Glycoconj J (2011) 28:99–110
DOI 10.1007/s10719-011-9327-4

Regulation of glycosyltransferases and Lewis antigens expression by IL-1β and IL-6 in human gastric cancer cells

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Received: 22 November 2010 / Revised: 13 January 2011 / Accepted: 17 February 2011 / Published online: 2 March 2011

Abstract Inflammation of stomach mucosa has been postulated as initiator of gastric carcinogenesis and the presence of pro-inflammatory cytokines can regulate specific genes involved in this process. The cellular expression pattern of glycosyltransferases and Lewis antigens detected in the normal mucosa changed during the neoplassic transformation. The aim of this work was to determine the regulation of specific fucosyltransferases and sialyltransferases by IL-1β and IL-6 pro-inflammatory cytokines in MKN45 gastric cancer cells. IL-1β induced significant increases in the mRNA levels of FUT1, FUT2 and FUT4, and decreases of FUT3 and FUT5. In IL-6 treatments, enhanced FUT1 and lower FUT3 and FUT5 mRNA expression were detected. No substantial changes were observed in the levels of ST3GalIII and ST3GalIV. The activation of FUT1, FUT2 and FUT4 by IL-1β is through the NF-κB pathway and the down-regulation of FUT3 and FUT5 by IL-6 is through the gp130/STAT-3 pathway, since they are inhibited specifically by panepoxydone and AG490, respectively. The levels of Lewis antigens after IL-1β or IL-6 stimulation decreased for sialyl-Lewis x, and no significant differences were found in the rest of the Lewis antigens analyzed, as it was also observed in subcutaneous mice tumors from MKN45 cells treated with IL-1β or IL-6. In addition, in 61 human intestinal-type gastric tumors, sialyl-Lewis x was highly detected in samples from patients that developed metastasis. These results indicate that the expression of the fucosyltransferases involved in the synthesis of Lewis antigens in gastric cancer cells can be specifically modulated by IL-1β and IL-6 inflammatory cytokines.

Keywords Fucosyltransferases · Inflammatory cytokines · Lewis antigens · IL-1β · IL-6

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Introduction

Lewis blood group antigens, associated with glycoproteins and glycolipids, are oligosaccharides present in hematopoietic and epithelial cells [1–4]. They are terminal structures synthesized by the sequential action of different glycosyltransferases, in particular fucosyltransferases (FUTs) and sialyltransferases (STs), on the precursor type 1 (Galβ1→3GlcNAc-R) or type 2 (Galβ1→4GlcNAc-R) chains. In the first step, FUT2 and ST3GalIII act preferentially in type 1 structures whereas for type 2 chains FUT1 and ST3GalIV and ST3GalVI are the most specific enzymes, and the α1,3-4 fucosyltransferases add fucose in the last step of the Lewis antigens synthesis. These glycosyltransferases show a complex tissue- and cell type-specific expression pattern that varies during development and malignant transformation. In the normal gastric mucosa, cells of the foveolar epithelium express FUT2 and type 1 Lewis antigens (Lewis b, Lewis a and sialyl-Lewis a), deep gland cells express FUT1 and type 2 Lewis antigens (Lewis y, Lewis x and sialyl-Lewis x), and the α1,3-4 fucosyltransferase FUT3 is detected in both populations [5]. In gastric tumors, co-expression of both types of Lewis antigens has been observed as a consequence of a

de-regulated expression pattern of FUTs and an increase in the expression of STs, such as ST3GalIII, ST3GalIV or ST3GalVI [6–8].

The fucosylated oligosaccharides located at the cell membrane participate in biological processes as implantation, embryogenesis, tissue differentiation, tumor metastasis, inflammation and bacterial adhesion [9–12]. In the stomach, the colonization of the gastric mucosa by *Helicobacter pylori* (*H. pylori*) is a risk factor for gastric cancer development. *H. pylori* binds to the gastric cells through the bacterial adhesins: BabA mediates the adhesion to the gastric mucosa binding Lewis b and type 1 structures [13], while SabA binds sialyl-Lewis x [14]. Also, the association of sialylated Lewis antigens with increased invasion and metastasis of gastric tumors [15–18] is related to their capacity to bind to Selectins [19]. The infection of the stomach mucosa with *H. pylori* induces chronic gastritis that is the first step in the progression to gastric cancer. In this process, different pro-inflammatory cytokines are released, such as TNF-α, IL-1β and IL-6 [20–23], which can activate genes involved in the gastric neoplassic transformation, like the highly glycosylated intestinal mucins, MUC2, regulated by TNF-α and IL-1β through the NF-κB signaling pathway [24], and MUC4 activated by IL-6 through gp130/STAT3 pathway [25].

In this work we have analyzed, in the MKN45 gastric cancer cell line, the effect of the pro-inflammatory cytokines IL-1β and IL-6, on the expression of the fucosyl- (FUT1, FUT2, FUT3, FUT4, and FUT5) and sialyltransferases (ST3GalIII and ST3GalIV) and the signaling pathways associated to their regulation. Also, the changes on the levels of Lewis antigens induced by the cytokines treatments in these cells and in a mouse xenograft model have been evaluated. In addition, Lewis antigens expression has been also analyzed in human intestinal-type gastric tumors and related to specific clinical characteristics of the patients.

Materials and methods

Reagents and antibodies

IL-1β and IL-6 were purchased from PeproTech EC. The specific inhibitor of STAT3 activation, AG490, and the specific inhibitor of IκBα phosphorylation, panepoxydone, were obtained from Calbiochem and Alexis Biochemicals, respectively. The primary antibodies used in this study were: T-174 (Lewis a), T-218 [26] and 2.25 (Lewis b), 19-0le (H-type 2) [27], 77/180 (Lewis y), 57/27 (sialyl-Lewis a) [28], CsLex-1 [29] and KM93 (Chemicon Int.) (sialyl-Lewis x). Anti-p-STAT3 (Tyr705) and anti-p-IkBα (Ser32/36) were purchased from Cell Signaling. Anti-β-actin was obtained from Sigma-Aldrich. Secondary anti-mouse and anti-rabbit HRP (Dako) were used for immunohistochemistry, and Alexa Fluor 488 (Invitrogen) for flow cytometry.

Cell culture and treatments

The human gastric cancer cell line MKN45 was obtained from ATCC, and cultured in DMEM-10%FBS under standard conditions. For cytokine treatments, 60–70% confluent cells were rinsed in phosphate-buffered saline (PBS) before the incubation with 40 ng/ml IL-1β or IL-6 diluted in DMEM, for 5, 10, and 20 h (for glycosyltransferases mRNA detection) or 40 h (for Lewis antigens detection). All treatments were performed in duplicate and at least three independent experiments were done.

