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MUC1 modulates gastric epithelial immune response to bacteria or inflammatory stimuli

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ABSTRACT

BACKGROUND

Cell surface mucin glycoproteins are expressed on the mucosal surface. One of these cell surface mucins is mucin-1 (MUC1), which plays a role as a physical barrier and limits inflammation. However, its functional role in modulating responses to pathogens, particularly with respect to intracellular signaling, needs to be investigated. Therefore, the aim of this study was to characterize the modulation of responses of human gastric epithelial cells by MUC1 to common mucosal pathogens and inflammatory stimuli.

METHODS

Human gastric epithelial cell lines (MKN7) were co-cultured with *Campylobacter jejuni (C. jejuni)*. In order to investigate the effect of MUC1 expression on *C. jejuni*-induced cytokine production, MKN7 cells were transfected with 1:1 small interfering RNA (siRNA) to knock down the MUC1 gene using MUC1 targeting siRNA or non targeting siRNA as a control. The read out of the experiment was interleukin (IL)-8 concentration as a result of the inflammation process. This cytokine concentration was measured using ELISA and compared between the two groups.

RESULTS

This study demonstrated that by using siRNA transfection, knockdown of MUC1 expression in MKN7 human gastric epithelial cells suppressed IL-8 production at the early phase of incubation, but promoted an increase in IL-8 production at the late phase, in response to *C. jejuni.*

CONCLUSION

Knockdown of the MUC1 gene in MKN7 cells reduced IL-8 levels both in the cells with and without exposure to *C. jejuni*. This study provides direction for exploration of the intracelular mechanisms by which cell surface mucins modulates inflammation in the response of gastrointestinal epithelial cells to pathogens or other inflammatory stimuli.

Keywords: MUC1, epithelial cells, pathogen, inflammation

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MUC1 memodulasi respon imun sel epitel lambung akibat stimulus bakteri dan peradangan

ABSTRAK

LATAR BELAKANG

Glikoprotein musin seluler adalah musin yang diekspresikan di permukaan mukosa jaringan epitel. Musin-1 (MUC1) adalah musin seluler yang banyak diekspresikan di permukaan mukosa lambung dan berperan penting sebagai sawar fisik serta mencegah inflamasi. Meskipun demikian, peran fungsional dalam memodulasi respon terhadap patogen, perlu diteliti secara mendalam. Hal tersebut penting untuk memperoleh pemahaman yang baik tentang terjadinya respon alamiah terhadap infeksi. Tujuan penelitian ini adalah untuk menentukan modulasi respon imun sel epitel lambung dari MUC1 terhadap bakteri patogen dan stimulus inflamasi lainnya.

METODE

Penelitian in vitro menggunakan sel epitel lambung manusia berupa MKN7 cell line dilakukan ko-kultur bersama dengan Campylobacter jejuni (C. jejuni). Untuk menilai pengaruh ekspresi gena MUC1 pada produksi sitokin yang diinduksi oleh C. jejuni, pada sel MKN7 dilakukan transfeksi small interfering *RNA (siRNA) menggunakan MUC1 targeting siRNA untuk membuat gena MUC1 non aktif dan transfeksi dengan non targeting siRNA digunakan sebagai kontrol. ELISA digunakan untuk mengukur konsentrasi* interleukin *(IL)-8 dan kemudian dibandingkan antara kedua kelompok yakni kelompok dengan gena MUC1 non aktif dan kelompok kontrol.*

HASIL

Dengan transfeksi siRNA, penonaktifan ekspresi gena MUC1 pada sel epitel lambung mengakibatkan produksi IL-8 dihambat pada awal fase inkubasi namun produksi IL-8 tersebut menjadi meningkat di akhir fase inkubasi ketika sel dipaparkan patogen C. jejuni.

KESIMPULAN

Gena MUC1 berpengaruh pada sekresi IL-8. Konsentrasi IL-8 menurun ketika gena tersebut di-non-aktifkan, baik pada kelompok tanpa perlakuan maupun kelompok yang dipaparkan patogen C. jejuni. Penelitian ini dapat memberikan gambaran awal untuk eksplorasi lebih lanjut tentang mekanisme intraseluler musin dalam mengubah respon inflamasi sel epitel gastrointestinal terhadap paparan patogen atau paparan inflamasi lainnya.

