

6-10-2011

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Recommended Citation

Radhakrishnan, Prakash; Chachadi, Vishwanath; Lin, Ming-Fong; Singh, Rakesh; Kannagi, Reiji; and Cheng, Pi-Wan, "TNF α enhances the motility and invasiveness of prostatic cancer cells by stimulating the expression of selective glycosyl- and sulfotransferase genes involved in the synthesis of selectin ligands." (2011). *Journal Articles: Biochemistry & Molecular Biology*. 42.
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Published in final edited form as:

Biochem Biophys Res Commun. 2011 June 10; 409(3): 436–441. doi:10.1016/j.bbrc.2011.05.019.

TNF α enhances the motility and invasiveness of prostatic cancer cells by stimulating the expression of selective glycosyl- and sulfotransferase genes involved in the synthesis of selectin ligands

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Abstract

Sialyl Lewis X (sLe^x) plays an important role in cancer metastasis. But, the mechanism for its production in metastatic cancers remains unclear. The objective of current study was to examine the effects of a pro-inflammatory cytokine on the expression of glycosyltransferase and sulfotransferase genes involved in the synthesis of selectin ligands in a prostate cancer cell line. Androgen-independent human lymph node-derived metastatic prostate cancer cells (C-81 LNCaP), which express functional androgen receptor and mimic the castration-resistant advanced prostate cancer, was used. TNF α treatment of these cells increased their binding to P-, E- and L-selectins, anti-sLe^x antibody, and anti-6-sulfo-sialyl Lewis x antibody by 12, 240, 43, 248 and 21%, respectively. Also, the expression of *C2GnT-1*, *B4GalT1*, *GlcNAc6ST3*, and *ST3Gal3* genes was significantly upregulated. Further treatment of TNF α -treated cells with either anti-sLe^x antibody or E-selectin significantly suppressed their *in vitro* migration (81 and 52%, respectively) and invasion (45 and 56%, respectively). Our data indicate that TNF α treatment enhances the motility and invasion properties of LNCaP C-81 cells by increasing the formation of selectin

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ligands through stimulation of the expression of selective glycosyl- and sulfotransferase genes. These results support the hypothesis that inflammation contributes to cancer metastasis.

Keywords

LNCaP cells; sLe^x; TNF α ; glycogenes; motility and invasion

1. Introduction

Prostate cancer is the second leading cause of cancer death of men in the United States [1]. At the time of initial diagnosis, more than 70% of prostate cancer patients have lymph node metastases [2–5]. Although metastasis is the primary cause of the fatalities of cancer patients, the mechanism is not completely understood. One key step in the cancer metastatic process is the adhesion of circulating cancer cells to the endothelium at distant sites. This step involves selectin-ligand interactions between host cells (selectins) and cancer cells (selectin ligands). Understanding how the production of these two key players is regulated during cancer progression could help develop cancer therapeutic strategy.

Selectins are a family of type II transmembrane proteins found on the surface of leukocytes, platelets and activated endothelial cells. They are calcium-dependent C-type lectins [6]. The three major types of selectins include E-, P-, and L-selectins, which are located on endothelium, platelets and activated endothelium, and leukocytes, respectively [6]. The naturally occurring vascular carbohydrate ligands for these selectins include E-selectin ligand-1 (ESL-1), P-selectin glycoprotein ligand-1 (PSGL-1), CD34, cutaneous lymphocyte-associated antigen (CLA), glycoprotein GlyCAM-1, MAdCAM-1, Podocalyxin-like protein and Sgp200 [7]. These selectin-ligand interactions were originally shown to be involved in the trafficking of immune cells to inflamed tissues [8] and lymphoid organs, and recently in the metastasis of cancer cells [9]. The naturally occurring vascular ligands for these selectin molecules are mostly mucin-type glycoproteins which carry sialylated, fucosylated and sulfated glycans [10] although some N-linked glycans also can participate in this process [11]. Increased expression and altered glycosylation of mucins are prominent features of carcinoma progression [10]. For example, sialyl Lewis x (sLe^x) and sialyl lewis a (sLe^a) are increased substantially in tumors [12], including prostate cancers [13, 14]. Elevated expression of sLe^x and sLe^a on mucin type O-glycans is highly correlated with lymphatic and venous invasion of cancerous cells [15, 16]. SLe^x and 6-sulfo-sLe^x also were elevated under *in vivo* inflammatory condition [9] and *in vitro* induction with proinflammatory cytokine, tumor necrosis factor α (TNF α) [17, 18]. Glycosyl and sulfotransferases responsible for the biosynthesis of the above mentioned epitopes were selectively upregulated in various cancers [19] and inflammatory conditions [9] and/or cells treated with TNF α [17, 18]. However, a concerted effort to correlate the effects of inflammatory cytokine on the expression of selectin ligands, glycosyl- and sulfotransferase genes responsible for the synthesis of these ligands, interactions of these ligands with selectins, and the acquisition of metastatic potential of the TNF α -treated cells was lacking.