To prevent the NF-κB activation, cells were incubated with 40 ng/ml of IL-1β in combination with 5 μg/ml of panepoxydone for 5 and 20 h. STAT3 phosphorylation was inhibited by incubating cells with 40 ng/ml of IL-6 with 60 μM of AG490 for 5 and 20 h. The doses and conditions for these treatments were established previously [24, 25].

RNA extraction and quantitative RT-PCR

Total RNA extraction was carried out from control and cytokine-stimulated cells using GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich). After rDNAse I (Ambion) treatment, mRNA levels of FUT1, FUT2, FUT3, FUT4, FUT5, ST3GalIII, and ST3GalIV, were quantified in triplicate using QuantiTect SYBR green RT-PCR (Qiagen). The primers used for amplification of FUT1, FUT2, FUT3 and FUT4 were described by Higai [30], FUT5 primers were: F: 5′-TGGGTGTGACCTCGGCGTGA-3′, and R: 5′-AAACCAG CCTGCACCATCGCC-3′, ST3Gal III primers were: F: 5′-GGTGGCAGTCGCAGTCGGCAGGATTT-3′, and R: 5′-CATGCGAACGGTCTCATAGTAGTG-3′, and ST3GalIV primers were: F: 5′-CGGGTGCGAAAGGGTTT-3′ and R: 5′-GGGCTCCGAGACCTGAGGGG-3′. Hypoxanthine-guanine phosphoribosyl transferase (HPRT) mRNA (GeneCardsdatabase, NCBI36:X) was analyzed as an internal control. Data collection was performed on the ABI Prism 7900HT systems according to the manufacturer's instructions. At least two independent experiments were performed. Gene levels are expressed normalized to HPRT.

Cell lysates and Western blot analysis

Cellular pellets were solubilized in 2X SDS gel sample buffer (20 mM dithiothreitol, 6% SDS, 0.25 M Tris-HCl pH 6.8, 10% glycerol, and bromophenol blue) and sonicated

using 2 bursts of 10 s each. Lysates were boiled at 95°C for 5 min and immediately cooled on ice. Protein extracts were electrophoresed on 10% (p-IkBα and β-actin) or 8% (p-STAT3) SDS-polyacrylamide gels. Separated proteins were blotted onto nitrocellulose membranes (Protran), blocked for 1 h at RT, and incubated overnight with the specific primary antibodies following the manufacturer's instructions. ECL Western Blotting Substrate, for p-IkBα and β-actin, or Supersignal West Femto, for p-STAT3, were used.

Flow cytometry

Cultured cells were treated for 20 and 40 h with IL-1β or IL-6. Untreated and treated cells were trypsinized, counted, and 2.5×10^5 viable cells were incubated with the primary antibodies (30 min at 4°C) diluted in PBS-1% bovine serum albumin (BSA). Cells were rinsed in PBS-1%BSA and incubated with the secondary antibody Alexa Fluor 488 (30 min at 4°C). After washing, fluorescent analysis was performed using a FACScan (Becton Dickinson).

Development of subcutaneous tumors in mice, and IL-1β and IL-6 treatments

Eight-week-old female BALB/c nude mice were purchased from Charles River Laboratories. For tumor development, mice were injected subcutaneously in both flanks with 10^6 MKN45 cells in a total volume of 200 μl PBS. When tumors became palpable (day 7), 50 μl PBS (n=3), 10 ng/50 μl IL-1β (n=6), or IL-6 (n=6) were intratumorally injected. Cytokine or PBS treatments were repeated every 3 days (4 times). All animals were sacrificed on day 24. Tumors were fixed in formol and embedded in paraffin for classification and immunohistochemical analysis.

Mice were given food and water *ad libitum* during all the experimental period. All the experiments involving mice were conformed to the Ethics Committee of Animal Experimentation of the IMIM-Hospital del Mar. Animal protocols, were previously approved by the Departament de Medi Ambient i Habitatge (DAMB) of the Generalitat de Catalunya (approval number 4078).

Human tissue samples and immunohistochemistry

Stomach cancer tissue samples (n=61) were obtained from the paraffin-embedded tissue bank of the IMIM-Hospital del Mar, and the study was approved by the Ethics Committee (CEIC) of the Institution. Tissue samples were processed in 4 μm sections and haematoxylin-eosin staining was used for diagnostic purposes. For immunohistochemical analysis, the primary antibodies were used as supernatant at a 1/2 dilution in PBS-1%BSA, except the 19-0LE MoAb that was used as ascites diluted at a 1/1000, and KM93 according to manufacturer's instructions. Indirect immunoperoxidase technique was performed as described [5] and sections were developed with DAB (Dako).

Statistical analysis

To compare the differences observed in the expression of fucosyl- and sialyltransferases and Lewis antigens after cytokine treatments the Student's t-test was used. Mann-Whitney U-test was utilized for the expression of Lewis antigens in mouse tumors, and in human tumor samples to evaluate the association between Lewis antigens expression and specific clinical parameters of the patients. Statistical analysis was performed with SPSS 15.0 (SPSS Inc.). Statistical significance was established when $p\leq0.05$.