Kata kunci : *MUC1, sel epitel, pathogen, inflamasi*

INTRODUCTION

Being a portal of entry to large numbers of bacteria, mucosal epithelial cells lining the gastrointestinal tract have mucins which can act as a physical barrier and play a role in modulating the immune response.[1] Mucin 1 (MUC1) is expressed by most mucosal tissues including stomach and intestinal tissue, and expressed by hematopoietic cells.[2] The MUC1 molecule has been estimated to be 200-500 nm in length, so that it will exceed other molecules attached to the plasma membrane. The molecule are surface contains a complex array of O-linked oligosaccharides that have been shown to bind to microbial molecules. For example, distinct sets of carbohydrates play an important role in adhesion of *Helicobacter pylori* (*H. pylori*) to epithelial cells, attenuation of *H. pylori* colonisation and facilitation of inflammatory

response following *H. pylori* infection.[1,3] There is evidence that MUC1 can limit infection and inflammation. The extracellular domain of MUC1 protects the epithelial cells by inhibiting bacterial adhesion to the mucosal surface by steric hindrance or by acting as a releasable decoy so that the severity of pathology caused by pathogen invasion such as *H. pylori* and *Campylobacter jejuni* (*C. jejuni*) can be reduced.[4-6] In addition to limiting adhesion of bacteria to the cell surface, MUC1 can modulate immune responses to bacterial components and toll-like receptor (TLR) ligands.[7-9] Such immune modulation has been demonstrated in relation to the involvement of phosphorylated cytoplasmic domains in NF-κB activation and attenuation of TLR-signalling.[8,10-12] The severity of pathology following pathogen infection is known due to the increased bacterial binding to the cells in the absence of MUC1.

In a mouse model study, it has been demonstrated that Muc1 limits *H. pylori* colonisation.[5,9] Mice lacking Muc1 had more bacterial colonization compared to wild type mice. Additionally, following *H. pylori* infection the degree of gastric inflammation was compared between mice lacking Muc1 and wild type and the results were consistent showing that mice lacking Muc1 developed more severe gastric inflammation. Co-culture of primary gastric epithelial cells with *H. pylori* showed that there was an increased adherence of *H. pylori* to murine primary gastric epithelial cells which lack Muc1. Muc1 appears to have a key role in limiting *H. pylori* colonisation and gastritis-associated pathologies.[5]

However, it is possible that there is an alteration of immune response resulting from altered cell signalling as a result of interaction between pathogen and host epithelial cells. Therefore, it is important to explore the immune response modulation by MUC1 *in vitro*. The objective of the present study was to characterize the response of gastrointestinal epithelial cells to live pathogenic bacteria in the presence and absence of MUC1.

METHODS

Research design

This was an experimental in vitro study using co-culture technique between human gastric epithelial cell lines and *C. jejuni* and was conducted from February to October 2010. Moreover, TNF á was also co-cultured with the cell lines as a trigger for inflammatory stimuli other than pathogenic bacteria. Small interference RNA transfection technique was used to knockdown the gene of interest (MUC1).

Cell culture

The human gastric epithelial cell line MKN7 (RIKEN Cell Repository, Japan) was stored in liquid nitrogen. The cells were then propagated in a complete culture media with added antibiotics, containing RPMI-1640 media, 10% heat-inactivated Fetal Bovine Serum (FBS), 2 mM L-glutamine (Invitrogen), 100 units/mL penicillin G sodium and 100µg/mL streptomycin (Invitrogen).

MUC1 knockdown

MUC1 was knocked down using small interfering RNA transfection technique. Small interfering RNA (siRNA) specific for MUC1 as well as non-targeted control siRNA were chemically synthesized. MKN7 cells were transfected with either 100nM of MUC1 siRNA or control siRNA using lipofectamine 2000 (Life technologies, Carlsbad, CA) according to the manufacturer's instructions. After 48 h of transfection, the cells were treated with *C. jejuni* and TNF-á respectively for the indicated time. The level of knockdown of MUC1 protein was measured using flow cytometry.

