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Influence of TNFα on the sialylation of mucins produced by a transformed cell line MM-39 derived from human tracheal gland cells

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In order to investigate the influence of inflammation on the peripheral glycosylation of airway mucins, a human respiratory glandular cell line (MM-39) was treated by TNFα. The expression and the activity of sialyl- and fucosyl-transferases, involved in the biosynthesis of peripheral carbohydrate determinants like sialyl-Lewis x, were investigated by RT-PCR and by HPAEC respectively. The mRNA steady-state level of sialyl- (ST3Gal III) and of fucosyl- (FUT3) transferases was moderately up-regulated by TNFα; a 52% increase of α2,3-sialyltransferase activity was also observed in TNFα-stimulated MM-39 cells. After metabolic radio-labelling with [^{3}H]glucosamine and [^{3}H]fucose, the mucins released in the culture supernatant were purified by Sepharose CL-4B, density-gradient centrifugation and treatment with glycosaminoglycans-degrading enzymes. The mucins, released in the culture supernatant from control MM-39 cells, were constituted by two populations of molecules having the same 1.39−1.44 mg/ml density but carrying either high or low amounts of sialic acid residues at their periphery. TNFα was able to increase the sialylation of the weakly sialylated mucins. This effect and the enhancement of the α2,3-sialyltransferase activity by TNFα argue in favour of a regulation of the mucin sialylation by this pro-inflammatory cytokine. Despite the moderate overexpression of *FUT3,* no fucosylation of mucins produced by MM-39 cells was induced by TNFα. In conclusion, the influence of TNFα on the sialylation of mucins could explain why the mucins from infected patients suffering either from cystic fibrosis or from chronic bronchitis are more sialylated.

Keywords: glycosylation, cytokines, mucus, fucosyl-transferases, sialyl-transferases

Abbreviations: mAb, monoclonal antibody; BCA, bicinchoninic acid; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; *FUT*, Fucosyltransferase; *C2-GnT*, UDP-GlcNAc:Gal β1-3-GalNAc-R (GlcNAc to GalNAc) β1-6GlcNAc transferase; *I-GnT*, β1-6N-acetylglucosaminyltransferase; *ST3Gal*, Galactose-α2-3 sialyltransferase; *ST6Gal*, Galactose-α2-6 sialyltransferase; *GnT*, *N*-acetylglucosaminyltransferase; HPAEC, High Performance Anion Exchange Chromatography; PAD, Pulsed Amperometric Detection; PAPS, Adenosine 3′-phosphate 5′-phosphosulfate; RT-PCR, Reverse Transcription-Polymerase Chain Reaction; PBST, phosphate buffered saline; GdmCl, Guanidinium chloride.

Introduction

In relation to the secretion of cytokines, several recent reports have indicated possible glycosylation alterations of acute phase glycoproteins, such as an increased expression of sialyl-Lewis x epitope [1−5]. This selectin ligand [6] could modulate the inflammation response in human sera by inhibiting the adhesion of leukocytes to the endothelial cells [6]. The mucins from infected patients suffering either from cystic fibrosis or

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from chronic bronchitis [7−10] are more sialylated and contained more sialyl-Lewis x epitopes than the mucins from not severely infected patients [11]. This determinant can also act as receptor for *P. aeruginosa* [12−13]. In order to precise the role of inflammation in the glycosylation modifications of mucins, which have been described in respiratory disorders, the effects of Tumor Necrosis Factor-α (TNFα) on respiratory mucin glycosylation have to be determined. This pro-inflammatory cytokine has been chosen since it is able to initiate the inflammatory reaction. TNFα-mediated alterations of the glycosylation level of small bowel mucins from rabbit [14] and of the sulfation degree of mucins secreted by intestinal goblet cells [15] have been already described.

The aim of this study was to investigate the TNFα effects on the mucins produced by the respiratory MM-39 cell line [16]. This cell line is considered as having a mixed, both serous and mucous, phenotype. It derives from human submucosal tracheal glands. Mucins originate predominantly from the mucous part of airway glands. The present work was designed to determine the TNFα effects (i) on the expression of *MUC* genes, (ii) on the expression and the activity of the different fucosyl- and sialyl-transferases involved in the peripheral glycosylation of mucins and (iii) on the glycosylation of the mucins produced by the MM-39 cells.

The peripheral glycosylation of some mucins biosynthesised by MM-39 cells has been modified by TNFα. The α2,3-sialyltransferases are up-regulated by TNFα and this effect is associated to the release in the culture supernatant of mucins which are more sialylated.

Material and methods

Material

Culture materials were obtained from Life Technologies (Paisley, U.K.). TNFα was purchased from PeproTech (London, England). [6-^{3}H]glucosamine (40 Ci/mmol) and [6-^{3}H]fucose (40 Ci/mmol) were from Amersham Pharmacia Biotech (Saclay, France).

RNase A, amyloglucosidase from *Aspergillus niger* were from Boehringer Mannheim (Mannheim, Germany). Benzamidine hydrochloride, EDTA, N-ethylmaleimide, α-aminohexanoic acid, iodoacetamide, PMSF, micrococcal endonuclease from *Staphylococcus aureus* (Foggi strain), hyaluronidase from bovine testes, heparitinase I (Heparinase III) from *Flavobacterium heparinium,* the Galβ1-4GlcNAc substrate, the Galβ1-4[Fucα1-3]GlcNAc, Fucα1-2Galβ1-4GlcNAc and Fucα1-2Galβ1-4[Fucα1-3]GlcNAc standards were from Sigma (St Louis, MO, USA). Chondroitin ABC lyase was from Seikagaku Corp (Tokyo). CDCl and guanidinium chloride (GdmCl) were from Merck (Darmstadt, Germany). Amplify™ solution and Hyperfilm™-MP film were from Amersham Pharmacia Biotech. The NeuAcα2-3Galβ1-4GlcNAc and NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAc standards were from Toronto Research Chemicals (Ontario, Canada). [^{14}C]CMP-NeuAc (0.294 Ci/mmol) was from Amersham Pharmacia Biotech. CarboPac PA-100 column (4 × 250 mm) and the high performance anion exchange chromatography with pulsed amperometric detection apparatus (PAD 2 model) (HPAEC/PAD) were from Dionex Corp. The high performance liquid radioactivity detector LB 506 C-1 was from EG & G, Berthold (Wildbad, Germany). The BCA Protein Assay was from Pierce (Rockford, Il, USA). YM-30 membrane was from Amicon (Danvers, MA, USA).