Results

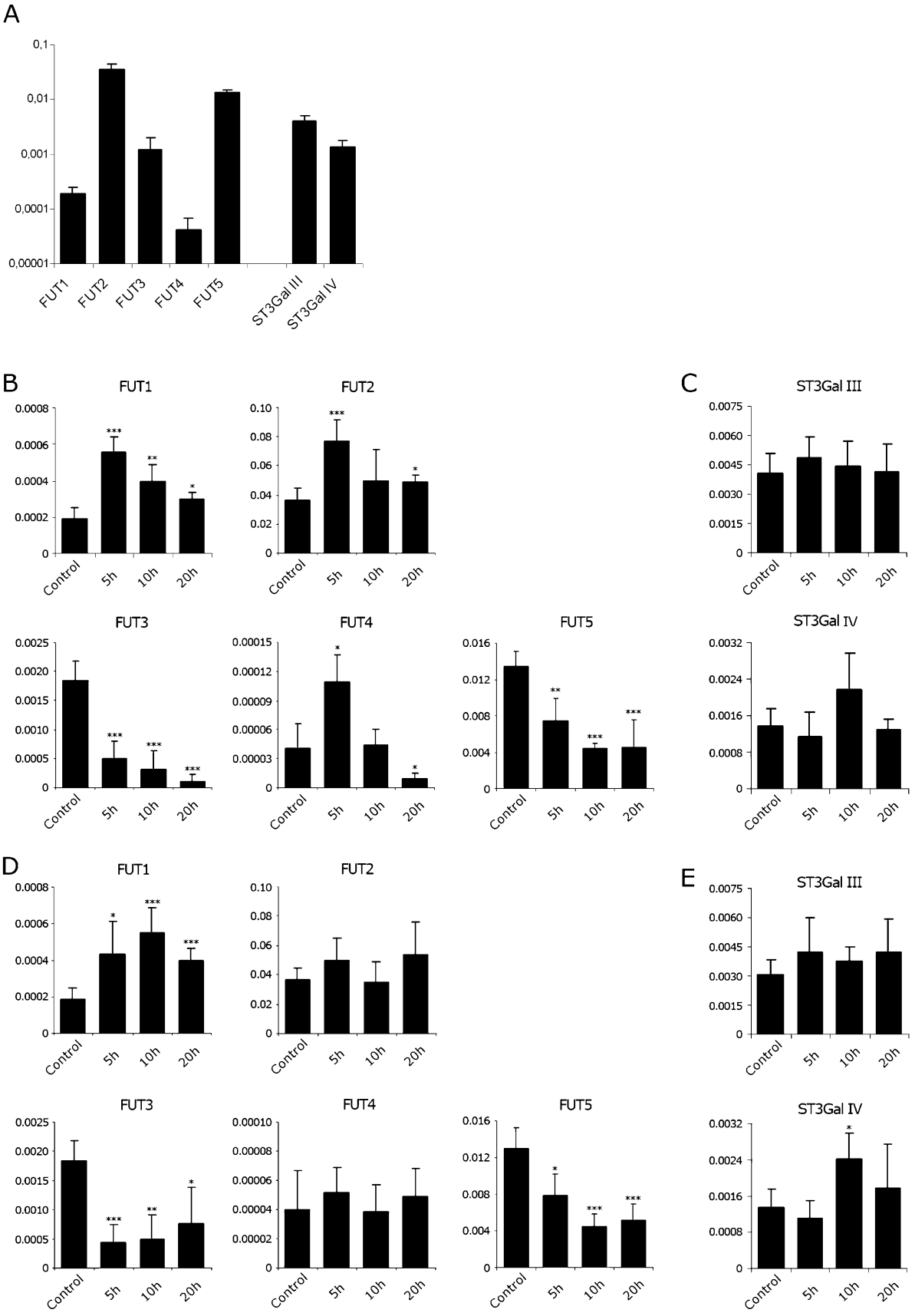
Expression of fucosyl- and sialyltransferases in MKN45 gastric cancer cells induced by IL-1β or IL-6 treatments

The MKN45 cells express high levels of FUT2 and FUT5, intermediate levels of FUT3, ST3GalIII and ST3GalIV, and low amounts of FUT1 and FUT4. The mRNA expression levels of these glycosyltransferases are shown in Fig. 1a.

To asses if the pro-inflammatory cytokines IL-1β and IL-6 induce changes in the expression of the fucosyl- and sialyltransferases, the mRNA levels of FUT1, FUT2, FUT3, FUT4 and FUT5, and ST3GalIII, and ST3GalIV enzymes were analyzed by qRT-PCR after 5, 10 and 20 h, and 40 ng/ml of IL-1β or IL-6 stimulation.

After IL-1β treatment, FUT1 mRNA levels increased significantly after 5, 10, and 20 h, although they decreased gradually (2.95, 2.12, and 1.60 folds, respectively). Similar results were observed for FUT2 mRNA after 5 h stimulation (2.12 folds), whereas at 10 and 20 h the levels were similar to the untreated cells. The mRNA expression levels of the α1,3-4 fucosyltransferases, FUT3 and FUT5, decreased significantly at 5, 10, and 20 h (16.59 folds for FUT3 and 2.97 folds for FUT5 at 20 h). The levels of FUT4 mRNA increased after 5 h of IL-1β stimulation, at 10 h were similar to the untreated cells, and at 20 h a significant decrease (4.08 folds) was observed (Fig. 1b). Regarding the STs expression, no significant changes in the mRNA levels of ST3GalIII and ST3GalIV were detected. These results are shown in Fig. 1c.

When the MKN45 cells were stimulated with IL-6, again significant increases in the mRNA expression levels of FUT1 were found after 5, 10, and 20 h of treatment, being the highest levels (2.95 folds) observed at 10 h. No statistically significant differences were found in the


A
0,1
0,01
0,001
0,0001
0,00001
FUT1
FUT2
FUT3
FUT4
FUT5
ST3Gal III
ST3Gal IV
B
FUT1
FUT2
C
ST3Gal III
FUT3
FUT4
FUT5
ST3Gal IV
D
FUT1
FUT2
E
ST3Gal III
FUT3
FUT4
FUT5
ST3Gal IV
Control
5h
10h
20h

◀ **Fig. 1** **a** mRNA expression levels of fucosyl- and sialyltransferases detected by quantitative RT-PCR in MKN45 cells. **b-e** mRNA levels of fucosyl- and sialyl-transferases in MKN45 cells treated for 5, 10 and 20 h with IL-1β (B,C) or IL-6 (D,E) (40 ng/ml). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$

expression of FUT2 and FUT4 mRNA (Fig. 1d). Significant decreases at 5 (4.15 folds), 10 (3.78 folds) and 20 h (2.41 folds) of IL-6 stimulation were observed in the mRNA levels of FUT3, and comparable results were obtained for FUT5, that at 5, 10 and 20 h was detected at lower levels (1.66, 2.90, and 2.51 folds, respectively) (Fig. 1d). The ST3GalIII levels were not changed, whereas a slight and significant increase (1.78 folds) in the ST3GalIV mRNA expression was detected after 10 h of IL-6 treatment. These results and the statistically significant differences are shown in Fig. 1e.

Changes in the mRNA expression levels of fucosyltransferases after IL-1β stimulation associated to the activation of the NF-κB signaling pathway

MKN45 cells express neither p-IκBα nor nuclear p65 transcription factor, and after IL-1β treatment they can be detected, indicating the activation of the NF-κB signaling pathway [24]. These cells were incubated with the specific inhibitor of IκBα phosphorylation, panepoxydone, simultaneously with IL-1β for 5 h to determine the implication of this pathway in the regulation of the fucosyltransferases. First, the expression of p-IκBα was determined by western blot in MKN45 cells after IL-1β or IL-1β plus panepoxydone treatments. Results indicate that IL-1β induced the phosphorylation of IκBα that was reverted by the addition of panepoxydone, as it shown in Fig. 2a.

