations speak in favour of a coordinated regulation depending on the respective signalling and their functional consequences during vessel formation and

endothelial adhesive interactions. For instance, during inflammatory processes, several enzymes of the endothelial glycome are upregulated such as B4GALT1 [36], αST6Gal1 [37] and FUT1 [9].

In a first step, we analysed the glycome status of non-treated HBMEC-60. In order to demonstrate the expression level of glycan-related genes, we displayed the genes in an expression profile after microarray hybridisation in ascending order. The number of glycome genes in relation to the amount of total genes expressed in HBMEC-60 was approx 1.9%, which is comparable to assessments made earlier for eukaryotic cells [7]. Genes with high expression levels belong to the group of house keeping genes of several glycosylation pathways. Mutations or dysfunctions of these genes involved in lysosomal glycan degradation, e.g. hexosaminidase B, the galactosaminidase A (AGAL) and LAMP2 lead to GM2 gangliosidosis (Fabry disease) [38, 39]. In a similar way, dysfunctions in GT ALG3, EXT1, DPGAT1 and B4GALT7 may lead to a severe disease phenotype [40]. Most of glycosyltransferase and glycosidase genes strongly expressed in HBMEC-60 cells were also found to be expressed at high transcription levels in native HUVEC after TNF stimulation [12].

Our investigation exhibited a major influence of TNF on the endothelial glycome as evidenced by a large number of glycan relevant genes undergoing up- or down-regulation. In contrast, the effects of VEGF treatment with regard to the glycome regulation were much smaller both in numbers and extent of regulation.

In order to predict changes in endothelial glycosylation patterns induced by specific cytokine signalling, we assembled gene expression data for glycosyltransferases, glycosidases and sulfotransferases according to their affiliation to glycosylation pathways. The strength of their expression and the extent of their up- or down-regulation may give insights into the glycome of activated endothelial cells. As a general impression, we conclude that VEGF and TNF may induce modifications of glycans rather than enhanced de novo synthesis. Transcription of enzymes for initial N-linked oligosaccharide synthesis was slightly diminished. However, it has to be considered that their transcription was relatively high under non-treated conditions. After both stimulatory modalities, an increase in complex glycans may occur with an expected increase in multiantennary complex glycans after TNF treatment. According to the respective gene expression, core fucosylation by FUT8 may be slightly increased after VEGF but significantly reduced after TNF stimulation. Reduction in FUT8 activity may entail a deregulated TGF-β 1 receptor and concomitantly an increase in metalloproteinases [41]. In terms of gene expression, a marked difference between VEGF and TNF stimulation was a preponderance towards α2,3 sialylation after VEGF stimulus and more significantly towards α2,6 sialylation of N-linked glycans after TNF treatment. An increased α2,6 sialylation of TNF activated endothelial cells had been observed earlier and was associated to enhanced binding of the B lymphocyte-specific lectin CD22 (Siglec 2) to endothelium [37]. In our study, we could show that after TNF stimulation α2,6 sialoglycans are largely enhanced in their expression as evidenced by binding of specific antibodies and lectins in microscopic and flow cytometric analysis. Also, the sialyltransferase ST6Gal1 responsible for α2,6 sialylation of lactosamine glycan structures was enhanced in their intracytoplasmic expression after TNF treatment.

With regard to genes relevant for O-linked glycosylation only moderate modification were visible after VEGF whereas after TNF treatment enzymes for type 2 chain elongation and 3-O-sulfation at GalNAc sites were enhanced in their gene expression. GAG chains of endothelial proteoglycans may preferentially undergo pronounced changes in their heparin sulfate moieties. For both stimuli, gene expression for enzymes acting on elongation of HS chains were enhanced, the 3-O-sulfotransferase gene was significantly decreased after TNF treatment.

Changes in the GSL compartment were observed for the ganglio- and the neolacto-series. The ganglioside GM3 was reported to be the major GSL of HUVEC [42]. We observed a slight increase in ST3Gal5 gene expression, responsible for sialylation of lactosaminyl sequences leading to GM3, and a decrease of this enzyme under TNF signalling. GM3 was described as a ganglioside with anti-angiogenic activity [43, 44]; however, the presence of GT1a may nullify this effect [43]. In this respect, it must be noted that the gene for ST6GalNAc6 characterizing longer chain gangliosides such as GT1a, is strongly expressed in HBMEC-60 cells though not modified under stimulation. Also HEXB encoding for the degrading enzyme β-hexosaminidase shows a very strong gene expression in this endothelial cell line. It must be assumed that ultimately the balance between different patterns of gangliosides is decisive for the influence of GSL on the angiogenic process. Neolacto-series oligosaccharides may show a marked enhancement after TNF treatment. It may be that polylactosaminyl structures and expression of I/i-antigen are enhanced in endothelial cells during inflammation. Also the gene encoding FUT4, the key enzyme for synthesis of Le^x (CD15), was enhanced after TNF stimulation. This finding could be corroborated by an significantly enhanced $Lewis^x$ surface expression. CD15 and its sialylated form (CD15s) are major ligands for selectins. High expression of CD15/CD15s on activated vascular endothelium is a prerequisite for binding of leucocytes expressing selectin L CD62L) [7].

The POFUT1 gene (FUT12) was strongly expressed in HBMEC-60 cells. This gene encodes a member of the

fucosyltransferase O-Fuc family which are described to be functional in modifications of the Notch receptor [45]. Notch is also involved in first steps of vascular endothelial tube formation [10].

On the other side, a number of GBP genes were expressed on HBMEC-60 cells. Regulation of lectins was seen after TNF stimulation as, e.g. upregulation of ICAM-1. Galectin-1 was strongly transcribed on HBMEC-60 as previously described for activated tumour endothelium [46] while expression of galectin-9 was strongly induced by TNF. No members of the siglec family were identified on HBMEC-60 with our GEP system.

Whereas changes in the glycosylation machinery and lectin expression after VEGF stimulation can be ascribed to events taking place during angiogenesis, those after TNF treatment may either be relevant for homing processes or angiogenesis during inflammatory signalling. Certainly, modifications of the glycome such as fucosylation, sialylation, sulfation and core 2 elongation as described here are related to homing of immune cells to vascular endothelium [47].

Our observations on glycome gene expression gave insights into the transcriptional regulation of endothelial glycosylation during angiogenesis and possibly homing events.

Acknowledgments The study was supported by a grant to R. S.-A. from the "Deutsche Carreras Leukämie-Stiftung" (project DJCLS R 08/13).

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