C. jejuni culture

The pure glycerol stocks of bacteria (*C. jejuni* strains 81-176 and ΔCDT81-176) stored at -75°C were thawed and then mixed with the appropriate volume of Brucella broth. Twenty µL of the bacterial suspension was aseptically spread using a bacterial spreader onto selective

agar plates (*C. jejuni* plates: 2% Columbia agar, 1% bacteriological agar, 5% defibrinated horse blood, Skirrow selective supplement (Oxoid), incubated under microaerophilic conditions (5% O2, 15% CO2, 80% N2) in an anaerobic jar using CampyGen, a microaerophilic gas-generating kit (Oxoid) for 2 days at 42°C. The mutant strain 81-176 ΔCDT was also grown under the same conditions as the parental strain.

Co-culture

The co-culture experiments were performed on confluent MKN7 cells with *C. jejuni.* The cells were transferred to microaerobic conditions (5% O2, 15% CO2, and 80% N2) at the start of the co-culture. Before introducing bacteria, the media was changed into antibiotic-free media containing RPMI-1640, 10% FBS and 2 mM L-glutamine.

Analysis of IL-8 by ELISA

The concentrations of IL-8 secreted by MKN7 in various treatments were determined by Human IL-8 ELISA (BD Biosciences, San Jose, CA, USA) following the manufacturer's instructions.

Flow cytometry

MKN7 cells were harvested using trypsine and incubated with the anti-MUC1 antibody BC2 (CT2) or isotype control 401.21 at 3 µg/mL in 5% FCS for 60 min at 4°C and then with anti-mouse antibody conjugated to Alexa fluor 488 (Life Technologies, Carlsbad, CA). After staining, all cells were fixed with 1% paraformaldehyde. Assessment of staining was performed on FCS 500 flow cytometer (BD Biosciences, San Jose, USA).

Data analysis

The Mann-Whitney U-test was used to compare means of IL-8 concentrations in the presence and absence of MUC1. All analyses were conducted using GraphPad Prism version 5. P values less than 0.05 were considered significant.

RESULTS

MUC1 gene was first knocked down to investigate the effect of MUC1 expression on *C. jejuni*-induced cytokine production. Figure 1 shows the expression of cell surface MUC1 after knockdown using non-target siRNA and MUC1 siRNA. In this experiment, transfection of MUC1 siRNA into MKN7 cells reduced cell surface MUC1 expression by 91%.

Then, IL-8 levels in culture media was measured after co-culture with *C. jejuni*. Initially, the IL-8 concentration was measured after 4 h and 24 h-bacterial exposure. It seems that at the short term co-culture, a higher bacterial density is needed to elicit significant IL-8 production. It was also demonstrated that at the same lower bacterial density but with a longer incubation time, the IL-8 production was much higher (Figure 2). The knockdown of MUC1 gene in MKN7 cells had an effect on IL-8 secretion. The IL-8 levels decreased in the absence of MUC1 both in cells with and without exposure to *C. jejuni*.

The production of IL-8 after *C. jejuni* treatment could be partially due to the presence of microbial toxin. Therefore, the next experiment was done using parent *C. jejuni* 81-176 and ΔCDT-*C. jejuni* 81-176. In this experiment, MKN7 cells transfected with a MUC1-targeting siRNA exhibited 92% reduction in MUC1 protein levels as depicted in Figure 3.

Comparison between parent and ΔCDT *C. jejuni* 81-176 demonstrated that there is no significant difference in IL-8 level between these two treatment groups either in MUC1-expressing cells or in MUC1-knocked down-cells. The timing of incubation also does not influence the difference in IL-8 concentration between treatment with parent and treatment with Δ cytolethal distending toxins (CDT) *C. jejuni* 81-176 (Figure 4). Therefore, probably CDT is not involved in inducing IL-8 production in response to this particular bacterial strain.