Guanidinium thiocyanate was from Fluka (Buchs, Switzerland). DNase I, oligodeoxythymidilic acid$_{12-18}$ primer for cDNA synthesis and restriction enzymes were from Life Technologies (MD, USA). Expand™ Reverse Transcriptase, the oligonucleotides used as primers for the PCR reactions are obtained from Genset (La Jolla, CA 92037, USA). Amplifications were performed using Minicycler™ (MJ Research Inc., Model PTC 150-16, MA, USA).

mAbs anti-Lewis a, anti-Lewis y, and anti-sialyl-Lewis a and *Maakia amurensis* agglutinin (MAA) were from Seikagaku Corp (Tokyo, Japan). mAb anti-sialyl-Lewis x was from Kamiya Biomed. Comp (Seattle, WA). Maxisorp immunoplates were from Nunc (Roskilde, Denmark). The sialyl-lactosamine-polyacrylate, corresponding to a polymeric neoglycoconjugate, was obtained from Syntesome (Munich, Germany) [17].

Culture, metabolic radio-labelling and TNFα stimulation of the MM-39 cells

Cells were cultured as previously described [16]. Cells were passaged at a split ratio of 1 : 3. Four days after trypsinisation, cells plated on T75 flasks were incubated with 5 μCi/ml [^{3}H]glucosamine for one day; a change was then performed with a freshly prepared medium containing 5 μCi/ml [^{3}H]-glucosamine, superoxide dismutase (250 U/ml), catalase (100 U/ml) [18] and 20 ng/ml TNFα, this last one being exclusively added in the "stimulated" flasks. The duration of the stimulation was 16 h. In a second experiment, 20 μCi/ml [^{3}H]fucose in the presence of 1 mM mannose was used rather [^{3}H]glucosamine.

RNA isolation and cDNA synthesis

Total RNA was isolated from cultured cells using the guanidinium thiocyanate/CsCl method according to Chirgwin et al. [19]. Total RNA was treated with DNase I kit and cDNA was prepared using Expand™ Reverse Transcriptase with oligodeoxythymidilic acid$_{12-18}$ primer as described by the manufacturer's manual.

Semi-quantitative analysis of *MUC* gene expression by PCR

PCR was performed on 5 μg of cDNA using specific pair of primers for *MUC1*, *-2*, *-3*, *-4*, *-5AC*, *-5B*, *-6* and *-7*. The oligonucleotide primers were designed from the published sequence in GenBank™ [20]. The operating conditions were

previously described [21]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Amplified products were quantified for each sample using the Gel Analyst 3.01 software suite.

Semi-quantitative analysis by PCR of glycosyltransferase expression

The oligonucleotides used as primers for the PCR reactions are given in Table 1. Most of the primers were previously described (Table 1), except those used for the amplification of *FUT*6, *FUT*7, *C2-GnT* and *I-GnT*. These ones were designed from cDNA sequences reported by Koszdin et al. [25], Sasaki et al. [26], Bierhuizen et al. [27] and Ropp et al. [28], respectively.

Amplifications were performed using 2 µl cDNA template in a total volume of 25 µl of a reaction mixture containing 20 mM Tris-HCl (pH 8.4 at 25°C), 50 mM KCl, 0.4 µM of both sense and antisense oligomer primers, 0.2 mM dNTP, 0.625 U of *Taq* DNA Polymerase and $MgCl_2$ at 1 mM for β-actin and the fucosyltransferases, or at 1.5 mM for the GlcNAc- and α2-3-sialyltransferases.

PCR for analysing the expression of fucosyltransferases FUT1, FUT2, FUT3, FUT4 and FUT5 was carried out under conditions described by Emery et al. [30]. For FUT6 and FUT7, the same conditions were used as for the other fucosyltransferases. The amplification conditions for each α2,3-sialyltransferases were performed as indicated by Recchi et al. [29]: 94°C for 2 min, 1 cycle; 94°C for 1 min, 58°C for 1 min, 72°C for 1 min 30 s, for 35 cycles; and 72°C for 7 min. The conditions for *N*-acetylglucosaminyltransferases were: 94°C for 2 min, 1 cycle; 94°C for 1 min, 62°C for 1 min, 72°C for 1 min 30 s, for 35 cycles for the C2-GnT and for the I-GnT; and 72°C for 7 min. For β-actin, the conditions were described by Hamosh et al. [22].

Eleven µl of the PCR products were subjected to electrophoresis on a 2% agarose gel equilibrated in TBE containing ethidium bromide. Gels were photographed under UV light and analysed by computerised scanning of the image using Gel Analyst 3.01 program. The amplified fragments were verified by digestion with restriction enzymes according to Emery et al. [30]: moreover *Dde* I was used for the digestion of the amplified fragment from *FUT6*, *Pst* I was used for *FUT7*, *Msp* I for *C2-GnT* and *Bgl* I for *I-GnT*. PCR products were quantified following the procedure described for the *MUC* gene expression [20], but in this case, the expression of glycosyltransferases was compared to the expression of β-actin.