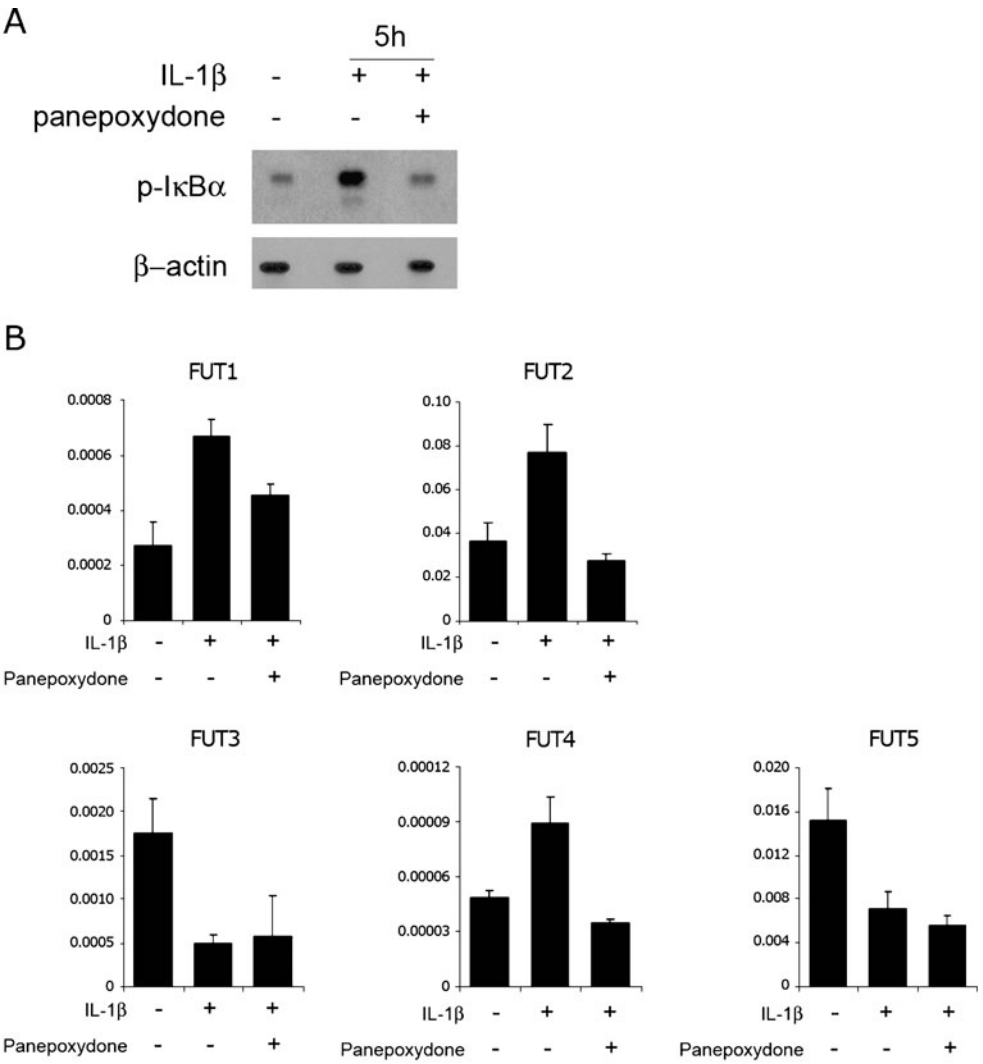


Fig. 2 MKN45 cells treated with IL-1β and panepoxydone for 5 h to block IκBα phosphorylation. **a** p-IκBα and β-actin detected by western blot. **b** mRNA levels of fucosyltransferases (FUT1, FUT2, FUT3, FUT4, and FUT5), detected by qRT-PCR. *$p \leq 0.05$, **$p \leq 0.01$

The IL-1β treatments induced significant changes in the expression levels of the FUT1, FUT2, FUT3, FUT4, and FUT5 fucosyltransferases in the MKN45 cells. The addition of panepoxydone to the 5 h IL-1β treatments reverted the effect in the mRNA levels of FUT1, FUT2 and FUT4, whereas this combination did not have effect in the expression of FUT3 and FUT5 (Fig. 2b). These results indicate that FUT1, FUT2 and FUT4 are activated through the NF-κB pathway after the IL-1β stimulation, whereas alternative pathways must be regulating the expression of FUT3 and FUT5.

Implication of the gp130/STAT3 signaling pathway in the regulation of fucosyltransferases by IL-6

MKN45 cells express the IL-6 receptor gp130, associated to the cell membrane, and the STAT3 transcription factor is constitutively active in these cells, as we have previously reported [25]. The IL-6 treatment increased p-STAT3 levels whereas the combination of IL-6 and AG490, that blocks the STAT3 activation through the inhibition of the Jak tyrosine kinase family, for 5 h induced decreased levels of p-STAT3 as it is shown in Fig. 3a.