Focusing on IL-8 production, it is likely that at short term incubation, the MUC1

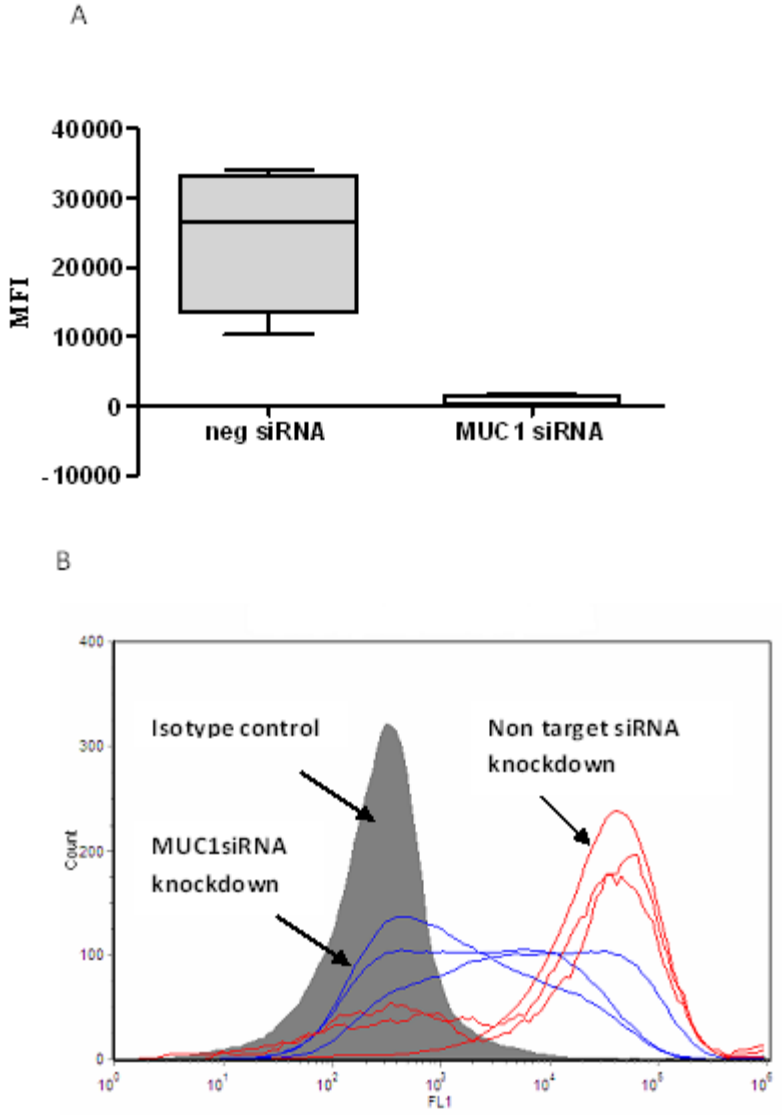

Figure 1. Cell surface MUC1 expression after knockdown using 1:1 siRNA transfection. Representative histograms from the flow cytometry analysis showing median fluorescence intensitiy (MFI). MFI was determined after subtraction of the MFI using a negative control antibody 401.21. Results from 3 replicates

knockdown causes decrease in IL-8 secretion but with the longer incubation time, the concentration of IL-8 increases although in some conditions these changes are not statistically significant (Figure 4).

TNF-α was used as a positive control and it was shown that the concentration of IL-8 induced by this pro-inflammatory cytokine varies with length of incubation time in the absence of MUC1. TNF-α induces a higher IL-8 concentration at 4 h but a lower IL-8 concentration at 24 h, although in some conditions such changes were not significant.

DISCUSSION

Although there are some variations in the results obtained from this study, it has been demonstrated that MUC1 knock down in MKN7 cells tends to cause a decrease in IL-8 production

