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Regulation of sialyl-Lewis x epitope expression by TNF-α and EGF in an airway carcinoma cell line

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Sialyl-Lewis x epitopes and MUC5AC protein are known to be overexpressed in mucins secreted by patients suffering from various respiratory diseases. To investigate the mechanisms by which airway inflammatory agents mediate the expression of sialyl-Lewis x epitopes and MUC5AC mucin, we examined the effects of tumor necrosis factor (TNF)-α and epidermal growth factor (EGF) in the human lung carcinoma cell line, NCI-H292. Basal expression levels of hST3GalIV, FUT3 and C2/4GnT mRNA, involved in the biosynthesis of sialyl-Lewis x, were higher than those of other glycosyltransferases in NCI-H292 cells. TNF-α induced expression of hST3GalIV, FUT3, C2/4GnT and MUC5AC mRNAs in NCI-H292 cells. When cells were pretreated with U73122, a phosphatidylinositol-phospholipase C (PI-PLC) inhibitor, the expression of these glycosyltransferase mRNAs was suppressed. Treating cells with EGF induced the down-regulation of these glycosyltransferase mRNAs and sialyl-Lewis x epitopes, while inducing an increase in expression of MUC5AC mRNA. These EGF-mediated effects on the glycosyltransferase and MUC5AC mRNAs were blocked when cells were first exposed to AG1478, an EGF receptor tyrosine kinase inhibitor. These findings suggest that the expression of sialyl-Lewis x epitopes, which is regulated separately from the expression of MUC5AC protein, may be controlled through pathways such as the EGF receptor tyrosine kinase and PI-PLC signaling cascades in NCI-H292 cells.
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Keywords: **sialyl-Lewis x, sialyltransferase, fucosyltransferase, MUC5AC**

Introduction

Human bronchial mucins have been traditionally defined as a family of high-molecular weight glycoproteins, which are secreted from the epithelial lining of the airway tract, creating a mucus layer to protect it from the threat of external pathogens [1]. The mucin family expressed by the airway epithelia includes at least eight gene products (MUC1, MUC2, MUC4, MUC5AC, MUC5B, MUC7, MUC8, and MUC13) [1]. Numerous previous studies have reported that MUC5AC mRNA, associated with goblet cell hyperplasia and metaplasia of the airway surface, is the predominant mucin gene overexpressed in patients suffering from asthma [2], chronic bronchitis [3], chronic obstructive pulmonary disease (COPD) [4] and cystic fibrosis [5]. Regulation of mucin mRNA expression by various mediators has also been reported with most studies performed in lung cancer epithelial cell lines, such as NCI-H292 cells, thus providing a useful model for mechanistic studies. Nadel [6] and Takeyama *et al.* [7] showed that exposure to tumor necrosis factor (TNF)-α caused epidermal growth factor receptor (EGFR) expression, and that binding of EGFR ligands, such as epidermal growth factor (EGF) to EGFR results in tyrosine kinase phosphorylation and subsequent activation of downstream signaling cascades, leading to MUC5AC expression.

Mucins secreted by patients suffering from respiratory diseases such as chronic bronchitis and cystic fibrosis also show increased levels of sialyl-, fucosyl-structures and sialyl-Lewis x epitopes, which are synthesized via complex core 2 or core 4 type O-glycan branch formations, on the mucin carbohydrate chains [5,8,9]. The regulation of sialyl-Lewis x epitopes on MUC5AC is performed by a number of glycosyltransferases such as hST3GalIV, FUT3 and C2/4GnT [10]. Cytokines and growth factors such as TNF-α and EGF are often found in the airways of patients with respiratory disease, and are thought to affect the glycosylation of MUC5AC. Delmotte *et al.* [11] reported that airway exposure to TNF-α caused an up-regulation

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in the expression of hST3GaIIII, hST3GalIV, FUT3 and FUT4 mRNAs, and this increased expression was able to amplify sialyl-Lewis x epitopes in the airways of patients suffering from respiratory disease. Beum *et al.* [12] showed that by treating airways with EGF, the mRNA expression of C2GnT, an enzyme involved in the formation of core 2 *O*-glycan structures during mucin glycan biosynthesis was significantly decreased preventing the formation of core 2 structures in NCI-H292 cells. Even with these results, little is known about the molecular mechanisms implicated in regulating sialyl-Lewis x epitope expression when the airway is exposed to inflammatory molecules that are involved in respiratory diseases.

We thus investigated the mechanisms by which TNF-α and EGF are able to mediate the biosynthesis of both sialyl-Lewis x and MUC5AC, using NCI-H292 cells as a model of respiratory pathology. Our results show that the expression of sialyl-Lewis x epitopes is controlled through EGFR tyrosine kinase and phosphatidylinositol-phospholipase C (PI-PLC) signaling pathways in NCI-H292 cells, a different regulatory pathway than for the expression of MUC5AC protein.

Materials and methods

Cell culture and treatment with cell-signaling molecules

NCI-H292 cells (ATCC, Manassas, VA), a human pulmonary mucoepidermoid carcinoma cell line, were cultured at 37°C in a humidified atmosphere of 5% CO_2 in RPMI-1640 medium (Sigma), supplemented with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 1.5 g/l sodium carbonate, 4.5 g/l glucose, 20 U/ml penicillin, 20 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (JRH Bioscience, Lenexa, KS). After reaching confluency, cells were incubated with 20 or 40 ng/ml TNF-α (Chemicon, Temecula, CA), 25 ng/ml EGF (Oncogene, San Diego, CA), or both 40 ng/ml TNF-α and 25 ng/ml EGF, for varing periods of time. For inhibition studies, cells were pretreated for 30 min with 10 μM AG1478 (Calbiochem, San Diego, CA), a selective EGFR tyrosine kinase inhibitor, or 10 μM U73122 (Calbiochem, San Diego, CA), a PI-PLC inhibitor.