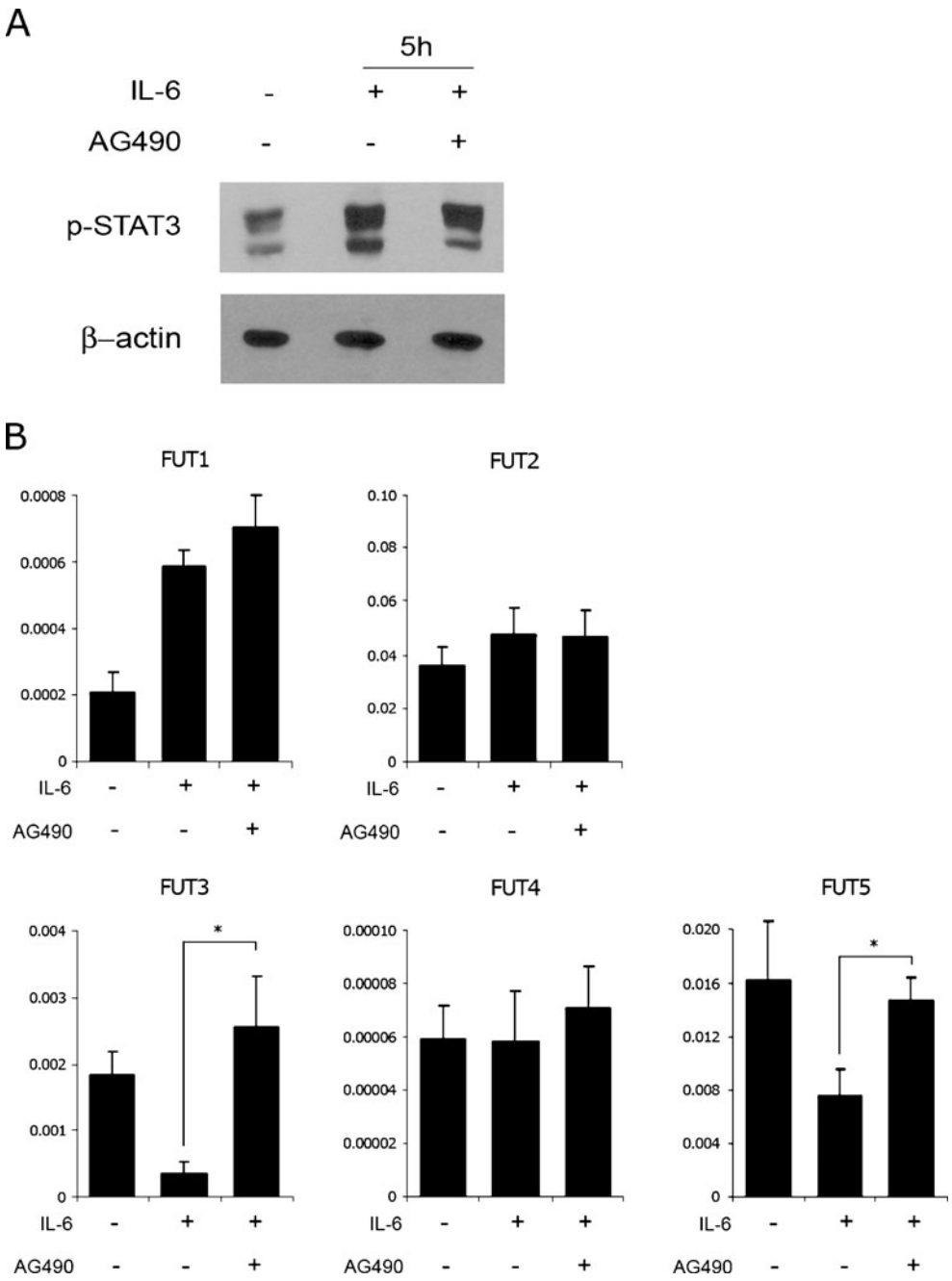


Fig. 3 MKN45 cells treated with IL-6 and AG490 for 5 h to block STAT3 phosphorylation. **a** p-STAT3 and β-actin detected by western blot. **b** mRNA levels of fucosyltransferases (FUT1, FUT2, FUT3, FUT4, and FUT5) detected by qRT-PCR. *$p \leq 0.05$

To demonstrate the direct implication of the gp130/p-STAT3 signaling pathway in the regulation of FUT1, FUT3 and FUT5, the MKN45 cells were treated with IL-6 and AG490 for 5 h. As it is shown in Fig. 3b, the mRNA levels of FUT3 and FUT5 were recovered by the addition of AG490 to the IL-6 treatment. In contrast, no differences were detected in the mRNA levels of FUT1 between the cells treated only with IL-6 or with IL-6 and AG490. As IL-6 did not alters the expression levels of FUT2 and FUT4, no effect was induced by the AG490 treatment (Fig. 3b). These results demonstrate that FUT3 and FUT5 share the same pattern of regulation by IL-6 through the STAT3 activation, whereas FUT1, FUT2 and FUT4 expression are not affected by this pathway.

Expression levels of Lewis antigens after IL-1β or IL-6 treatments in MKN45 gastric cancer cells

After treatments with 40 ng/ml of IL-1β or IL-6 for 5 to 40 h, no morphological and proliferative changes were observed between treated and non-treated MKN45 cells.

The expression of Lewis antigens at the cell membrane was evaluated in untreated MKN45 cells and after 20 and 40 h of IL-1β or IL-6 treatment (40 ng/ml), by flow cytometry, to detect if the changes observed in the expression levels of glycosyltransferases correlate with an altered pattern of Lewis antigens expression. The analysis of Lewis antigens expression in MKN45 cells show undetectable or very low levels of Lewis b and Lewis y; Lewis a and H-type 2 antigens are detected at low levels and high levels of the sialylated antigens sialyl-Lewis a and sialyl-Lewis x are expressed (Fig. 4). After 20 h of cytokine stimulation, no remarkable changes are observed in the levels of Lewis antigens (data not shown). At 40 h, the IL-1β treatment induced slight increases for Lewis a, sialyl-Lewis a, and H-type 2 antigens, and a decrease in sialyl-Lewis x expression. When the MKN45 cells were treated with IL-6 under the same conditions, the most relevant differences were found in the increase of H-type 2 and the decrease of sialyl-Lewis x expression levels. A representative flow cytometry profile of these results is shown in Fig. 4. When these results were statistically analyzed comparing the differences in the number of positive cells a significant decrease from 41.59±5.64% to 21.54±8.13% of the sialyl-Lewis x positive cells (p=0.042) induced by the IL-6 treatment was found.