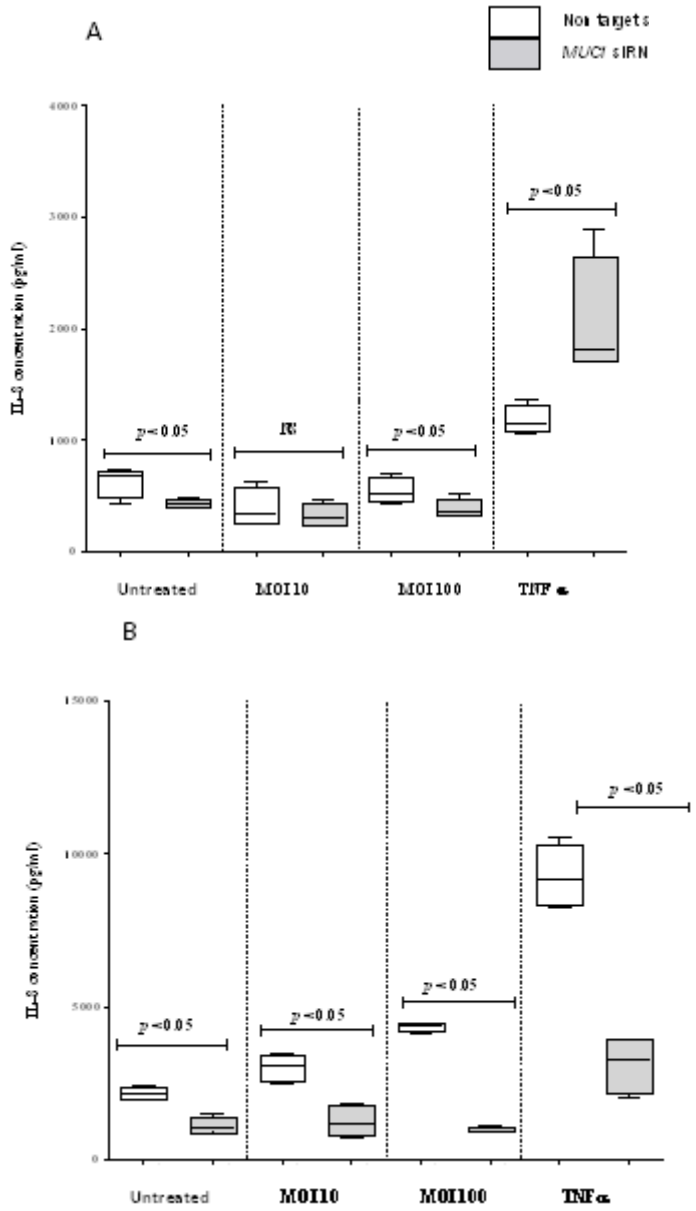

Figure 2. The effect of MUC1 knock down on IL-8 production in response to pathogenic bacteria. MKN7 cells were transfected with a MUC1 targeting siRNA or non-targeting control siRNA for 48 hours and then treated with *Campylobacter jejuni* (MOI 10 and 100) or TNF-α (100ng/mL) for a further 4 h (A) or 24 h (B) and IL-8 levels in culture media were measured by ELISA. Statistics: box plots show mean, quartiles and range. Mann-Whitney U-test, p values shown, n=4

in the short term but that a higher concentration is achieved in the long term. Because in this study the IL-8 production was only measured at end-points (4 h and 24 h), the probable fluctuation of IL-8 secretion over the incubation time cannot be determined accurately. Therefore the factual timing of IL-8 production by epithelial cells in a certain period needs to be elucidated further by observing IL-8 secretion over a full time course.

The variations in IL-8 production may also be influenced by actual bacterial growth. This hypothesis is based on a certain set of data of this study. In the same experiment with the same time course (24 h) and same multiplicity of