Real-time RT-PCR to quantify glycosyltransferase and MUC5AC mRNAs

Total cellular RNAs were extracted from harvested cells, with an RNeasy Mini Kit (QIAGEN, Hilden, Germany). Approximately 4 μg of total RNA was reverse-transcribed to prepare cDNA with a Super Script First-standard Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's suggested protocol. An ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) was used for real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) to measure the glycosyltransferase and MUC5AC mRNAs. Real-time RT-PCR was performed using qPCR™ Mastermix plus SYBR Green I QuickGoldStar reagents (Eurogentec, Seraing, Belgium). Primers used (Table 1), were designed with the Primer Express software program (Applied Biosystems), based on sequence data from previous reports [13–25]. PCR amplification and fluorescence detection were performed using procedures and conditions recommended by the manufacturer. All glycosyltransferase and MUC5AC mRNAs expression data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels for each individual sample.

SDS-PAGE and Western blotting to detect sialyl-Lewis x antigen and MUC5AC protein levels

SDS-PAGE and Western blot analysis were performed by methods, as previously described [26]. Briefly, each harvested cell sample was lysed in buffer at 4°C for 15 min. Cell lysates containing 25 μg protein, determined using the method of Lowry *et al.* [27], were denatured in reducing sample buffer and electrophoresed on a 8% polyacrylamide gel for 150 min at 20 mA with α_2-macroglobulin (Sigma) used as molecular weight standard. After electrophoresis, samples were transferred to PVDF membranes in a transfer buffer composed of 10 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (Dojindo, Kumamoto, Japan), and 10% methanol for 90 min at 100 mA. The membrane was then blocked with 1% tween phosphate buffered saline and incubated with mouse monoclonal anti-sialyl-Lewis x antibody (CSLEX1, 1:150, BD Biosciences Pharmingen, San Diego, CA) or mouse monoclonal MUC5AC antibody (1:100, Zymed, South San Francisco, CA) for 24 h. Antibody-treated membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgM and IgG (1:500) for the anti-sialyl-Lewis x monoclonal antibody and the monoclonal MUC5AC antibody for 1 h, respectively. Spots were color-developed with 0.02% 3,3′ diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan) containing 4.5×10^{-3}% hydrogen peroxide and analyzed using the National Institutes of Health Image program (NIH-Image, version 1.59, the U.S. National Institutes of Health).

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Data were statistically analyzed using Student' *t*-test or Dunnett's-type multiple comparison test. Probability of less than 0.05 was regarded as a significant level.

Results

Effects of TNF-α on glycosyltransferases and MUC5AC mRNA expression

In order to determine the mechanism by which sialyl-Lewis x epitopes are overexpressed during airway inflammation, we investigated the basal expression levels of glycosyltransferase and MUC5AC mRNAs as well as changes in the expression of the

Table 1. Oligonucleotide primers for amplifying glycosyltransferases, MUC5AC and GAPDH mRNAs. (F, forward; R, reverse)

Oligo name	*Sequence*	*Ref. No.*
hST3GalI	F: 5′ CAGCAGGAGTGAAGTCGAGCTTA 3′ R : 5′ ATCAAAAGGGATGGCAGGAAT 3′	[13]
hST3GalII	F: 5′ GTGGACGGGCACAACTTCAT 3′ R : 5′ TGCCAACATCCTGCTCAAAG 3′	[14]
hST3GalIII	F: 5′ TGGACAAACACTAGGCTCAGAGTATG 3′ R : 5′ CAGCAGGCAGTTTAGAGTCCAGAT 3′	[15]
hST3GalIV	F: 5′ ATGCAACAGCCACGGAAGAT 3′ R : 5′ GGAGGGCCAGCGTGATG 3′	[16]
hST3GalVI	F: 5′ GCCAGCCATGAGAGGGTATCT 3′ R : 5′ GTTCCCCATAATATGCAATGCA 3′	[17]
hST6GalI	F: 5′ CGCAGTCCTGAGGTTTAATGG 3′ R : 5′ CAGGCGAATGGTAGTTTTTGTG 3′	[18]
FUT1	F: 5′ CCAGGGCGATGTGACGTT 3′ R : 5′ TGTGTGAGCAGGGCAAAGTC 3′	[19]
FUT2	F: 5′ CCAGCTAACGTGTCCCGTTT 3′ R : 5′ GGAAAGGAGAAAGGCATCTGAA 3′	[19]
FUT3/5/6	F: 5′ AAGCCCTGGACAGATACTTCAATC 3′ R : 5′ AGCCGTAGGGCGTGAAGAT 3′	[20]
FUT4	F: 5′ TGAAATAGCTTAGCGGCAAGAAG 3′ R : 5′ ACAAATATGGCCTGTGGCAGTT 3′	[20]
FUT7	F: 5′ ATCTGCGGGTGGATGTCTTT 3′ R : 5′ TAGTCGCGGTGCTGAGGAGTTC 3′	[21]
FUT9	F: 5′ CAAATCCCATGCAGTTCTGATC 3′ R : 5′ TGGCCTAGCTTGCTGAGGTAA 3′	[22]
C2/4GnT	F: 5′ GACATCGATAAGGGTGCTCCTT 3′ R : 5′ AGCCCCATAAACGCAGATAGC 3′	[23]
MUC5AC	F: 5′ GCGTGGAGAATGAGAAGTATGCT 3′ R : 5′ CAAACATGCAGTTCGAGTAGTAGGTT 3′	[24]
GAPDH	F: 5′ CCCCCACCACACTGAATCTC 3′ R : 5′ GCCCCTCCCCTCTTCAAG 3′	[25]