Sialyltransferase assay

After trypsinization, 10^7 cells were sonicated for 4 min in a 50 mM Tris-HCl buffer, pH 7.4, containing 25 mM KCl, 250 mM saccharose, 5 mM β-mercaptoethanol, 5 mM magne-

Table 1. Oligonucleotide primers for amplifying glycosyltransferases and β-actin mRNA

mRNA detected	*Size (bp)*	*Primers used (5′ → 3′)*	*Tm (°C)*	*Ref.*
β-actin	228	S GCACTCTTCCAGCCTTCC AS GCGCTCAGGAGGAGCAAT	58	[22]
FUT1	374	S GCAGCTTCACGACTGGATGTCGGAGGAGTA AS TACACCACTCCATGCCGTTGCTGGTGACCA	72	[23]
FUT2	368	S GCGGCTAGCGAAGATTCAAGCCATGTGGGA AS AGCAGGGGTAGCCGGTGAAGCGGACGTACT	72	[23]
FUT3/5	447/486	S CTGCTGGTGGCTGTGTGTTTCTTCTCCTAC AS CAGCCAGCCGTAGGGCGTGAAGATGTCGGA	72	[24]
FUT4	319	S GGTGCCCGAAATTGGGCTCCTGCACAC AS CCAGAAGGAGGTGATGTGGACAGCGTA	72	[24]
FUT6	404	S CTCAAGACGATCCCACTGTGTAC AS CAGCCAGCCGTAGGGCGTGAAGATGTCGGA	72	[25]
FUT7	231	S TGCCACCTGAGTGCCAACCGAAGCCTGCTG AS GGGCACAAAGATGTCCGAGTCGCGCCGGTA	72	[26]
C2-GnT	374	S GTGCCTACTTCGTGGTCAG AS TCCCAAAGAGGTCAACATCC	62	[27]
I-GnT	632	S TCAACACCTGTGGGCAAGAC AS GGGTGGTATCGCAGTTTCACTC	62	[28]
ST3Gal I	537	S TCAGAGTGGTGCCTGGGAATGT AS TAGTGGTGCCAGTTCCCTTTGC	58	[29]
ST3Gal III	300	S CGGATGGCTTCTGGAAATCTGT AS TTGTGCGTCCAGGACTCTTTGA	58	[29]
ST3Gal IV	458	S CCCAAGAACATCCAGAGCCTCA AS CGTGGTGGGCTTCTGCTTAATC	58	[29]

sium acetate, and used for microsomal preparation as described by Lo-Guidice et al. [31]. The protein content of microsomal fractions was determined by BCA Protein assay [32]. The α2-3-sialyltransferase assay was performed according to the slightly modified method of Majuri et al. [33]: microsomal proteins (50−100 μg) were incubated with 0.5 μCi [^{14}C]CMP-NeuAc, 5 mM of Galβ1-4GlcNAc or Galβ1-4[Fucα1-3]GlcNAc in a Tris/acetate buffer, pH 6.7, containing 0.1% Triton X-100 and 1 mM AEBSF. Incubation was performed 5 h at 37°C and stopped as described by Lo-Guidice et al. [31].

Fucosyltransferase assays

α1,2- and α1,3-fucosyltransferase activities were measured according to the procedure of Goupille et al. [34]. Adherent cells from a T-75 flask were lysed in 200 μl of 50 mM potassium phosphate buffer, pH 6.0, containing 2% (v/v) Triton X-100 and 1 mM AEBSF on ice for 30 min. The protein content of cell lysates was determined by BCA Protein assay [32]. The reaction was performed with 50 μg of microsomal proteins, 20 μM GDP-L-[^{14}C]fucose (1.83 kBq), 20 μM N-acetyllactosamine, 10 mM L-fucose, 7.7 mM $MgCl_2$, 1.9 mM ATP at 37°C. The incubation time was 2 hours.

Identification by HPAEC of radio-labelled products obtained in sialyl- and fucosyltransferase assays

Dry samples of sialylated or fucosylated radio-labelled products were dissolved in water and directly injected onto a CarboPac PA-100 column (4 × 250 mm) for HPAEC. The elution of neo-synthesised products was monitored both by pulsed amperometric detection and by radioactivity on line. It was performed at alkaline pH at a flow rate of 1 ml/min in 0.1 M NaOH for 10 min followed by a linear gradient of sodium acetate to 0.1 M NaOH/0.07 M sodium acetate at 16 min, to 0.1 M NaOH/0.1 M sodium acetate at 30 min and to 0.1 M NaOH/0.45 M sodium acetate at 80 min. The standards used for the identification of the α2-3-sialylated products were NeuAcα2-3Galβ1-4GlcNAc and NeuAcα2-3Galβ1-4 [Fucα1-3]GlcNAc. The standard used for the identification of neo-synthesised fucosylated products by HPAEC were Galβ1-4[Fucα1-3]GlcNAc, Fucα1-2Galβ1-4GlcNAc and Fucα1-2Galβ1-4[Fucα1-3]GlcNAc. The activity of the different enzymes was expressed as picomoles of radio-labelled nucleotide sugar transferred/mg of protein/min.

Purification of mucins released in the culture supernatant

Culture supernatants were supplemented with a proteinase inhibitor cocktail [35]. They were concentrated at 4°C by ultrafiltration through a YM-30 membrane. The gel-filtration chromatography was performed on a Sepharose CL-4B column (2.5 × 50 cm) equilibrated with 67 mM phosphate saline buffer, pH 6.7, containing the proteinase inhibitor cocktail [35]. The flow rate was 20 ml/h.