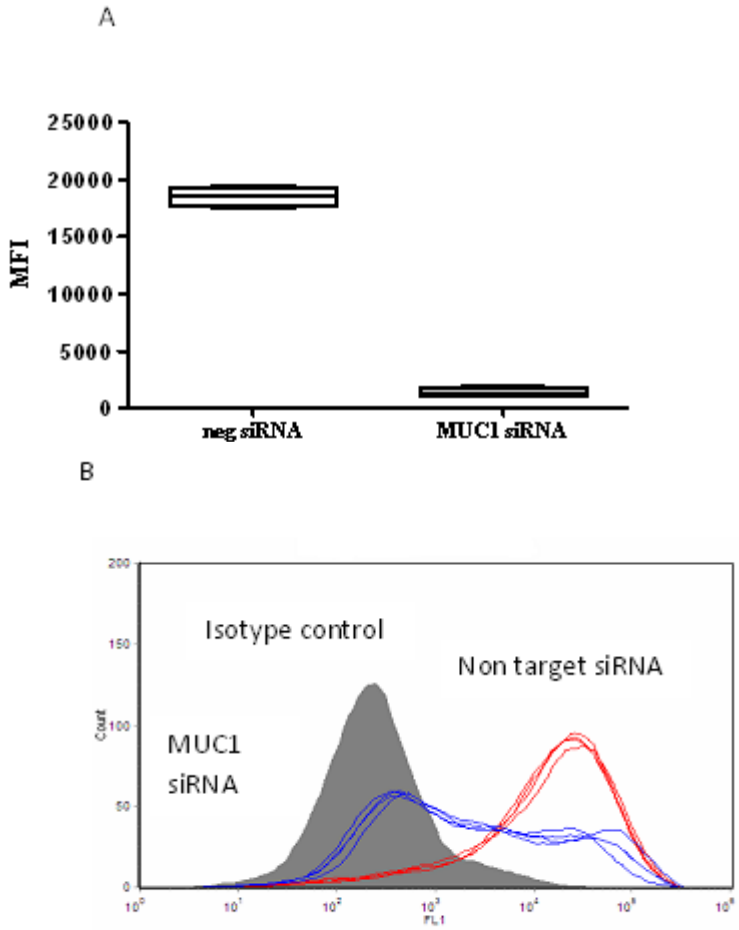

Figure 3. Cell surface MUC1 expression after knockdown using 1:1 siRNA transfection. Results from 4 replicates

infection (MOI 100), the results are opposite. Knockdown of MUC1 expression causes an increase in IL-8 production in one experiment and a decrease in IL-8 production in the other (Figures 2B and 4B). This difference may be due to the variation of actual bacterial growth. Although the MOI provides the average number of bacteria per cell, the actual number of bacteria that infect any given epithelial cells can vary in different experiments, for example, if the MOI is 1, some cells will get infected with one bacteria but some cells may be infected with 0 bacteria and other cells infected with 2 bacteria. Hence, the proportion of cell population infected by a specific number of bacteria should have been controlled to get the number of bacteria evenly in each experiment. However, in dealing with the dynamic nature of bacterial culture, it is a challenge to control the same actual bacterial growth with the number of MOI. A variation in the number of actual bacterial growth compared to the MOI may be one of the limitations of this study that might influence the variation in IL-8 production. The increase in IL-8 production in the absence of MUC1 in the late phase of incubation suggests that MUC1 has a role in this proinflammatory cytokine expression in response to *C. jejuni*.

With regard to cytolethal distending toxins (CDTs) and its role in inducing an inflammatory response, it seems that there is no strong evidence that this toxin plays a role in inducing IL-8 production, given that there was no significant difference in IL-8 levels between parent *C. jejuni* 81-176 and Δ CDTs *C. jejuni* 81-176-treated