corresponding mRNAs induced by TNF-α in NCI-H292 cells using real-time RT-PCR. For determination of FUT3, FUT5 and FUT6 mRNA expressions, however, it is extremely difficult to design their specific primers for real-time RT-PCR, because the cDNA sequences of FUT3, FUT5 and FUT6 show a high homology among these genes [28]. We thus determined whether NCI-H292 cells would express FUT3, FUT5 and FUT6 mRNAs instead by using specific primers designed for reverse transcription-polymerase chain reaction (RT-PCR). As a result, FUT3 mRNA was found expressed in basal and TNF-α-treated NCI-H292 cells, whereas FUT5 and FUT6 mRNAs were not detected (data not shown). Therefore, FUT3/5/6 primers, which are designed even for real-time RT-PCR, could have determined FUT3 mRNA level in NCI-H292 cells.

As shown in Figure 1, basal expression levels of hST3GalIV, FUT3, C2/4GnT and MUC5AC mRNAs involved in the regulation of sialyl-Lewis x epitopes, were higher in NCI-H292 cells, as compared to hST3GalI, hST3GalII, hST3GalIII, hST3GalVI, hST6GalI, FUT1, FUT2, FUT4, FUT7 and FUT9 mRNAs. The expression levels of the hST3GalIV, FUT3, C2/4GnT and MUC5AC mRNAs, when treated with TNF-α at 40 ng/ml for 24 h, were significantly increased when compared to control or basal levels. When cells were treated with TNF-α (20 and 40 ng/ml) for 0-, 4-, 12-, 24-, 36- or 48-h, the expression levels of these mRNAs increased in a time-dependent manner, reaching maximal values after application of 40 ng/ml for 24 h (data not shown). Expression levels of hST3GalI, hST6GalI, FUT7 and FUT9 mRNAs were also significantly increased, when treated with TNF-α at 40 ng/ml for 24 h. Because, however, the basal levels of their expression were much lower than those of hST3GalIV, FUT3, C2/4GnT and MUC5AC mRNAs, their maximally increased levels were still correspondingly lower. Gene expression levels for hST3GalII, hST3GalIII, hST3GalVI, FUT1, FUT2, and FUT4 mRNAs were not altered by treatment with TNF-α. These results indicate that hST3GalIV, FUT3 and C2/4GnT mRNAs, which are related to the biosynthesis of sialyl-Lewis x epitopes, are the principal glycosyltransferase genes expressed in NCI-H292 cells and that TNF-α treatment causes an up-regulation in their transcription.