High molecular mass fractions were submitted to micrococcal endonuclease from *Staphylococcus aureus* (Foggi strain) (1.25 U/ml) and RNase A (50 μg/ml) treatment for 1 h at 37°C. The pH was secondly adjusted to 6.0 with citric acid and hyaluronidase from bovine testes (10 U/ml) was added for 24 h at 37°C. The pH was adjusted to 4.6 with citric acid before a third enzymatic treatment with amyloglucosidase from *Aspergillus niger* (1 U/ml) for 16 h at 37°C. After a dialysis against 100 mM Tris/acetate buffer, pH 7.3, in which the proteinase inhibitor cocktail was included, the fractions were concentrated to 1 ml using a YM-30 ultrafiltration membrane and then incubated with chondroitin ABC lyase (0.2 U/ml) for 5 h at 37°C, followed by heparitinase I (Heparinase III) from *Flavobacterium heparinium* (5 U/ml) for 5 h at 37°C. Before each enzymatic treatment, 1 mM PMSF was added extemporaneously.

The recovered material was subjected to a density-gradient centrifugation according to Carlstedt et al. [36] with a buffer containing 67% (w/v) CsCl and 4 M GdmCl. The fractions obtained after density-gradient centrifugation were reduced [35] and re-chromatographied on Sepharose CL-4B (1 × 50 cm) equilibrated in 6M GdmCl. The high molecular mass material was extensively dialysed against water and lyophilised.

Control of [^{3}H]fucose metabolic radio-labelling specificity

An aliquot of radiolabelled material corresponding to 1000 dpm was incubated with 0.1 mg of α-L-fucosidase from bovine kidney in 0.1 M citrate-phosphate buffer pH 4.5 for 16 h. The digestion products were loaded on a Sepharose CL-4B column (1 × 50 cm) equilibrated in 6M GdmCl.

SDS-PAGE analysis and fluorography

5−15% gradient polyacrylamide slab gel electrophoresis was performed as previously described [37]. The samples were treated with 2% (w/v) SDS-5% (v/v) β-mercaptoethanol overnight before being loaded onto the gel. Gels run with radio-labelled products were immersed in Amplify™ solution for 30 min, dried, and then exposed to Hyperfilm™-MP film at −80°C.

Enzyme-linked immunosorbent assay of carbohydrate determinants

The purified mucin samples were dissolved in 6M GdmCl (2 mg/ml) before being diluted 1 to 20 in PBS pH 7.2 (stock solution). Aliquots were diluted in PBS in order to obtain a concentration ranging from 1 to 0.1 μg/ml before being coated in duplicate on 96-well Maxisorp immunoplates. Only the fractions containing mucins without contaminating bands in SDS-PAGE were investigated. For this reason, the later tubes of the second gel-filtration in GdmCl were not tested. The immunoassays were performed as previously described [10].

Competition assay was performed for the binding of MAA lectin. Coated mucins were incubated with biotinyled MAA lectin in the presence of a 100 molar excess of sialyl-lactosamine-polyacrylate.

Results

TNFα-mediated overexpression of glycosyltransferases

When the cells were cultured without TNFα, *ST3Gal I*, *ST3Gal III*, *ST3Gal IV*, *FUT2*, *FUT3*, and *C2-GnT* mRNAs were easily detectable (Figure 1A), whereas *FUT1*, *FUT4* and *I-GnT* were weakly expressed (data not shown). *FUT5*, *FUT6* and *FUT7* mRNAs were not detected, even when PCR experiments were performed with a higher number of cycles (data not shown).

Three experiments of cell stimulation by TNFα were performed. For each of them, a TNFα mediated overexpression of the three α2,3-sialyltransferase genes (*ST3Gal I*, *ST3Gal III* and *ST3Gal IV*) and of the α1,3/α1,4-fucosyltransferase *FUT3* (coding Lewis enzyme) was observed (Figure 1A). TNFα effects were dose- and time-dependant; they peaked at 20 ng/ml for 16 hours. Above these two values, a plateau appeared (data not shown). The expression of *ST3Gal III* and *FUT3* were more particularly increased [+80% ± 25% for *ST3Gal III* (Figure 1B); +90% ± 27% for *FUT3* (Figure 1C)]. The expression of *FUT4, C2-GnT* and *I-GnT* did not vary in cells stimulated with different concentrations of TNFα (data not shown).

The primers used to amplify *FUT3* cDNA (447 bp) also amplified *FUT5* cDNA (486 bp). In our experiments, a single band appeared after migration on a 8% polyacrylamide gel. This RT-PCR product was cleaved into 282- and 165-bp fragments after digestion by *Dde I* [30], indicating that only *FUT3* had been amplified.

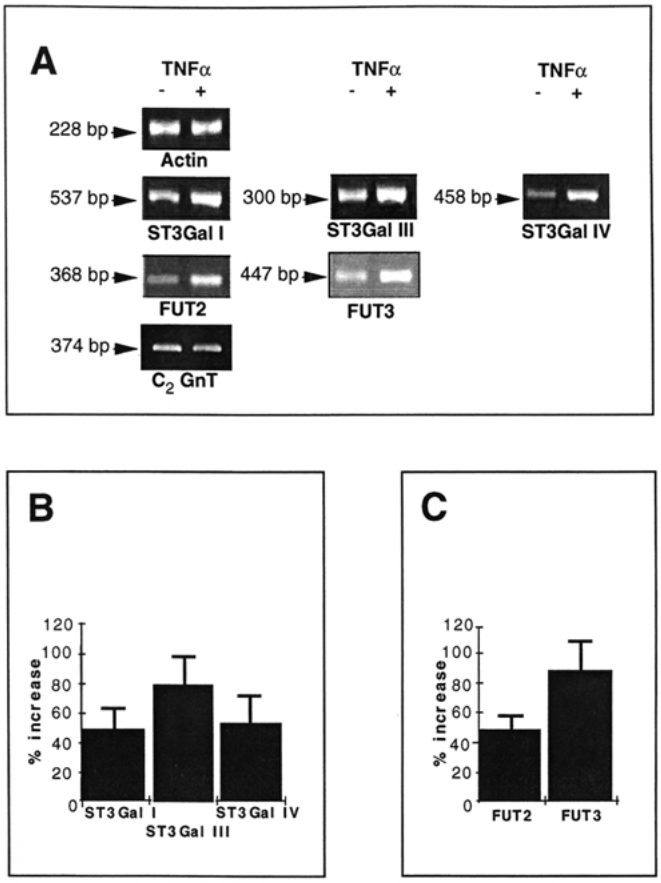

Figure 1. Expression of sialyl- and fucosyl-transferases in MM-39 cell line treated with 20 ng/ml TNFα for 16 h. (Panel A) analysis by RT-PCR of the glycosyltransferase expression in control cells (−) and in cells treated by TNFα (+). (Panel B) values of sialyl-transferase variations are representative of 3 separate experiments. (Panel C) values of fucosyl-transferase variations are representative of 3 separate experiments.