Recently, serum TNF α level was found to be increased in prostate cancer patients with metastatic disease and elevated PSA [20]. This observation prompted us to examine whether TNF α could enhance the metastatic potential of prostate cancer cells via stimulating the production of sLe^x, an important determinant of cancer metastasis. To carry out a more comprehensive study on the effect of TNF α on the acquisition of the sLe^x-related metastatic potential in prostate cancer, we employed LNCaP C-81 cells, which exhibit properties mimicking the clinical characteristics of advanced prostate cancers [21–23]. C-81 cells are androgen independent, grow faster and have high colony-forming property on soft agar as compared to LNCaP parental C-33 cells [22]. We found that treatment of the C-81 LNCaP cells with TNF α increased the motility and invasiveness, which accompanied an increase in selectin ligands resulted from elevated expression of selected glycosyl- and sulfotransferase genes.

3. Materials and methods

2.1 Cell lines

The human Prostate cancer cell line LNCaP was originally purchased from the American Type Culture Collection ATCC (Rockville, MD). The LNCaP cell model including C-33 and C-81 cells used in the present study was developed by Lin *et al* [22] and further characterized by Igawa *et al* [21]. The cell line was maintained in RPMI 1640 medium supplemented with 5% Fetal bovine serum, 1% L-glutamine and 1% Penicillin-Streptomycin.

2.2 Flow Cytometry analysis of selectin interactions with ligands on LNCaP cells

Flow cytometry analysis of selectin-ligand interaction on cancer cells was carried out as described previously [24]. Briefly, LNCaP cells of both control and TNF α (20 ng/ml, 36 h) treated were detached from plates after incubation with PBS containing 2 mM EDTA for 5 min at 37 °C, washed three times with Hanks' balanced salt solution (HBSS), and then treated with 0.5% BSA in HBSS for 30 min to block nonspecific binding. Chimeras of selectins P (50 μ g/ml), E (50 μ g/ml), and L (50 μ g/ml) fused with Fc region of human IgG were purified from human 293 cells (transformed embryonic kidney fibroblast cell line) of Psel IgG, ESIG B3293 and LSRg293 Cytel, respectively [24]. These selectins were pre-incubated with a goat-anti human IgG conjugated with FITC for 1 h at room temperature. Similarly, sLe^x (KM93) (10 μ g/ml) and 6-sulfo-sLe^x (G152) (1:10 diluted) [25] mouse monoclonal antibodies were pre-incubated with goat anti-mouse antibody conjugated with FITC before use. These complexes (Selectin, KM93 Ab, or G152 Ab complexed with respective FITC-conjugated secondary antibodies) were incubated with tumor cells at 4 °C for 2 h and then washed successively with HBSS/BSA and HBSS. Then cells were fixed with 2 % (wt/vol) paraformaldehyde in HBSS at room temperature for 15–30 min. The cells were then washed with HBSS and resuspended in 1 ml of HBSS/BSA for flow cytometry analysis. Controls were cells stained in a presence of 5 mM EDTA (calcium chelation) or 30 mM EDTA in the case of P-selectin. In some instances, tumor cell surfaces were pretreated before probing for selectin ligands. Cells treated with FITC-conjugated secondary antibody alone served as an antibody control.

2.3 Real Time PCR analysis of the expression of glycosyl- and sulfotransferase genes

RT-PCR analyses on LNCaP C-81 cells were performed as described previously [26]. Total cellular RNAs were isolated from TNF α treated (20 ng/ml, 3 h) and control LNCaP cells using the TRI-REAGENT (Molecular Research Center, Inc, USA) according to the manufacturer protocol. cDNA was synthesized by using 2 μ g of total RNA as the template in a 20 μ l RT reaction mixture by using Verso™ cDNA kit (ABgene, UK) according to the manufacturer instruction. Quantitative real time PCR was performed on a Master cycler ep realplex² (Eppendorf AG, Hamburg) using SYBR Green PCR kit (Takara Bio Inc, USA). The forward and reverse primers used for amplification of selective glycosyl- and sulfotransferase genes are listed in Table 1. The data were analyzed using Eppendorf realplex software, version 1.5 (Eppendorf). The amounts of various glycogene transcripts were normalized to the amount of GAPDH transcript in same cDNA sample and expressed as % of GAPDH. Relative fold differences in transcript expression were determined using the following comparative CT method: $2^{-[\Delta C_t(TNF\alpha) - \Delta C_t(Control)]} = 2^{-\Delta\Delta C_t}$, where $\Delta C_t = C_t(Target) - C_t(GAPDH)$ as described previously [26]. The results were expressed as the amount (%) relative to that (100 %) of GAPDH and plotted as mean fold changes \pm SEM.