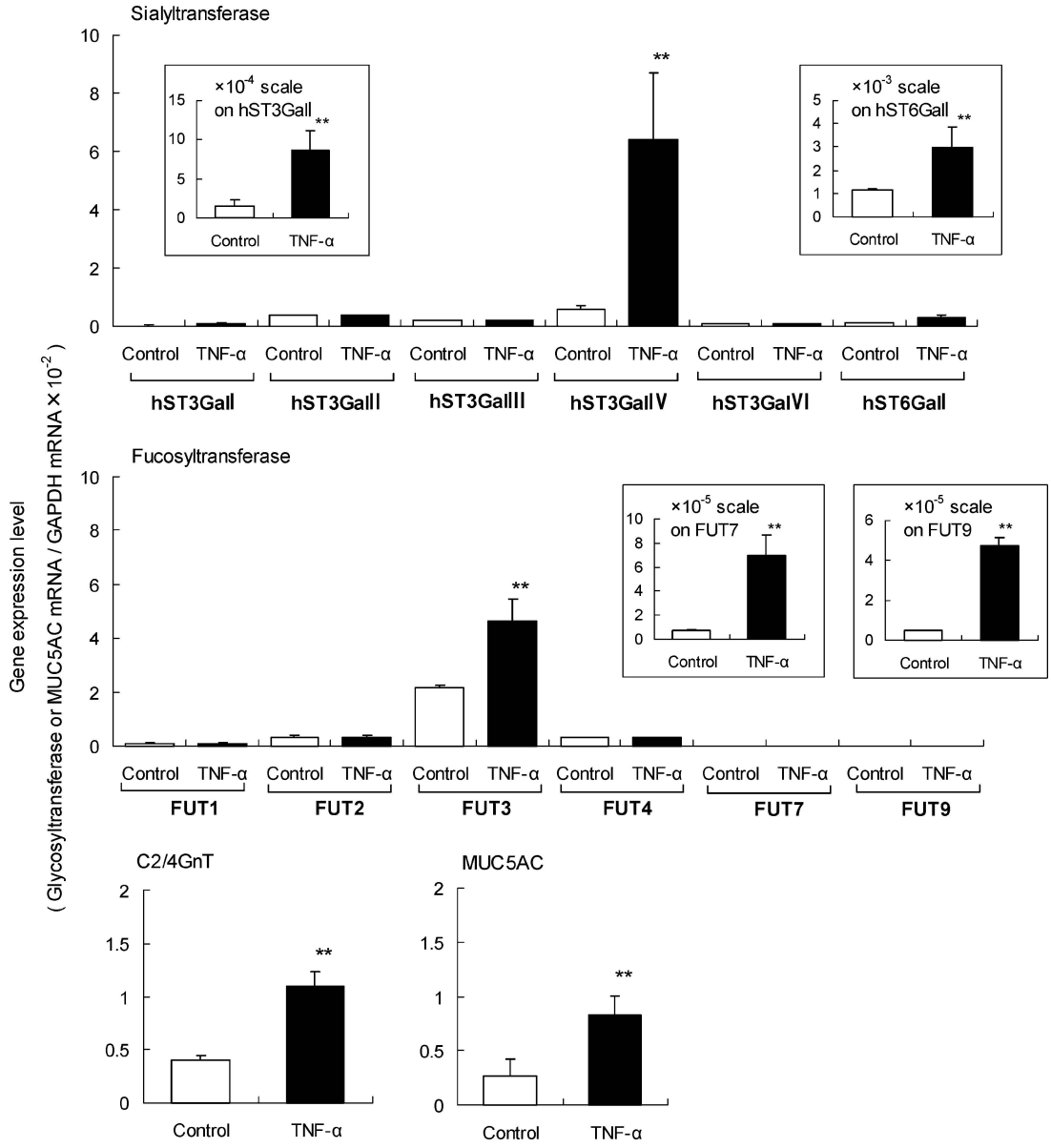

Figure 1. Expression of glycosyltransferases and MUC5AC mRNAs in NCI-H292 cells treated with 40 ng/ml TNF-α for 24 h. Gene expression was measured using real-time RT-PCR method. Squared areas represent enlargements for comparison between TNF-α and PBS-treated control groups. Data are presented as mean $\pm$ SD ($n = 4$). $^{**}P < 0.01$ vs Control (Student's t-test).

Effects of EGF and TNF-α/EGF on glycosyltransferase and MUC5AC mRNA expression

We also examined the effects of EGF on the mRNA expression of the various glycosyltransferase and MUC5AC genes. As shown in Figure 2A, EGF induced significant down-regulations of the expression levels of hST3GalIV, FUT3 and C2/4GnT, which are the consequences of principal glycosyltransferase genes expression in NCI-H292 cells, as compared to control. hST6GalI, FUT7 and FUT9 mRNA expression levels, which were originally very low in basal NCI-H292 cells, were significantly down-regulated by EGF, as compared to control. hST3GalI mRNA expression level was not affected even after the treatment of EGF (Figure 2B). Indeed, It is known that hST3GalI mRNA has little relation to the biosynthesis of sialyl-Lewis x epitopes in carcinoma cell lines [23]. Furthermore, administration of AG1478, an EGFR tyrosine kinase inhibitor, blocked the EGF-induced effect on the glycosyltransferase mRNAs (excluding hST3GalI mRNA). In contrast, EGF increased the expression of MUC5AC mRNA and as expected, AG1478 was also able to completely block this EGF-induced effect.

To further investigate the effects on mRNA expression in NCI-H292 cells, we administered both TNF-α and EGF concurrently (Figure 3). The combination of TNF-α and EGF was