TNFα-mediated increase of activity from peripheral glycosyltransferases

The α2-3-sialyltransferase activity was tested with Galβ1-4GlcNAc. When microsomal fractions (from control cells) were incubated with [^{14}C]CMP-NeuAc and Galβ1-4GlcNAc, the HPAEC elution profile showed three radio-labelled peaks (Figure 2). Two of them (peak I and peak III) were present when incubations were performed without any acceptor; they were identified by co-elution with non-labelled standards (which are detected by PAD) whose retention times are known. The first radio-labelled peak (peak I), which was eluted at

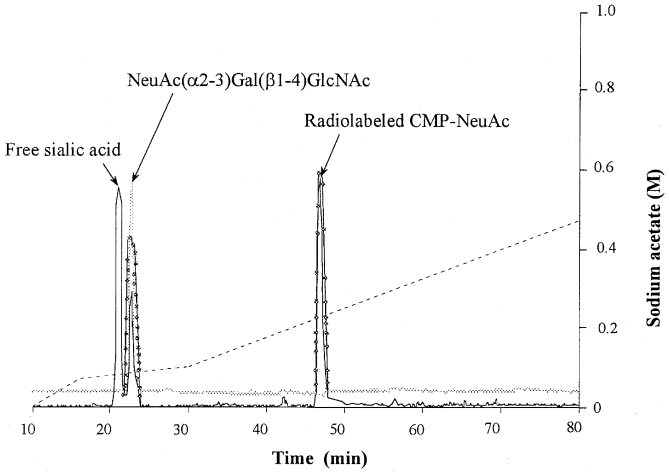

Figure 2. HPAEC elution profile of a mixture containing radio-labelled products synthesised when a same amount of microsomal proteins (from cells treated with TNFα or not) were incubated with [^{14}C]CMP-NeuAc and Galβ1-4GlcNAc, and non-labelled NeuAcα2-3Galβ1-4GlcNAc. The elution was monitored by both pulsed amperometric detection and radioactivity detection. The radiolabelled products synthesised during incubations with [^{14}C]CMP-NeuAc and Galβ1-4GlcNAc are represented by a solid line (———) for untreated cells and a solid line with circles (-●-●-) for treated cells. The unlabelled standards, visualised by PAD, are represented by a dashed line (- - - - -).

21 min 4 s, corresponded to free labelled sialic acid. The third one (peak III) was eluted at 47 min 30 s and was identified as being non degraded [^{14}C]CMP-NeuAc. The characteristic peak, which was eluted at 23 min 24 s, co-eluted with unlabelled NeuAcα2-3Galβ1-4GlcNAc, showing the presence of an α2-3-sialyltransferase activity in MM-39 microsomal fractions. When microsomal fractions from cells stimulated with TNFα were incubated with [^{14}C]CMP-NeuAc and Galβ1-4GlcNAc, the same three peaks were obtained, but there was an increased incorporation of [^{14}C]CMP-NeuAc into Galβ1-4GlcNAc (+52%) (Table 2). This value was representative of the TNFα-mediated increase of the α2-3-sialyltransferase activity observed for each of the 3 experiences performed.

The α1-3-fucosyltransferase activity from MM-39 cell lysates was tested using Galβ1-4GlcNAc acceptor. A basal activity was found (Table 2), which decreased weakly (−26.9%) in TNFα-treated cells; nevertheless this variation cannot be taken in consideration since the activity was very low in the control cells (Table 2).

Effect of TNFα on the expression of *MUC* genes

In agreement with the results obtained by Northern blot [16], only *MUC1* and *MUC4* transcripts were amplified by RT-PCR in the MM-39 cell extract (data not shown). The expression of these two *MUC* genes did not vary when the cells were stimulated by TNFα (data not shown).

Characterisation of the different forms of mucins released in the culture supernatant of the MM-39 cell line

The [^{3}H]glucosamine radio-labelled material present in the culture supernatant from the MM-39 cell line was separated by a first Sepharose CL-4B chromatography in 4 peaks (I, II, III and IV) (Figure 3) as previously described [16]. Peak IV corresponded to free radioactivity and peak III to low molecular mass glycoproteins secreted by the cells [16]. Among the mucins secreted by MM-39 cells, some of them are partially included in Sepharose CL-4B [16]. Consequently, the material contained in the peak I and also in the peak II were further studied (Figure 3). Each peak was treated by enzymes degrading nucleic acids, glycogen, hyaluronic acid and proteoglycans before being submitted to isopycnic centrifugation. Most of the radio-labelling was recovered in the density-gradient centrifugation tubes 7 to 12. The components having a 1.45−1.50 mg/ml density (tubes 7−9) were totally included in the second gel-filtration performed in denaturing condition and as such they were not investigated any further.