2.4 Motility and Invasion assays

Motility and invasion assays were performed as previously described [23]. For motility assays, 5×10^4 cells/ml of control, TNF α treated (20 ng/ml, 36 h), and TNF α treated cells further incubated with sLe^x monoclonal antibody KM93 (10 μ g/ml) or E-selectin (100 μ g/ml) were placed on the top chamber of non-coated polyethylene teraphthalate membranes. After incubation for 24 h, the cells that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. Cells that transversed the membranes were stained with a Diff-Quick cell staining kit (Dade Behring, Inc., Newark, DE). Invasion assay of prostate cancer cells was carried out as described above except that 24 well BD BioCoat™ Matrigel™ invasion chambers (8 μ m pore size; Becton Dickinson) were used and the incubation time was 22 h. Cells at three random fields per insert were counted with 40 \times magnification and expressed as the average number of cells per field of view. Three independent experiments were performed in triplicates in each condition. The data were presented as the mean of three independent experiments \pm SD.

3.5 Statistical Analysis

The Student's t-test was used for analysis of the significance of differences in means between control and tested samples. P<0.05 was considered significant.

4. Results

3.1 TNF α enhancement of selectin binding to LNCaP cells

LNCaP C-81 cells with and without TNF α treatment were assessed for their binding to P-, E-, and L-selectins by flow cytometry. These cells exhibited a decreasing order of selectin binding, P-selectin > L-selectin > E-selectin, in the untreated controls (Figs. 1A–C). TNF α treatment increased the number of cells that bind to E, P, and L-selectins by 240% (p<0.001), 12% (p<0.05), and 43% (p<0.05), respectively (Fig. 1A–C).

3.2 TNF α enhancement of sLe^x and 6-sulfo-sLe^x epitopes on LNCaP cells

The E- and L-selectin ligands, sLe^x and 6-sulfo-sLe^x, on C-81 LNCaP cells were assessed with KM93 and G152 antibodies, respectively. As shown in Fig. 2A and 2B, TNF α treatment increased the binding of LNCaP cells to KM93 and G152 antibodies by 248% ($p < 0.001$) and 21% ($p < 0.05$), respectively.

3.3 TNF α modulation of the expression of glycosyl- and sulfotransferase genes involved in the synthesis of selectin ligands

The TNF α -induced changes in the expression of the glycosyl- and sulfotransferase genes involved in the synthesis of mucin-type selectin ligands were assessed by real time PCR analysis. As shown in Fig. 3, TNF α treatment of LNCaP cells significantly increased the expression of the following genes (% increase over the control): *C2GnT1* (340%) ($p < 0.01$), *B4GalT1* (68%) ($p < 0.05$), *GlcNAc6ST3* (760%) ($p < 0.01$) and *ST3Gal3* (240%) ($p < 0.05$). Although *C1GALT1* gene expression was not affected by TNF α , it did express at a high basal level, i.e. 0.11% of *GAPDH*. However, the gene expression level of core 1 extension enzyme, *B3GnT3*, was very low, i.e. 0.00006% of *GAPDH*. *C2GnT1* is the highest expresser among the three *C2GnT* isozymes. All *B4GalTs* were expressed at very high levels. After TNF α treatment, *GlcNAc6ST1* & 3 were expressed at modest levels, *ST3Gal4* was the highest expresser among the three *ST3Gal* isozymes and *FUT4* was the highest expresser among the *FUT* genes.

3.4 TNF α enhancement of *in vitro* motility and invasion of LNCaP cells

The motility and invasion properties of LNCaP cells treated with TNF α were analyzed by *in vitro* Boyden chamber assay. As shown in Fig. 4A–4C, TNF α treatment significantly increased the number of cells that migrated through polyethylene terephthalate membrane by 83% ($p < 0.001$) and those that invaded the matrigel by 44% ($p < 0.001$) when compared with the untreated cells. Incubation of the TNF α -treated cells with KM93 antibodies and E-selectin reduced the number of cells that migrated through polyethylene terephthalate membrane by 81% ($p < 0.001$) and 52% ($p < 0.001$), respectively, and those that invaded the matrigel by 45% ($p < 0.001$) and 56% ($p < 0.001$), respectively when compared with the cells treated with TNF α only.

4. Discussion

Metastasis is the primary cause of cancer death, and inflammation is a key contributor of cancer metastasis [27, 28]. Current study shows that TNF α , a proinflammatory cytokine, enhances the motility and invasiveness of human prostate cancer cells by stimulating the expression of selective glycosyl- and sulfotransferase genes involved in the synthesis of selectin ligands. These results support the role of inflammation in cancer metastasis [29].