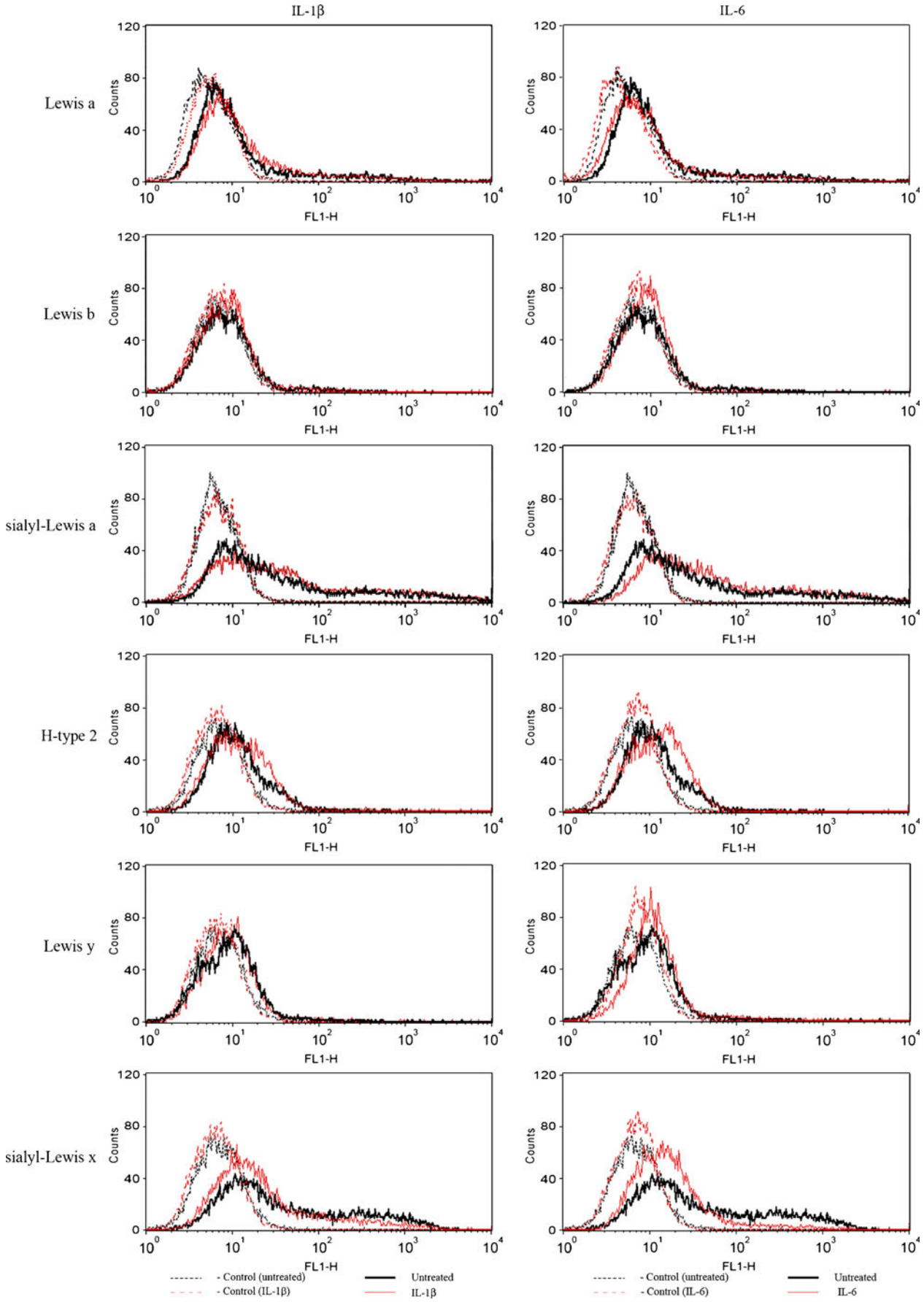
To analyze the effect of the inhibitors of IkBα and STAT3 phosphorylation, cells were treated for 20 h with IL-1β and panepoxydone or IL-6 and AG490, respectively. At this time, no changes in the expression levels of H-type 2 and sialyl-Lewis x were detected (data not shown). As the effects of IL-1β and IL-6 in specific Lewis antigens expression were only detected after 40 h of stimulation, H-type 2 and sialyl-Lewis x antigens were also evaluated in cells subjected for 20 h to IL-1β and panepoxydone or IL-6 and AG490 treatments and cultured in standard medium for additional 20 h. At this time, an increase in the expression of sialyl-Lewis x was detected after IL-1β and panepoxydone treatment, while in IL-6 and AG490 treated cells the sialyl-Lewis x level was reverted to the observed in MKN45 untreated cells, and no changes were detected in the levels of H-type 2 antigen (Fig. 5). The decrease in the number of sialyl-Lewis x positive cells after IL-6 treatment was statistically significant, and the addition of AG490 reverted significantly the number of sialyl-Lewis x positive cells to the levels of the untreated cells (p=0.0006).

IL-1β and IL-6 treatments of subcutaneous mice tumors from MKN45 cells induce changes in the expression pattern of Lewis antigens

To confirm the previous results, MKN45 cells were injected subcutaneously in BALB/c nude mice. Once the tumors developed, at day 7, they were treated by intratumor injections of IL-1β or IL-6, and Lewis antigens expression was analyzed by immunohistochemistry. The expression of Lewis antigens observed in the untreated subcutaneous tumors did not show remarkable changes comparing to the MKN45 cultured cells. No significant changes in the expression of Lewis a, Lewis b, Lewis y and sialyl-Lewis a were detected in the tumors treated with IL-1β or IL-6. However it was observed a slight increase in the H-type 2 levels, and a high detectable decrease in the levels of sialyl-Lewis x. The sialyl-Lewis x levels decrease from 45% of positive cells in the tumors of the control group, to 37.5% and 29.1% in the IL-1β and IL-6 treated tumors, respectively. The differences in the number of positive cells observed between the control and the IL-6 treated tumors regarding sialyl-Lewis x expression are statistically significant (p=0.027) (Fig. 6a and b).

Expression pattern of Lewis antigens in human intestinal-type gastric tumors and association with the nodal status and the development of metastasis

The expression of Lewis antigens was also evaluated in 61 human gastric intestinal-type adenocarcinomas. All of the Lewis structures were detected in most of the cases: from 96.72% of positive cases for Lewis y to 68.85% of Lewis a expressing tumors, whereas the number of positive cells was higher for type 2 Lewis antigens: 66.48% and 64.39% for H-type 2 and Lewis y, respectively. In these 61 patients, the nodal status and the development of distal metastasis were analyzed. Regarding to the nodal status, in 25 of the patients not ganglionar invasion (N0) was detected,


IL-1β
IL-6
Lewis a
Lewis b
sialyl-Lewis a
H-type 2
Lewis y
sialyl-Lewis x
Counts
FL1-H
- Control (untreated)
- Control (IL-1β)
Untreated
IL-1β
- Control (untreated)
- Control (IL-6)
Untreated
IL-6

◀ **Fig. 4** Profiles of Lewis antigens expression detected by flow cytometry in MKN45 cells treated for 40 h with IL-1β or IL-6

whereas several degrees of invasion (N1–N3) were found in 36 cases at surgery time. From these 61 patients, 31 developed distal metastasis after at least 15 months post-surgery. When the association between Lewis antigens expression and these clinical parameters (nodal status and presence of distal metastasis) was analyzed, significant high levels of sialyl-Lewis x were detected in the primary tumors of patients that developed metastasis (p=0.045). No other significant association was found with the rest of the Lewis antigens.