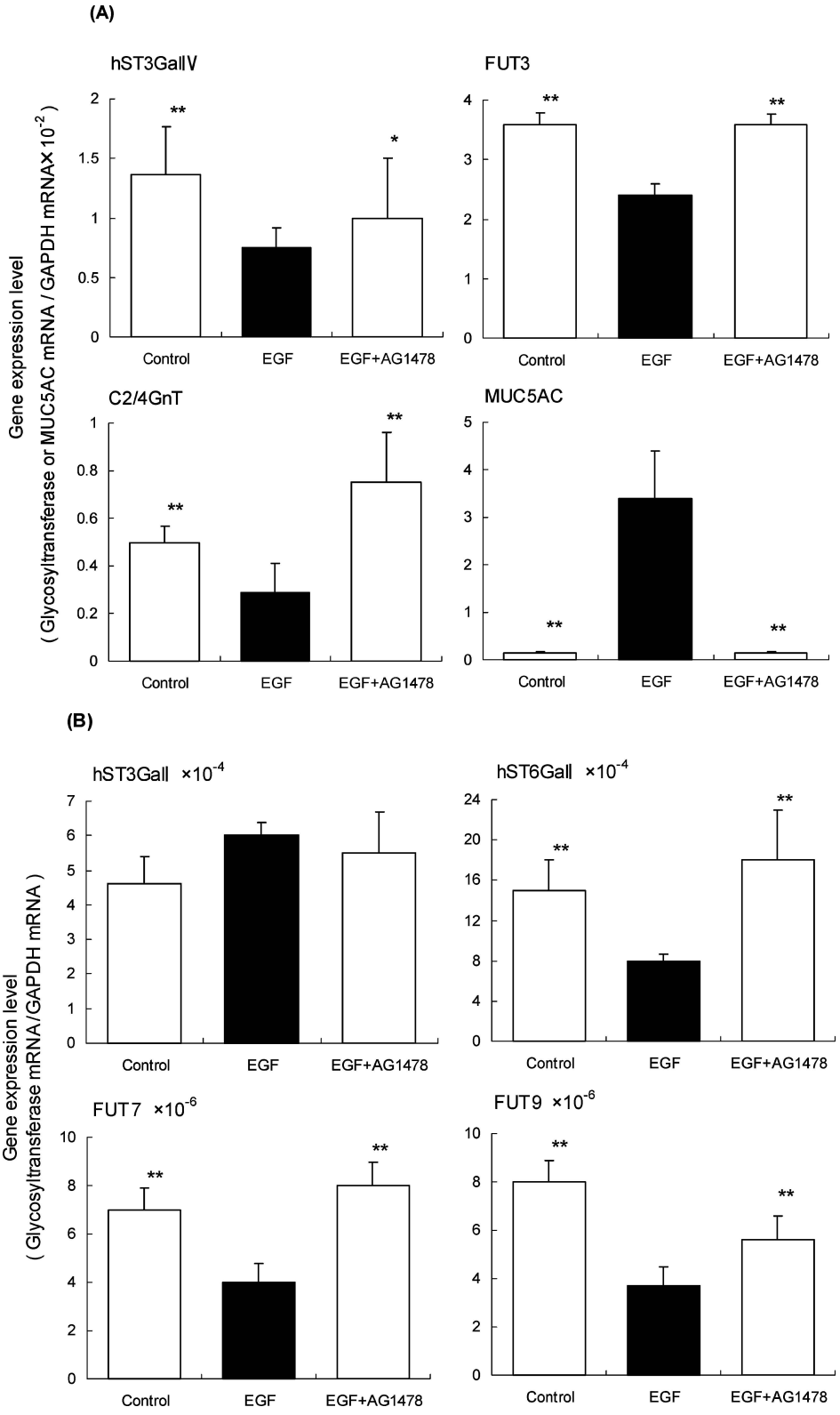

Figure 2. EGF-induced changes in expression of glycosyltransferase and MUC5AC mRNAs and the effects of AG1478, an EGFR tyrosine kinase inhibitor. Cells were incubated with 25 ng/ml EGF or pretreated with 10 μM AG1478 for 30 min prior to incubation with 25 ng/ml EGF. mRNAs expression was measured by real-time RT-PCR. hST3GalIV, FUT3, C2/4GnT and MUC5AC mRNA (A), hST3GalI, hST6GalI, FUT7 and FUT9 mRNA (B). Data are presented as mean ± SD ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs EGF-treatment (Dunnett's multiple comparison test).

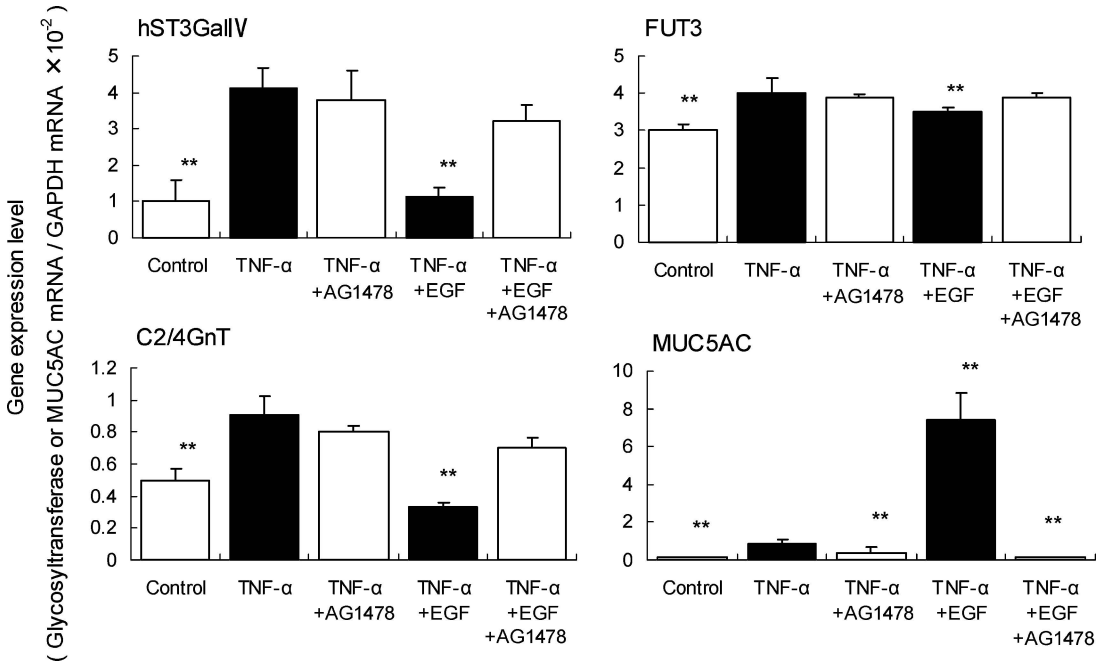

Figure 3. Effects of AG1478, an EGFR tyrosine kinase inhibitor, on the regulation of glycosyltransferase and MUC5AC mRNA expression induced by TNF-α and TNF-α in combination with EGF. Cells were incubated with 40 ng/ml TNF-α or 40 ng/ml TNF-α plus 25 ng/ml EGF. For tyrosine kinase inhibition experiments, cells were pretreated with 10 μM AG1478 for 30 min prior to incubation with 40 ng/ml TNF-α or 40 ng/ml TNF-α plus 25 ng/ml EGF. mRNAs expression was measured by real-time RT-PCR. Data are presented as mean $\pm$ SD ($n = 4$). $^{**}P < 0.01$ vs TNF-α-treatment (Dunnett's multiple comparison test).