Cancer metastasis involves several highly coordinated steps, including escaping of tumor cells from the primary sites, transport of these cells by circulation to the lymphoid organs and/or distant sites, adhesion of these cells to the inflamed endothelium at these sites, and then establishment of tumors. Interactions of selectins and their ligands play a crucial role in the second and third steps of the metastatic process as described above. The circulating

metastatic cancer cells provide sLe^x, an E-selectin ligand, to facilitate their arrest by the E-selectin present on the activated endothelium at distant sites [30,31]. The L-selectin ligand, 6-sulfo-sLe^x, expressed on the surface of these cancer cells help them adhere to the circulating leukocytes by binding to L-selectin expressed on the surface of leukocytes, which facilitates their transport to the lymphoid organs and inflamed endothelium at distant sites. The P-selectin ligand expressed on these cancer cells render these cells capable of adhering to P-selectin present on the activated endothelium at distant sites and on circulating platelets, which help these cancer cells to metastasize [30]. The increased expression of P-, E-, and L-selectin ligands on these cancer cells following treatment with TNF α could enhance the potential of these cells to target to the lymphoid organs and inflamed endothelium at distant sites. In this study, we have shown that TNF α enhances the motility and invasiveness of these prostate cancer cells. Confirmation of this observation *in vivo* remains to be made.

sLe^x and 6-sulfo-sLe^x can be found on core 1 and core 2 mucin glycans [31,32]. The formation of these glycotopes is controlled by the levels of these enzymes of which expression is subject to regulation [31,33]. For example, TNF α has been shown to increase the production of sialylated and/or sulfated Lewis x epitopes in the human bronchial mucosa by stimulating the expression of glycosyl- and sulfotransferases responsible for their synthesis [18]. The enzymes responsible for the enhancement of these glycotopes have been shown to be FUT3 & 4, ST3Gal3 & 4, and GlcNAc6STs. TNF α also can enhance the production of sulfated N-acetyllactosamine, a precursor of 6-sulfo-sLe^x on CD44 of a SR91 myloid cell line [34]. In this report, we showed increased production of P-, E-, and L-selectin ligands on LNCaP cells treated with TNF α . Upregulation of *C2GnT-1*, *B4GalT1*, *GlcNAc6ST3* and *ST3Gal3* genes coupled with high expression levels of additional glycosyl- and sulfotransferase genes can explain the formation of these selectin ligands in response to TNF α treatment. However, the low expression level (0.00005% of GAPDH) of the core 1 extension enzyme (B3GnT3) suggests that the core 1 can not be extended to support the formation of these glycotopes on this core structure. Therefore, these selectin ligands are most likely localized to the core 2 branch. This prediction remains to be confirmed by glycan analysis.

We extended the biochemical and molecular biological observations by showing that TNF α -induced changes in glyco gene expression profile led to increased binding of these cells to P-, E-, and L-selectins, and increased their adhesion, motility, and invasion properties. This observation confirmed a previous report that TNF α increased migration and invasion of a human melanoma cell line [35]. Collectively, these results support the idea that inflammatory cytokines can enhance the metastatic properties of cancer cells and further substantiate the link between inflammation and cancer progression as previously described [27, 28].

In conclusion, the present study shows the TNF α effect on enhancement of prostate cancer cell migration and invasion through activation of selective glycosyl and sulfotransferase genes involved in the biosynthesis of sialylated and/or sulfated sLe^x. These genes may serve potential therapeutic targets.

Acknowledgments

We wish to acknowledge the research support from NIH RO1 CA88184 (MFL), RO1 HL48282 and R21 HL97238, the State of Nebraska-NRI cancer glycobiology program and LB506 (PWC), and the Department of Defense Postdoctoral Fellowship (PR), and expert technical assistance of Helen Cheng.

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Highlights

In this communication, we have made the following observations.

- > TNF α , a proinflammatory cytokine, enhances the motility and invasiveness of prostate cancer cells.
- > TNF α enhances the production of selectin ligands in prostate cancer cells, which results in increased binding to P-, E-, and L-selectins.
- > TNF α upregulates the expression of selective glycosyl- and sulfotransferase genes involved in the synthesis of selectin ligands.