Discussion

Several fucosyl- and sialyltransferases contribute to the synthesis of Lewis antigens and their expression determines the Lewis structures detected in a specific cell type or tissue. In cancer cells, an altered pattern of Lewis antigens expression have been observed, and several studies reported that the expression of sialyl-Lewis x correlates with poor prognosis in gastric cancer [31, 32]. sialyl-Lewis x is implicated in the union to the E-Selectin molecules promoting the extravasation of the tumor cells in a process that mimetizes the leukocyte rolling [19], and the relation between the expression of sialylated Lewis antigens and the invasive phenotype of the tumor cells has been well established. Here, we have confirmed that gastric adenocarcinomas developing metastasis have high levels of sialyl-Lewis x, and this result is in accordance with previous studies reporting that sialyl-Lewis x is over expressed in gastric cancer and its expression correlates with poor prognosis associated to the development of metastasis [31, 32].

As inflammation has been postulated as an initiation factor in the development of gastric cancer, here we have evaluated the implication of the inflammatory cytokines, IL-1β and IL-6, in the regulation of the glycosyltransferases involved in the synthesis of Lewis antigens in the MKN45 gastric cancer cell line. Our results indicate that the fucosyltransferases analyzed in this study can be differentially regulated by IL-1β and IL-6. FUT1 and FUT2 catalyze the addition of fucose in α1,2 position, and FUT1 is specific for type 2 precursor chains, whereas FUT2 can act in both, type 1 and type 2, precursor structures. These α1,2 fucosyltransferases are significantly up-regulated after IL-1β stimulation whereas IL-6 treatment only activates the expression of FUT1. In contrast, the α1,3-4 fucosyltransferases FUT3 and FUT5, are down-regulated by the IL-1β and IL-6 treatments; whereas FUT4 is regulated by IL-1β in a different way and its expression levels are not altered by the IL-6 stimulation. These results agree with the fact that FUT1 and FUT2 located in the same chromosome cluster (19q13.3), display a 67% of homology in the amino acid sequence, as it occurs for FUT3 and FUT5 (19p13.3) with 88% of homology [33, 34]. On the other hand, FUT4, located in the chromosome 11q21, does not display high levels of homology with other α1,3-4 fucosyltransferases (43% with FUT3 and FUT5) [35] and shows a different and specific regulation pattern. By

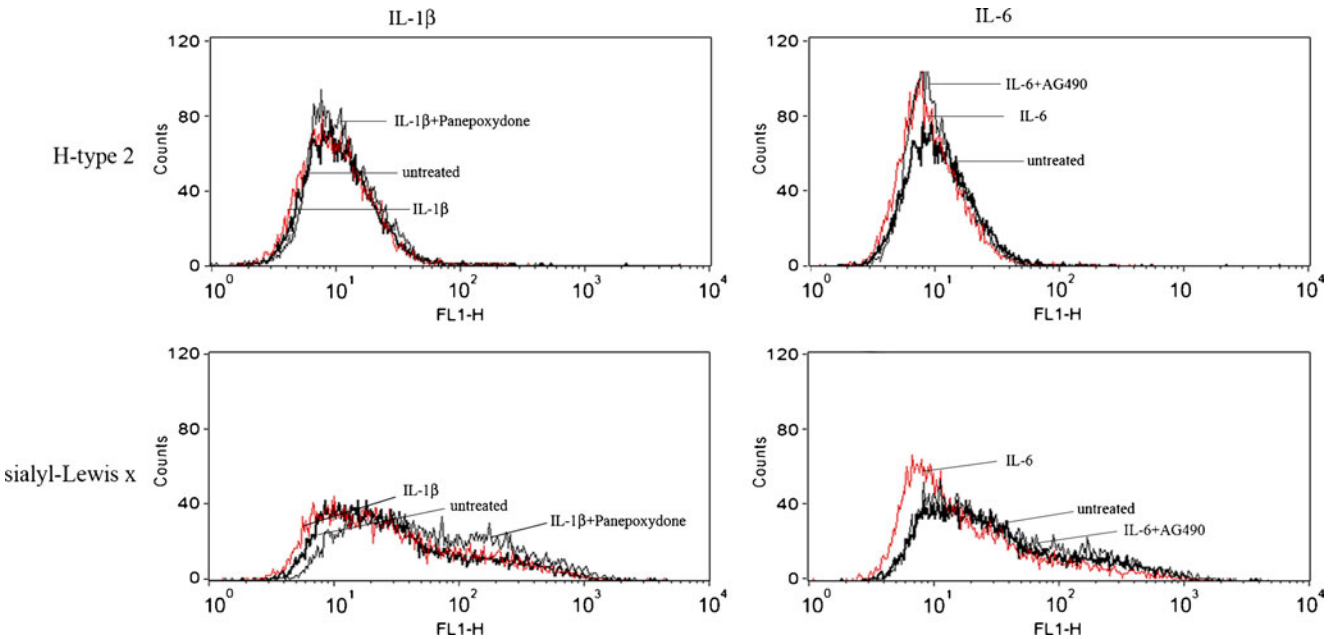


Fig. 5 Flow cytometry detection of H-type 2 and sialyl-Lewis x after 20 h of IL-1β and panepoxydone or IL-6 and AG490 treatments, and cultured in standard medium for additional 20 h