able to induce greater up-regulation of MUC5AC mRNA than either TNF-α or EGF alone, and once again, the tyrosine kinase inhibitor, AG1478 was able to completely block this amplified up-regulation of MUC5AC mRNA, results in agreement with a previous report [7]. In the cases of hST3GalIV, FUT3 and C2/4GnT mRNAs which are related to the biosynthesis of sialyl-Lewis x epitopes and the consequences of principal glycosyltransferase genes expressed in NCI-H292 cells, the previously observed increase in expression due to TNF-α was considerably diminished due to the presence of EGF. When AG1478 was then added, the down-regulation in gene expression related to EGF was completely abolished. However, in the case of solely TNF-α induced changes in gene expression, the addition of AG1478 had no effect (Figure 3). These results indicate that EGF had opposing effects on the expression of glycosyltransfease and MUC5AC mRNAs. We therefore suggest that EGF may control the expression of these mRNAs bi-directionally despite the mutual involvement of the EGFR tyrosine kinase signaling pathway in NCI-H292 cells.

Effect of U73122 on glycosyltransferase and MUC5AC mRNA expression

As shown earlier, the increased expression of glycosyltransferase mRNAs due to TNF-α was not related to EGFR tyrosine kinase signaling, as the inhibitor AG1478 did not have any effect on the amplified gene expression. To examine the mechanism by which TNF-α-induced increased expression of these glycosyltransferase mRNAs, we examined activated PI-PLC, which has previously been reported to be involved in the biosynthesis of sugar chains [29] and expression of sialyl-Lewis x epitopes in colorectal carcinoma cells [30]. Additionally, PI-PLC signaling has been previously shown to be activated by TNF-α in NCI-H292 cells [31]. In this study, we also confirmed that the PI-PLC activity in this NCI-H292 cells increased approximately 4-fold after treatment with TNF-α for 24 h (data not shown). U73122, a PI-PLC inhibitor, was used to determine whether it would be able to block the expression of hST3GalIV, FUT3 and C2/4GnT mRNAs that are involved in the synthesis of sialyl-Lewis x epitopes, in NCI-H292 cells treated with TNF-α. Results showed that U73122 significantly suppressed the expression of these glycosyltransferase mRNAs, as compared to solely TNF-α treatments (Figure 4), suggesting that TNF-α may induce the up-regulation of these glycosyltransferase mRNAs through the activation of the PI-PLC pathway in NCI-H292 cells.

Sialyl-Lewis x antigen and MUC5AC protein expression stimulated by TNF-α and TNF-α in combination with EGF

From our results showing that TNF-α could increase the expression of the hST3GalIV, FUT3 and C2/4GnT genes involved in the biosynthesis of sialyl-Lewis x epitopes, and that EGF reduced the expression of these mRNAs, we then investigated the effects of TNF-α and TNF-α in combination with EGF on glycosylation levels. To analyze the expression of sialyl-Lewis

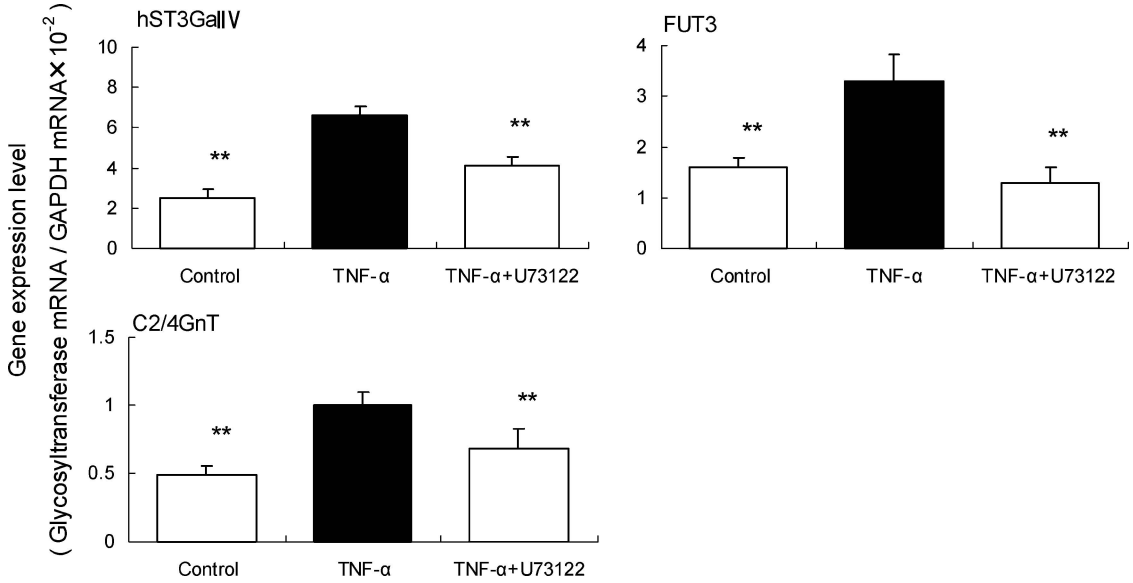

Figure 4. Effects of U73122, a PI-PLC inhibitor, on changes in expression of glycosyltransferase mRNAs induced by TNF-α. Cells were incubated with 40 ng/ml TNF-α or pretreated with 10 μM U73122 for 30 min prior to incubation with 40 ng/ml TNF-α. mRNAs expression was measured by real-time RT-PCR. Data are presented as mean $\pm$ SD ($n = 4$). $^{**}P < 0.01$ vs TNF-α-treatment (Dunnett's multiple comparison test).