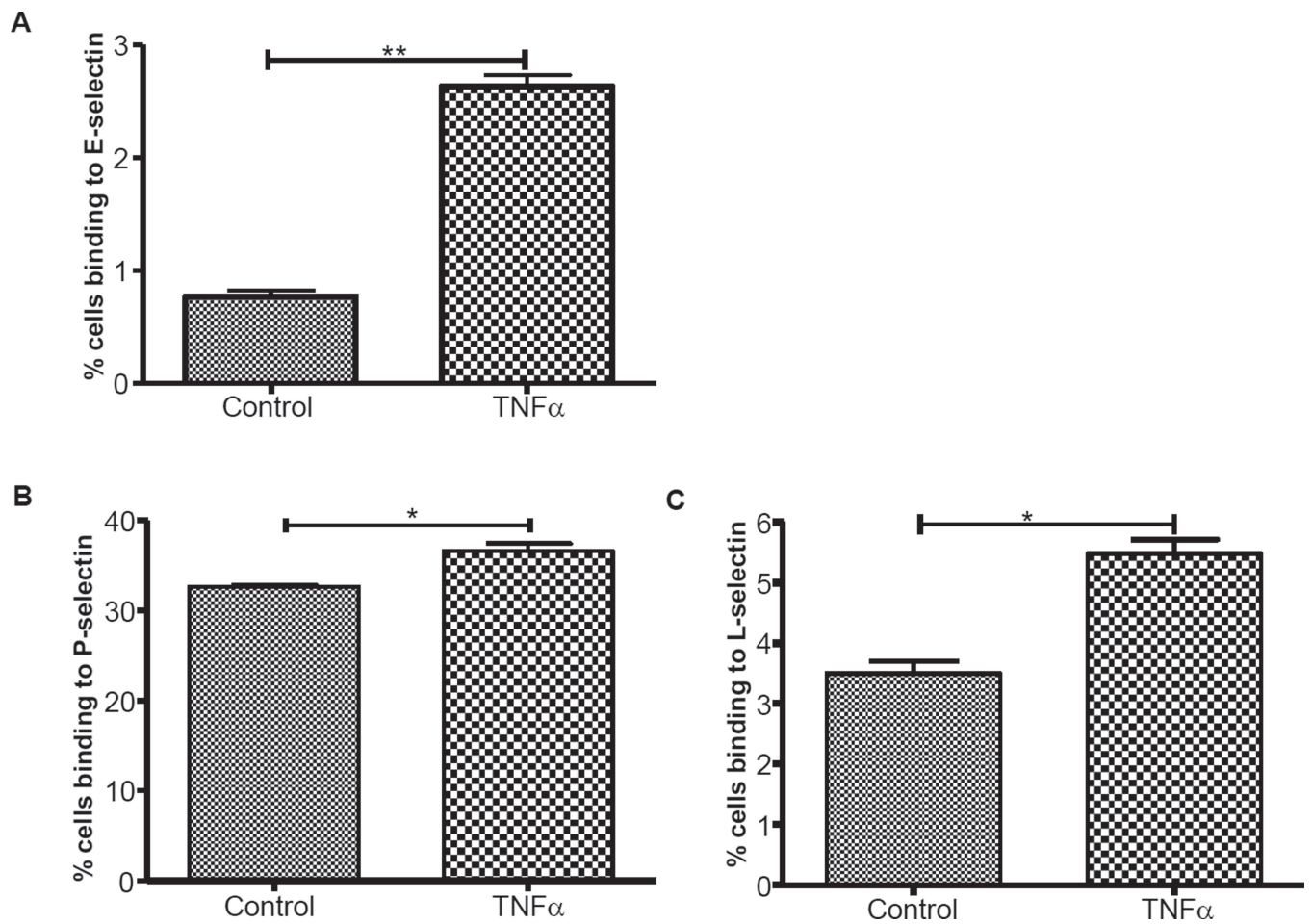


Fig. 1.

Interactions of selectins and ligands on LNCaP C-81 cells. The decreasing binding order of selectins with C-81 cells were P > L > E. Treatment of C-81 cells with TNF α (20 ng/ml, 36 h) significantly increased the binding of E-selectin (A), P-selectin (B) and L-selectin (C) when compared with control cells (*, $p < 0.05$; **, $p < 0.001$).