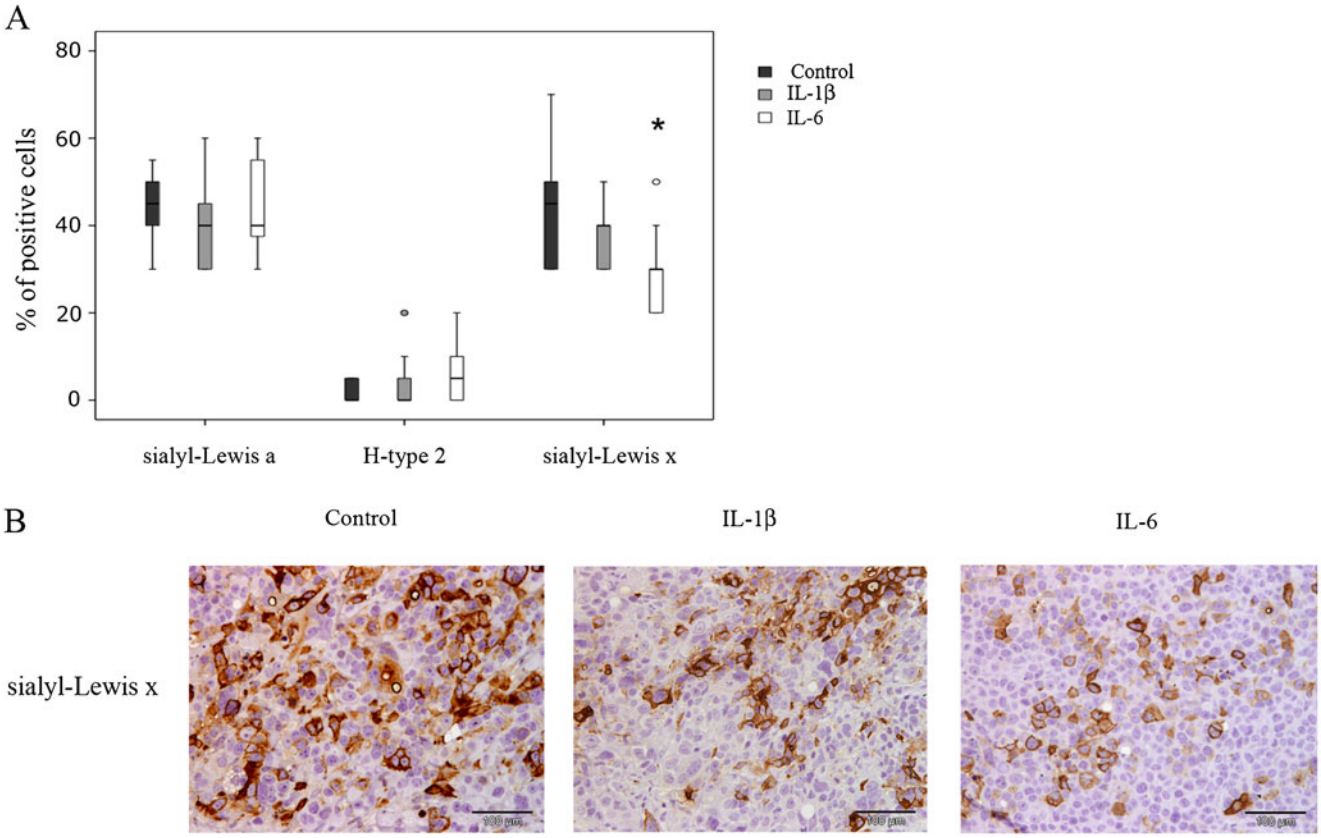


Fig. 6 Subcutaneous primary tumors from MKN45 cells, developed in BALB/c nude mice, were treated with PBS (n=3), IL-1β (n=6), or IL-6 (n=6) (4 doses of 10 ng). A) Levels of Lewis antigens expression in untreated, and IL-1β or IL-6 treated tumors. *$p \leq 0.05$. B) Immunohistochemical detection of sialyl-Lewis x. Decreased levels of s Le x were detected in IL-1β and IL-6 treated mice compared to control tumors

contrast, no important changes in the expression levels of the ST3GalIII and ST3GalIV enzymes are detected after the stimulation of MKN45 cells by IL-1β and IL-6.

We have also analyzed the implication of specific signaling pathways in the regulation of the fucosyltransferases. Specifically the α1,2 fucosyltransferases, FUT1 and FUT2, and the α1,3-4 fucosyltransferase FUT4 are regulated through the NF-κB pathway by IL-1β as it is demonstrated through the inhibition of the IκBα phosphorylation that reverts the activation of these glycosyltransferases. The regulation of the α1,3-4 fucosyltransferases FUT3 and FUT5 through the gp130/STAT3 signaling pathway is also demonstrated since the inhibition of STAT3 phosphorylation blocks the effects of IL-6 treatments. Alternative signaling pathways activated by IL-1β or IL-6 can mediate the regulation of FUT3 and FUT5 after IL-1β treatment, and the activation of FUT1 after IL-6 stimulation.

The observed up-regulation of FUT1 suggests that the balance in the amounts of the two glycosyltransferases (FUT1 and ST3GalIII/IV) competing for the type 2 precursor structure (Galβ1-4GlcNAc-R) can be disrupted after IL-1β or IL-6 treatments. This activation of FUT1, associated to low levels of FUT3 and FUT5 detected after IL-1β or IL-6 stimulation, induces a decrease in the levels of sialyl-Lewis x and an increase of the non-sialylated type 2 structures (H-type 2 and Lewis y). These results also agree with recent published data reporting that these α1,3-4 fucosyltransferases are implicated in the synthesis of the sialylated Lewis antigens in MKN45 cells [36]. In this sense, we have previously described that the ectopic expression of FUT1 in colon cancer cells, induced decreased levels of sialyl-Lewis x that correlated with changes in the metastatic capacities of the tumor cells [37].

Several studies have shown that inflammatory cytokines such as TNF-α, IL-1β, and more recently IL-6, can modulate the expression of specific glycosyltransferases in different cell types and tissues, although no data regarding gastric models have been published. In the hepatocellular carcinoma HuH-7 cells, IL-1β has been reported to enhance the expression of FUT6 and ST3GalIV inducing the synthesis of sialyl-Lewis x [38]; and in colon cancer cells,

TNF-α treatment activates the NF-κB pathway associated to the expression of p65 transcription factor and promotes biphasic and transient increases in the transcription levels of several glycosyltransferases among them FUT4 [30]. Also, it has been described that treatments of bronchial mucosa explants with IL-6 and IL-8 induce a significant increase in the expression of specific fucosyltranferases, FUT3 and FUT11, sialyltransferases, ST3GalVI and ST6GalII, and sulfotransferases, CHST4 and CHST6, implicated in the biosynthesis of sialyl-Lewis x and 6-sulfo- sialyl-Lewis x [39]. More recently, the stimulation of the bovine ST6GalII by IL-6 and not by IL-1β and TNF-α, has been reported [40]; and, the enhanced transcription of FUT1 associated to an increase in Lewis y expression was obtained after TNF-α stimulation of endothelial cells [41].

All together the data reported here indicate that inflammatory cytokines can determine the set of glycosyltranferases involved in the synthesis of the carbohydrate structures present in tumor cells which have been demonstrated to be important for the progression of gastric cancer.

Acknowledgements This work was supported by the AGAUR, Generalitat de Catalunya [2009SGR121], and it is part of M. Padró PhD project at Universitat Pompeu Fabra (Barcelona). Authors thank the scientists providing the antibodies, Dr. Òscar Fornas for flow cytometry analysis, Sergi Mojal for statistical analysis, and Laura Camacho for technical assistance.

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