x epitopes in NCI-H292 cells we used SDS-PAGE and Western blotting, with NIH-Imaging analysis for quantification studies. As shown in Figure 5, the band representing the sialyl-Lewis x antigen was present as a broad band at high molecular weight (>720 kDa) in control cell lysates. After treatment with TNF-α for 48 h, antigen levels were increased in cell lysates, as compared to control experiments. When treated with both TNF-α and EGF, the expression levels of the sialyl-Lewis x antigen in cell lysates was diminished compared to cells treated with only TNF-α. These results demonstrate that the changes in sialyl-Lewis x antigen correspond directly to the expression levels of the hST3GalIV, FUT3 and C2/4GnT glycosyltransferase genes when NCI-H292 cells were treated with TNF-α or TNF-α in combination with EGF.

In addition to production of sialyl-Lewis x epitopes, we also analyzed the protein expression of MUC5AC in NCI-H292 cells, when treated with TNF-α or TNF-α and EGF (Figure 5). Our results show that in control experiments without the addition of growth factors or cytokines, the band representing MUC5AC protein was faint and barely detectable. However, the presence of MUC5AC protein became evident as indicated by a broad band of high molecular weight (>720 kDa) when cells were treated with TNF-α for 48 h, results corresponding to the changes in the expression of the glycosyltransferases. MUC5AC protein from cell lysates after TNF-α and EGF treatment for 48 h was more obvious as a thicker, more predominant band than that in cell lysates treated with only TNF-α. Our results therefore suggest that stimulating NCI-H292 cells with TNF-α can induce increases in both sialyl-Lewis x epitopes and MUC5AC protein but that supplementation with EGF has contrasting effects, inducing the down-regulation of sialyl-Lewis x epitopes, while increasing MUC5AC protein expression.

Discussion

In this study we sought to examine the mechanism by which treatment with cytokines and growth factors such as TNF-α and EGF affected the expression of sialyl-Lewis x epitopes in NCI-H292 cells. TNF-α was able to increase the expression of hST3GalIV, FUT3, C2/4GnT and MUC5AC mRNAs, which are involved in the biosynthesis of sialyl-Lewis x epitopes and MUC5AC protein. Supplementation with EGF however, down-regulated the expression of these glycosyltransferase mRNAs while stimulating the expression of MUC5AC mRNA. We believe that EGFR tyrosine kinase phosphorylation leads to decreased expression of these glycosyltransferase mRNAs and sialyl-Lewis x epitopes in NCI-H292 cells, even though the expression of both MUC5AC mRNA and protein were increased.

It is also well known that TNF-α activates many signaling pathways including the nuclear factor (NF)-κB, protein kinase C, tyrosine kinase and PI-PLC [31] cascades. We were interested in examining the effects of TNF-α on the glycosyltransferase mRNA expression in our selected airway carcinoma cell line. In this study, we found that the TNF-α induced up-regulation of these glycosyltransfease mRNAs was not related to the EGFR tyrosine kinase pathway. When cells were treated with AG1478, an EGFR tyrosine kinase inhibitor, TNF-α induced changes in gene expression were not repressed. Furthermore, it has been reported that the hST3GalIV promoter lacks a binding site for NF-κB [32,33], and thus believed that