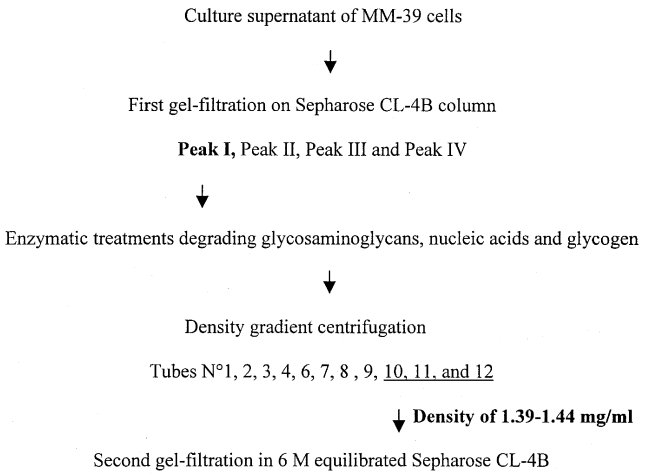

Figure 3. Schematic representation of the purification procedure of mucins released in the culture supernatant of MM-39 cells. They were isolated from peak I of the first gel-filtration on Sepharose CL-4B (example presented in Figure 3) and from peak II. The same protocol was applied in the two cases.

The material derived from peak I and having a 1.39−1.44 mg/ml density (density-gradient centrifugation tubes 10−12) (Figure 3) was separated in two fractions after the second gel-filtration (Figure 4A). The minor fraction, excluded from the Sepharose CL-4B column (Figure 4A, tubes 20−24) was located in 5−15% SDS-PAGE at the top of the stacking gel (Figure 4B). The preponderant fraction, partially included in the Sepharose CL-4B column (Figure 4A, tubes 25−35), migrated in the stacking gel of the 5−15% SDS-PAGE (Figure 4B). In ELISA, an intense staining was exclusively obtained with *Maackia amurensis* agglutinin (MAA), reacting with α2-3-sialic acid (Figure 4A, dashed line). The signal obtained with MAA disappeared when MAA was pre-incubated with sialyl-lactosamine polyacrylate before performing ELISA (data not shown).

The material derived from peak II and having a 1.39−1.44 mg/ml density (density-gradient centrifugation tubes 10−12) (Figure 3) was divided, after the second gel-filtration, in two partially included fractions (Figure 5A). The major fraction presented the same elution volume as the preponderant fraction coming from peak I (Compare Figure 5A

Table 2. Activities of sialyl- and fucosyl-transferases in MM-39 cells treated with TNFα. Values of sialyl-transferase and fucosyl-transferase variations are representative of 3 separate experiments

Glycosyl-transferase activities (pmol/mg of protein/min)	*Substrate*	*MM-39 Control*	*MM-39 + TNFα*
α2-3-sialyltransferase activity	Galβ1-4GlcNAc	1.54 ± 0.05	2.35 ± 0.05 (+52.6 %)
α1-3-fucosyltransferase activity	Galβ1-4GlcNAc	0.182 ± 0.05	0.133 ± 0.05

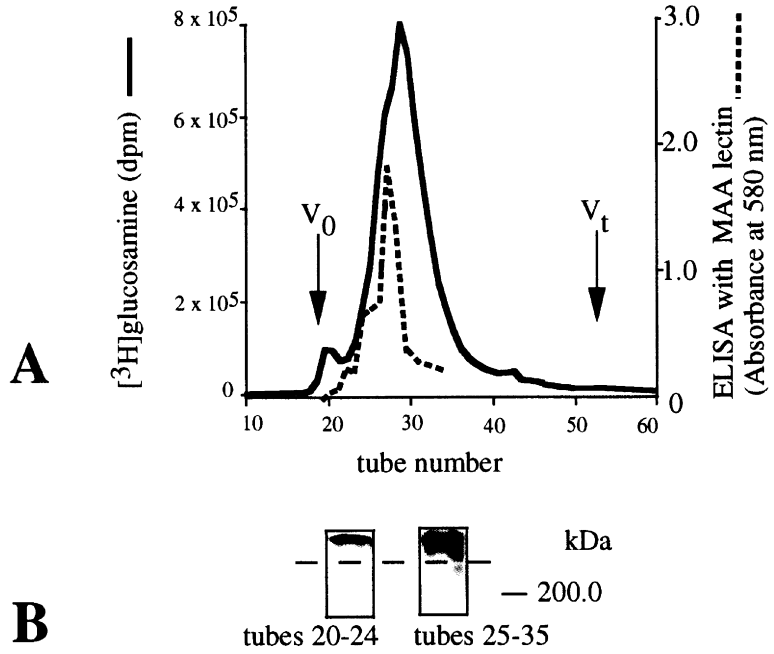

Figure 4. Characterisation of peak I-derived mucins, which were present in the supernatant of MM-39 cells cultivated without TNFα. (A) second gel-filtration on Sepharose CL-4B equilibrated in 6M GdmCl. Fractions were analysed for carbohydrate (———) [aliquots from the radio-labelled fractions were mixed with scintillation fluid and counted in an LKB 1214 β-scintillation counter] and for sialic acid by ELISA with MAA lectin (- - - - -). The binding of the MAA lectin to the mucins was tested by coating 0.1 μg of material per well in duplicate. Since the tubes 36–45 may be contaminated by low molecular mass glycoproteins, they have been discarded from sialic acid assay. (B) fluorography from the SDS-PAGE pattern of the [^{3}H]glucosamine radio-labelled mucins.

and Figure 4A). It contained material migrating in the 5% stacking gel of a SDS-PAGE and at the top of the 5–15% separating gel (Figure 5B). A weak reactivity with MAA lectin was also observed (Figure 5A, dashed line). The minor fraction, more included into the column (Figure 6A), was constituted by bands ranging from 200 kDa to 50 kDa (Figure 5B). They certainly corresponded to low molecular mass glycoproteins previously described by Lo-Guidice et al. [16]. When a [^{3}H]fucose metabolic radio-labelling of components secreted by the MM-39 cells was performed, only this fraction was markedly detected (data not shown). Its radio-labelling was destroyed after treatment with fucosidase.