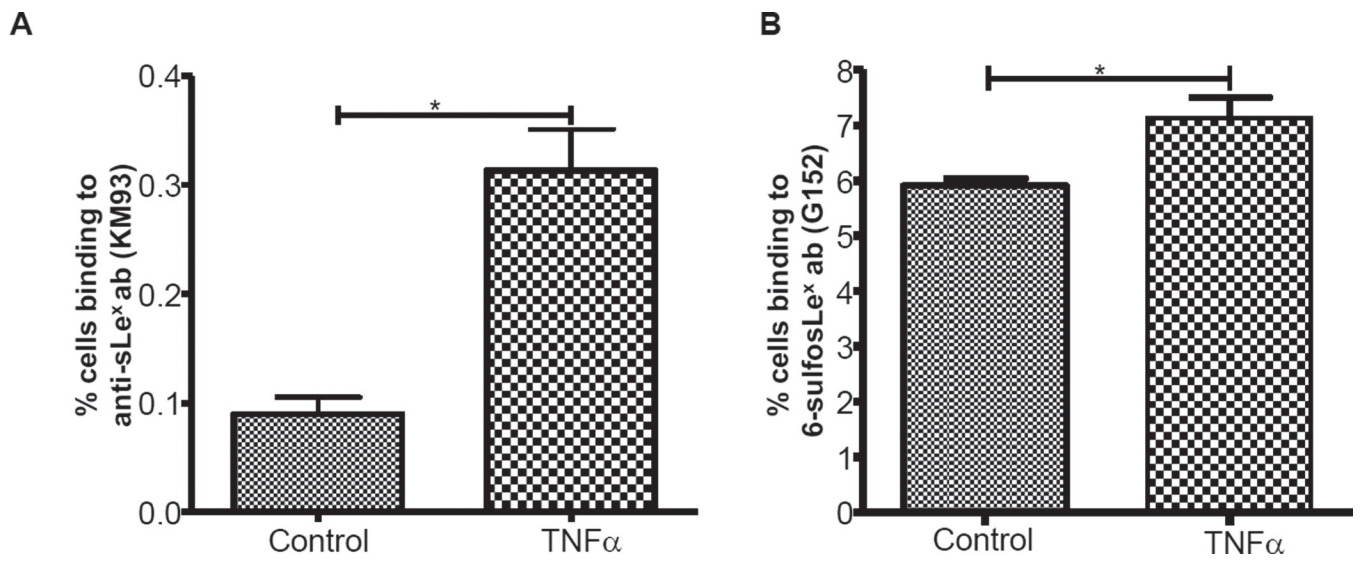


Fig. 2.

TNF α enhancement of sLe^x and 6-sulfo-sLe^x expression on LNCaP C-81 cells. Treatment of LNCaP cells with TNF α (20ng/ml, 36 h) significantly increased higher binding of these cells to anti-sLe^x (A) and 6-sulfo-sLe^x (B) antibodies (*, p<0.05; **, p<0.001).

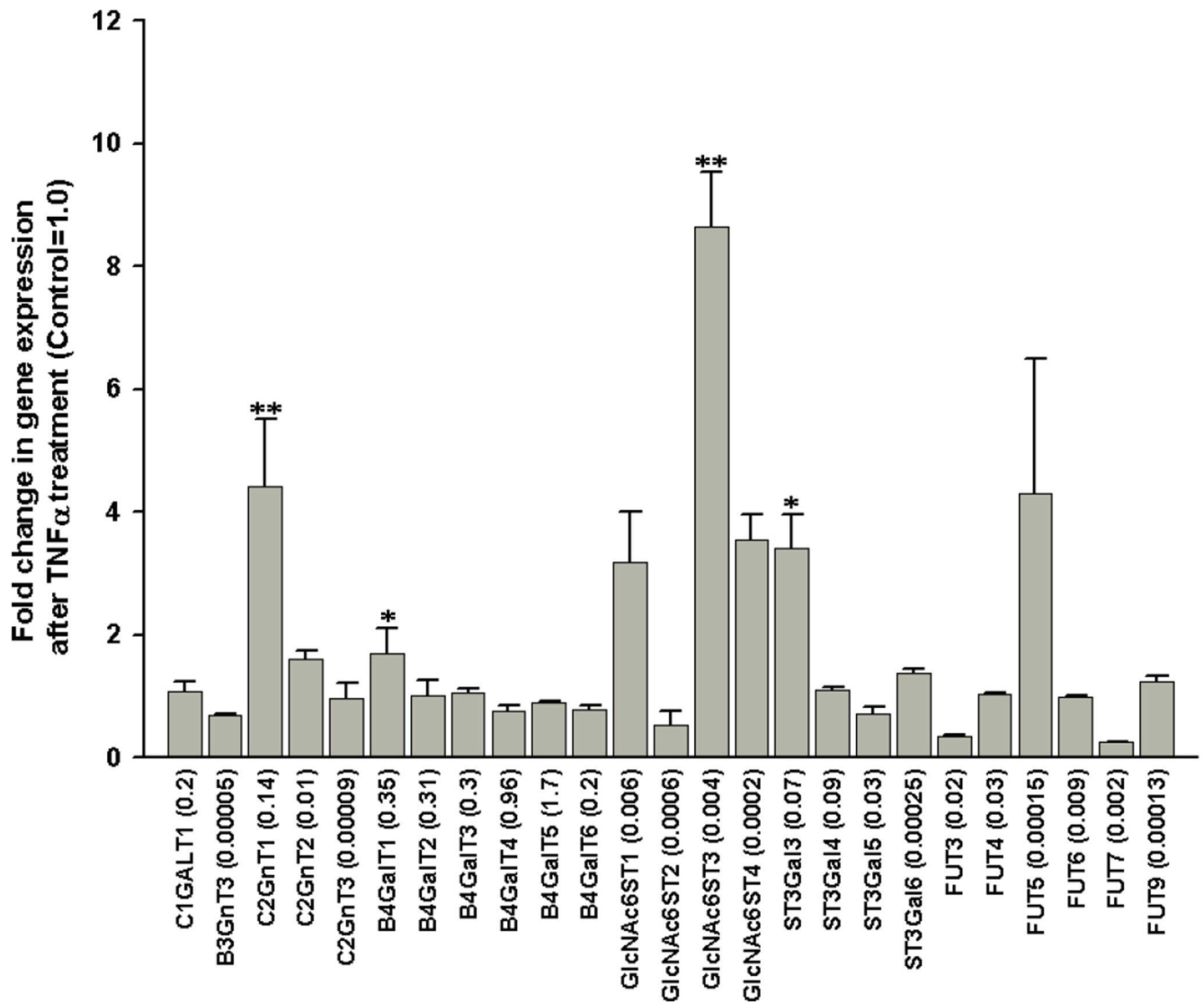


Fig. 3.