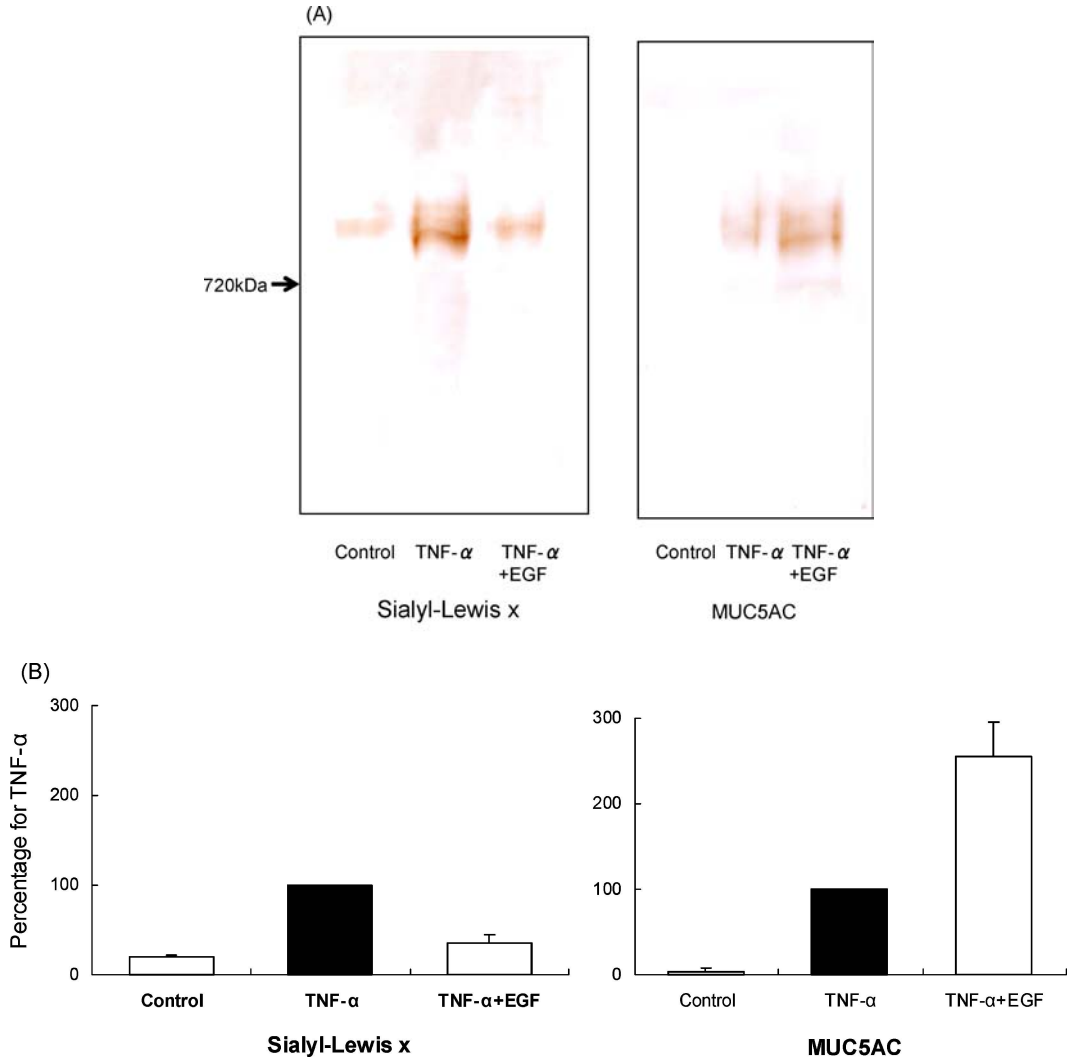

Figure 5. Western blot analysis of sialyl-Lewis x antigen and MUC5AC protein in NCI-H292 cells treated with 40 ng/ml TNF-α or 40 ng/ml TNF-α plus 25 ng/ml EGF for 48 h. Cell lysates were separated by SDS-PAGE on an 8% polyacrylamide gel, and then transferred to PVDF membranes. Sialyl-Lewis x antigen (left panel) and MUC5AC protein (right panel) present on membranes were evaluated by Western blot analysis with CSLEX1 and mouse MUC5AC antibody, respectively, and color-developed with horseradish peroxidase-conjugated goat anti-mouse IgM or IgG and 3,3′ diaminobenzidine tetrahydrochloride containing hydrogen peroxide. Results presented are representative of four experiments (A). Spots were analyzed using the National Institutes of Health Image program. Data are then shown as the average percentage for values with only TNF-α treatment ($n = 4$) (B).

NF-κB, a downstream mediator of TNF-α, cannot directly induce hST3GalIV gene expression.

When we pretreated cells with U73122, a PI-PLC inhibitor, TNF-α-mediated expression of the glycosyltransferase mRNAs was inhibited. We therefore presume that TNF-α produces these changes in the expression of glycosyltransferase mRNAs through the activation the PI-PLC pathway. Recently, Chen *et al.* reported that TNF-α could activate binding of NF-κB to DNA and subsequently stimulate the production of cyclooxygenase-2 (COX-2) through the activation of the gene promoter [31]. They also showed that U73122 was able to suppress these TNF-α-mediated effects in NCI-H292 cells. Kakiuchi *et al.* [34] showed that the activation of COX-2 caused an increase in the expression of both sialyl-Lewis a epitopes and hST3GalIV in colon cancer cells. Together, these findings suggest that in addition to the activation of the EGFR tyrosine kinase pathway, inflammatory mediators such as COX-2, which are regulated by PI-PLC pathways, may be involved in the expression of these glycosyltransferases (hST3GalIV, FUT3, and C2/4GnT).

Sialyl-Lewis x epitopes appear to have a very similar molecular weight to MUC5AC protein in Figure 5A. However, it is reported that other mucin proteins, such as MUC1, MUC4 and MUC5B protein were strongly expressed as well as MUC5AC protein in NCI-H292 cells, and the molecular weight of these mucin proteins were also similar range to MUC5AC protein

[35]. These finding suggest that sialyl-Lewis x epitopes are not only present on MUC5AC protein but also other mucin proteins, MUC1, MUC4 and MUC5B, in NCI-H292 cells.

It is known that some glycosyltransferase mRNA expression is regulated by the interaction between transcription factors and binding to corresponding promoters. For example, hST3GalIV mRNA is regulated mainly via AP2 binding sites of the hST3GalIV 5′-flanking region [32] and MUC5AC mRNA through Sp1 sites of the MUC5AC 5'-flanking region [36]. This suggests that the expression of these mRNAs, involved in the biosynthesis of sialyl-Lewis x epitopes and MUC5AC protein, may be regulated through opposing pathways differential transcription factor binding sites of their respective promoters. It is of great interest to further study and elucidate the regulatory factors influencing the expression of these glycosyltransferases and MUC5AC.

In conclusion, our results suggest that the expression of sialyl-Lewis x, with differing regulations from those involved in the expression of MUC5AC, is controlled by pathways comprising EGF receptor tyrosine kinase and PI-PLC signaling.

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