TNFα effects on the glycosylation of mucins released in the culture supernatant

When cells were grown in the presence of TNFα, only the [^{3}H]glucosamine radio-labelling of the partially included mucins derived from peak II increased up to 33% (Figure 6A). Their binding to MAA lectin was also markedly enhanced (Figure 6B). When superoxyde dismutase and catalase were omitted during the stimulation of the cells with TNFα, a decrease of the [^{3}H]glucosamine radio-labelling of the partially included mucins derived from peaks I and II was observed (data not shown).

No [^{3}H]fucose radio-labelling of mucins occurred when the cells were stimulated by TNFα (data not shown) whereas, the [^{3}H]fucose radio-labelling of the low molecular mass glycoproteins, more included into the column (Figure 5B), increased moderately (data not shown).

Discussion

In this study, biochemical criteria have been applied to define the mucins present in the culture supernatant from MM-39 cells. Firstly, these high-molecular-mass glycoproteins are resistant to digestion by hyaluronidase, chondroitin ABC lyase, heparitinase and amyloglucosidase. Secondly, most of them have a buoyant density ranging from 1.39 to 1.45 mg/ml in CsCl-density-gradient centrifugation. Thirdly, they are sialylated but not fucosylated. The characteristics of these mucins as well as their reactivity with antibodies directed against apomucin epitopes have been previously reported [16]. As already described in this article, they also have unusual features. They correspond to a population of molecules with different molecular size. A minor part of this population is excluded from a Sepharose CL-4B column and remains in the stacking gel of SDS-PAGE. A larger part of the mucins is formed by molecules that are partially included and appears in SDS-PAGE as a smear migrating from the stacking gel to the upper part of the separating gel.

According to the pattern of *MUC* gene expression in MM-39 cells [16], the mucins released in the culture supernatant are

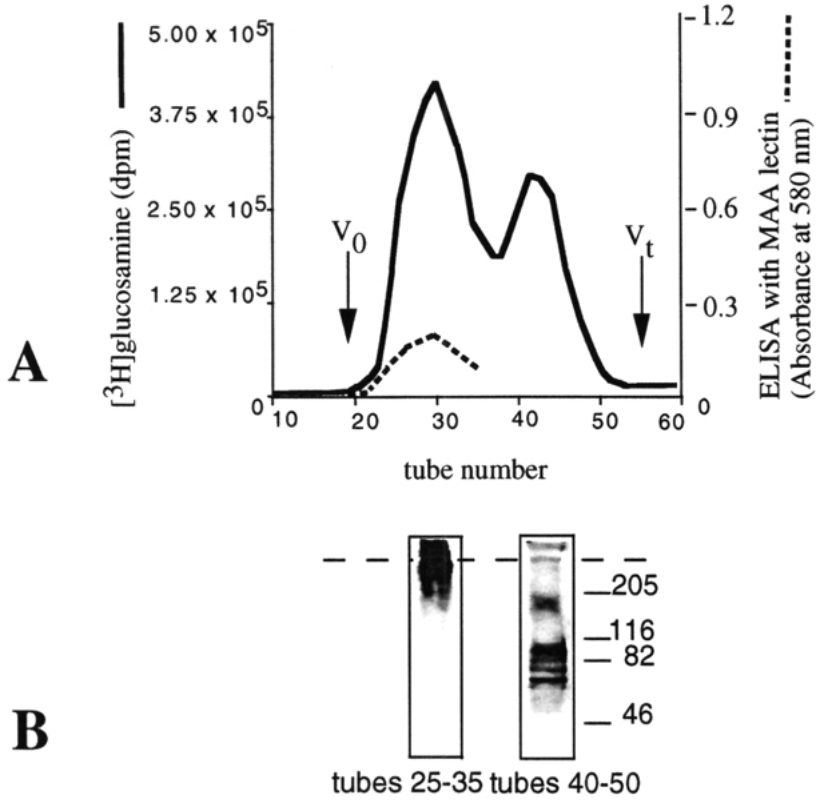

Figure 5. Characterisation of peak II-derived mucins, which were present in the supernatant of MM-39 cells cultivated without TNFα. (A) second gel-filtration on Sepharose CL-4B equilibrated in 6M GdmCl. Fractions were analysed for carbohydrate (———) and for sialic acid by ELISA with MAA lectin (- - - - -). The binding of the MAA lectin to the mucins was tested by coating 0.1 μg of material per well in duplicate. ELISA was performed as indicated in Material and Methods. The fraction which was the most included in Sepharose CL-4B (tubes 36−45 from figure 5A), was not incubated with MAA since it contained a majority of low molecular mass glycoproteins associated to few amounts of mucins. (B) fluorography from the SDS-PAGE pattern of the [^{3}H]glucosamine radio-labelled mucins.

probably constituted by a mixture of mucins from MUC1 and MUC4 type, which are not considered as representative of the mucous secretion from human respiratory glandular cells [38]. In the present status of their mucous phenotype [16], which is related to their culture conditions [39], the MM-39 cell line cannot be implicated as a good model to study the influence of inflammation on the respiratory tract mucosa. Nevertheless, these cells in standard culture conditions appear as a cellular tool to investigate the role of TNFα on the two major transmembrane mucins, MUC1 and MUC4 [16]. The effect of this cytokine proves to be an increase of their sialylation rather than a modulation of their expression.

Two complementary approaches indicate that TNFα is capable of influencing the glycosylation of mucin produced by MM-39 cells. Firstly, mRNA expression and activity of glycosyltransferases involved in the peripheral glycosylation of mucins vary in TNFα stimulated cells. Secondly, the mucins produced by TNFα treated MM-39 cells present alterations of their sialylation.