Quantitative RT-PCR analysis of glycosyl- and sulfotransferase gene expression. Quantitative real time PCR analysis was carried out on LNCaP cells with and without TNF α treatment (20 ng/ml, 3h). Relative expression level of each gene was sorted according to $\Delta\Delta C_t$ (see Materials and Methods) calibrated with GAPDH and expressed as % of GAPDH at basal levels and fold changes determined by calculating the ratio of the expression level of each gene in TNF α treated vs. vehicle-treated cells.

Relative amount of each gene versus that of GAPDH (100%) in untreated control cells was given in the parenthesis (n=3). Statistical analysis of the difference in means between treated and control cells for each gene was performed by the Student's t-test (*, p<0.05; **, p<0.01).

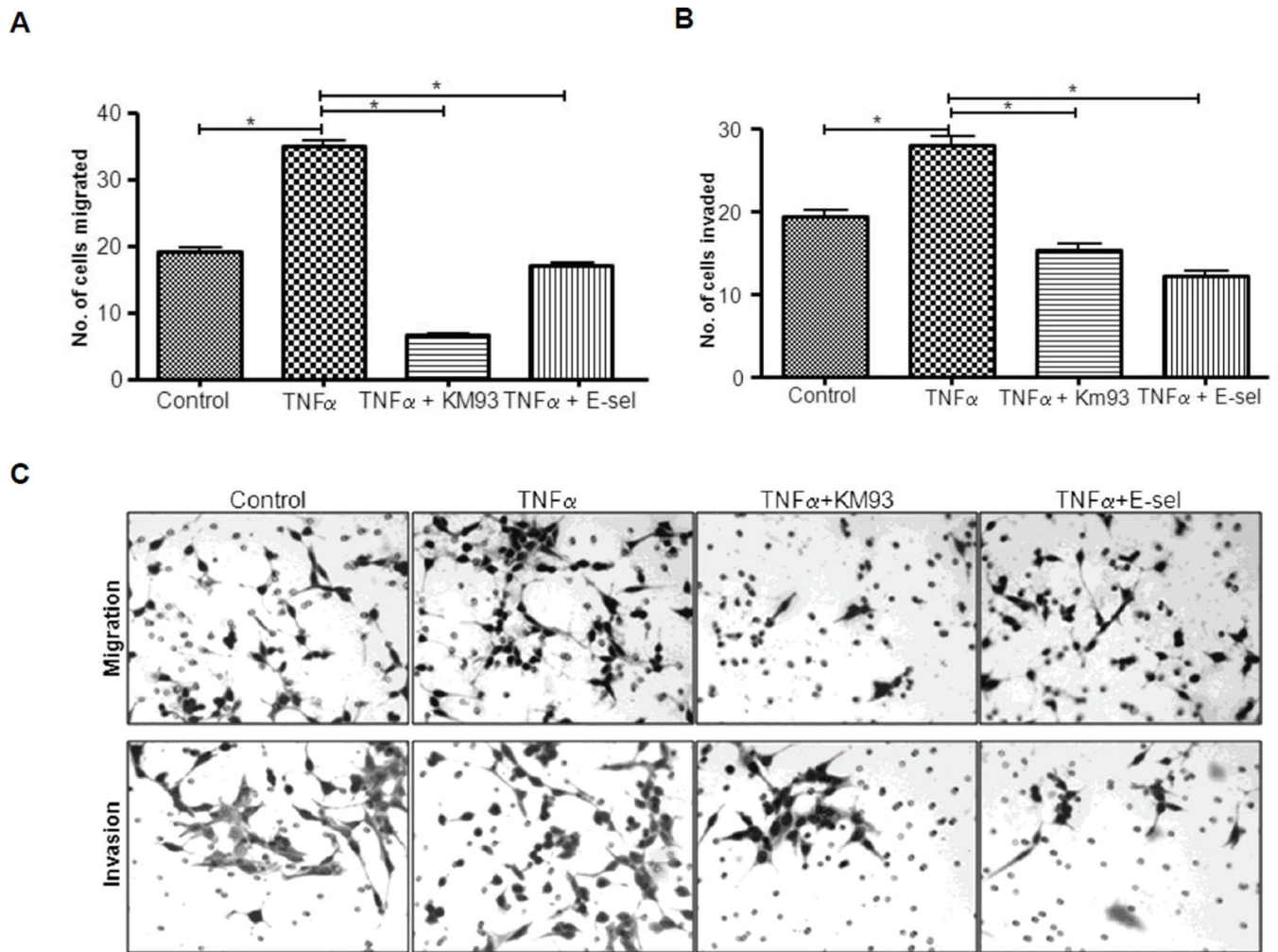


Fig. 4.

Migration and Invasion of LNCaP cells. LNCaP C-81 cells of 5×10^4 cells/ml in serum free medium was inoculated in the upper chambers of 24 well migration and matrigel invasion chamber plates. After 24 h, the cells that transverse to bottom side of the chamber plates were fixed, stained, and then counted under a phase contrast microscope (40 \times) in three independent fields for each insert. The TNF α -treated C-81 cells showed a significantly higher level of migration (A, C) and invasion (B, C) than control cells through polyethylene teraphthalate membrane and matrigel invasion chambers, respectively. Pre-incubation of TNF α treated cells with sLe^x specific antibody (KM93) or E-selectin, significantly reduced the number of cells that migrated (A, C) and invaded (B, C) when compared with cells treated with TNF α alone. The data represented an average of three independent experiments (*, $p < 0.001$).

Table 1

Oligonucleotide primers used for quantitative real-time PCR analysis of different glycosyl- and sulfotransferases

Genes	Forward primer sequences 5'-3'	Reverse primer sequences 5'-3'
<i>Core 1</i>	AGAAGCAAAGGTCACCAGTCCCAA	AGCCAGGATTTAGAGGCCATTTCC
<i>B3GnT3</i>	ATAAGGGAGTGCCAGGGAAGGTTT	ACAGTTGGAACCTTAGCACAGGCA
<i>C2GnT1</i>	GCATGGTTTCTGCAGAGCACAGTT	TTTCCACAATGCCTTGCTACCTGC
<i>C2GnT2</i>	AGAAGCGAGAGCCTTTCACAGACA	TACACAGCTCGCAGTAGCCTTCA
<i>C2GnT3</i>	GTTCCCTGGGCTTTCCTTGTTTGA	ACTCACCTAAGGCCAAAGGGAACA
<i>B4GalT1</i>	CTAGCAACTTGACCTCGGT	CATTGGGGTCTGCTTTGCC
<i>B4GalT2</i>	GCATAACGAACCTAACCTCAG	GCCCAATGTCCACTGTGATA
<i>B4GalT3</i>	GTAACCTCAGTCACCTGCC	ATTCCGCTCCACAATCTCTG
<i>B4GalT4</i>	ACTTCGTGGGTGCCATTCAAGAGA	AAGGAGACACAGAAGGGCAGTTGT
<i>B4GalT5</i>	TGGAACAGAGTACAGAATGCAG	CCTTGCCGTCTTTTGACTTC
<i>B4GalT6</i>	CTTCACATACTACCATACTCC	CCCCTGGCTCAATATCTAAATCC
<i>GlcNAc6ST1</i>	CGAAATGGAGCAGTTTGGCCGTGAA	ACTCCTCCACCTGTTTGATCTGCT
<i>GlcNAc6ST2</i>	ATCCCATGAATTTGCTGGGCTACC	AGATGTAGGCAAGGCTCAGAAGCA
<i>GlcNAc6ST3</i>	GCAGACACGCCAAGCTTTGAGAAA	AGCTGCAACGCTGATCACAATCC
<i>GlcNAc6ST4</i>	TAGTGCAACTTCTGTGAGCCAGT	TCCATCAAGCCTCACCTCACAACA
<i>ST3GAL3</i>	TGTTCTGGATGACTCCTTTCGCA	CTTGTTGGCAAGAACGCCTCCATT
<i>ST3GAL4</i>	ATGAGCAGATCACGCTCAAGTCCA	TCCCATCTCCAGCATCCGCTTAAT
<i>ST3GAL5</i>	GCACGGATTAGAACTGGG	CGCCTCTGGATAAGTCAT
<i>ST3GAL6</i>	TCTATTGGGTGGCACCTGTGGAAA	TGATGAAACCTCAGCAGAGAGGCA
<i>FUT3</i>	AGAGAGATCATCACGGCACGGTTT	ATAAGTGGTGGTCTGGGCTTGAA
<i>FUT4</i>	TGCAGGTGGGACTTTGTTGTTTGG	TCCTCCAAGGACAATCCAGCACTT
<i>FUT5</i>	TGCCACACTGAATGTCACCTGCTA	TGCTGATGGTCACACACATACCCT
<i>FUT6</i>	ATCACCGCACCGTTTCCAGATGTA	TCCCAAAGTGCTGGGATTACAGGT
<i>FUT7</i>	TTCGTGCATGTGGATGACTTTGGC	AGCGTTGGTATCGGCTCTCATTCA
<i>FUT9</i>	TCCCATGCAGTTCTGATCCAT	CAGCCTCAAGATCATCAGCA
<i>GAPDH</i>	TCGACAGTCAGCCGCATCTCTTT	ACCAAATCCGTTGACTCCGACCTT