A translational up-regulation of *ST3Gal III* mRNA (less than 2-fold) by TNFα has been demonstrated, but it must be appreciated as moderate. This result is in agreement with the TNFα mediated increase of the α2-3sialyltransferase enzymatic activity and the higher sialylation of some mucins produced by MM-39 cells. The expression of *FUT3* was also enhanced by TNFα (less than 2-fold). Nevertheless, no relationship has been found between *FUT3* overexpression and the corresponding enzymatic activity, which remains low in MM-39 cells stimulated by TNFα. Moreover, the mucins produced by the MM-39 cells, stimulated or not by TNFα, lack from structure H or from Lewis x derivatives. Consequently, they are substrates neither of α1,2- and α1,3-fucosyltransferases nor of the combined action of fucosyl- and sialyltransferases involved in the peripheral glycosylation of mucins. These results could be explained by the poorly differentiated status of the mucous phenotype in the MM-39 cells or in others words by a down-regulation of fucosyltransferases in the cells (lack of translation of mRNA to active enzyme, for example). Sialyltransferases and fucosyltransferases could also compete with one to another for the peripheral glycosylation of mucins. Moreover, others troubles could occur at the level of the assay (presence of fucosyl-

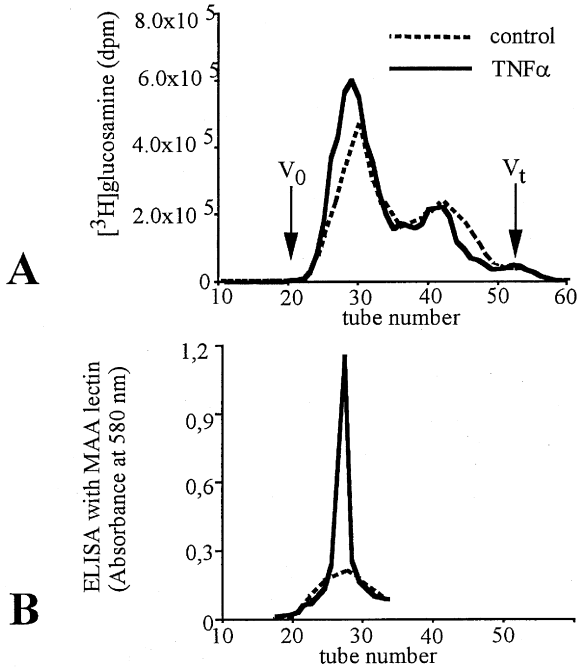

Figure 6. Effect of TNFα on the sialylation of mucins released in the culture supernatant from MM-39 cells. (A) second gel-filtration on Sepharose CL-4B equilibrated with 6M GdmCl of peak II-derived mucins, which were present in the culture supernatant of MM-39 cells stimulated (———) or not (- - - - -) by TNFα. (B) binding of the MAA lectin to the material contained in each collected tube of the second gel-filtration. 0.1 μg of material per well was coated in duplicate. ELISA was performed as indicated in Material and Methods. For the reason indicated in the legend of Figure 5, the tubes 36–45 were not tested.

transferase inhibitor in the cell lysats; inefficiency of the substrates used to perform the fucosyltransferase assay from which alternative substrates should be required). The metabolic [^{3}H]fucose labeling was limited to low molecular weight glycoproteins co-purified with mucins. Consequently, this defect of fucosyltransferase activities in MM-39 cells could concern more precisely enzymes engaged in the fucosylation of glycan chains from mucins.

The presence of sialyl-Lewis x has been effectively enhanced by TNFα in PC-9, PC-12, QG-56 and QG-95 lung cancer cell lines [40]. Concerning cultured human umbilical vein endothelial cells (HUVEC) [33] and human colon adenocarcinoma cell lines (COLO 205 and HT-29) [41], similarities are observed with our airway cell model: (i) in the unstimulated colon adenocarcinoma and HUVEC cell lines, mRNA of FUT3 is abundant, while ST3O (ST3Gal I) and ST3N (ST3Gal III) are strongly expressed; (ii) the α2,3-sialyltransferases and the α1,3/4-fucosyltransferases mRNAs are moderately modified (less than 2-fold) after TNFα treatment of COLO 205 and HT-29. Nevertheless, no significant increase of sialyl-Lewis x at the surface of TNFα stimulated COLO 205 and HUVEC cells, has been demonstrated.

Some mucins released in the culture supernatant become more sialylated when the MM-39 cells are stimulated by TNFα. As in our previous work [16], the mucins have been distinguished according to their provenance from peak I or from peak II. The choice of this purification procedure has allowed revealing more clearly the TNFα-upregulated sialylation of mucins. TNFα-mediated sialylation occurs preferentially on the partially included mucins derived from peak II. These mucins are weakly α2-3-sialylated and represent a better substrate for the TNFα up-regulated sialyl-transferases than the partially included mucins derived from peak I, which react strongly with *MAA* agglutinin. This effect of TNFα can only be demonstrated when anti-oxidants have been added in the culture supernatant during TNFα stimulation of the cells. Such a result suggests an oxidative attack of mucins through the production of reactive oxygen species by the TNFα-stimulated cells as already reported by Branka et al. [18]. These results argue in favour of a pro-inflammatory cytokine-dependent regulation of mucin sialylation. The effect observed is really due to TNFα and not to the presence of SOD and catalase in the culture supernatant of the stimulated cells since these two components have been also added in the "control" flasks as indicated in Material and Methods.

In conclusion, the expression and the activity of the sialyltransferases present in the human respiratory glandular cell line MM-39 are moderately up-regulated by TNFα. The consequence of this effect is that the whole mucins present in the culture supernatant of MM-39 cells raise to a high reactivity with *MAA* agglutinin. This effect of TNFα on the sialylation of mucins might explain why the airway mucins from severely infected patients are more sialylated [6–9].

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