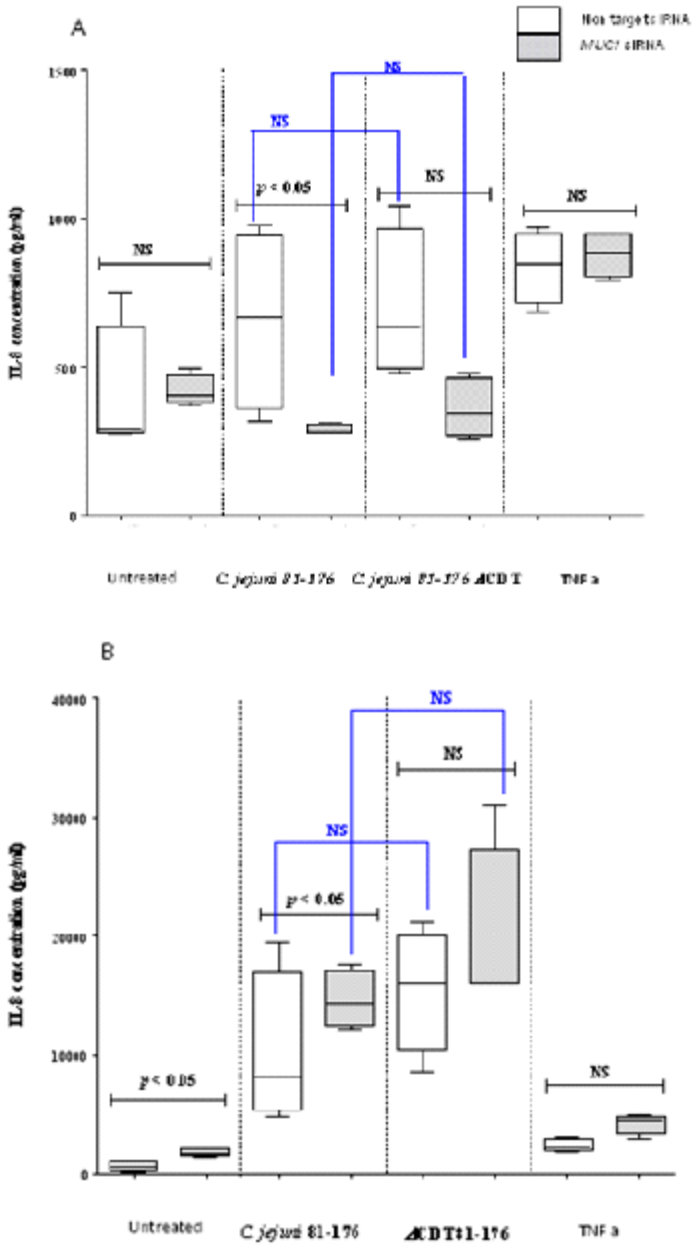

Figure 4. Knockdown of MUC1 expression tends to decrease IL-8 production in short term incubation but increases IL-8 secretion in long term incubation. Deletion of CDT does not change the IL-8 levels significantly (see blue line). MKN7 cells were transfected with a *MUC1* targeting siRNA or non-targeting control siRNA for 48 hours and then treated with *Campylobacter jejuni* (MOI 100) or TNF-α (5ng/mL) for a further 4 h (A) or 24 h (B) and IL-8 levels in culture media were measured by ELISA. Statistics: box plots show mean, quartiles and range. Mann-Whitney U-test, *p* values shown, n=4

cells. Other previous studies have established that the ability of *C. jejuni* to induce IL-8 production is determined by many factors such as a process that is related to bacterial adhesion or invasion and a process that depends on CDTs.[13] It has been demonstrated by Zheng et al. that *C. jejuni* can induce IL-8 secretion depending on CDT and NF-κB activation mediated by TLR.[14] However, the role of CDTs in inducing IL-8 production and the modulation of cellular response to CDTs by MUC1 needs further investigation.

With end-point analysis it is difficult to explain what is really happening in this pro-inflammatory secretion regulation and what action is performed by the mucins. Several possibilities may help us to understand such variation in IL-8 production with regard to incubation timing First, there is potential modulation by a steric hindrance mechanism or releasable decoy to inhibit bacterial attachment to the cell surface by the mucins[6] and second, there is potential intracellular signal transduction by the mucins.[8-10] Regarding the timing of these potential modulations, it is not known which one will happen at the early phase of incubation and which one will happen at later on. The timing of certain mechanisms of such modulation probably influences the variation of IL-production in response to pathogenic bacteria.

In untreated cells, the MUC1 knockdown causes variations in IL-8 production. There is a complex underlying mechanism of IL-8 production in epithelial cells. Epithelial cells are able to secrete IL-8 constitutively but such production can be enhanced once bacteria enter the cell.[15] However, in the absence of bacteria, the siRNA transfection itself causes not only a decrease in MUC1 expression but also influences bacterial adherence to the epithelial cells. This may explain why there is a variation in IL-8 secretion.

This study has demonstrated that MUC1 knockdown mostly causes an increase in IL-8 production in response to TNF-α, although one of the results was just the opposite. TNF-α is an important pro-inflammatory cytokine in both acute and chronic inflammation states and an activator of the transcription factors NF-κB and activator protein-1 (AP-1), two key modulators of the inflammatory response. The increase in IL-8 in the absence of MUC1, suggests that MUC1 has an anti-inflammatory effect on TNF-α.

In a previous in vivo study, it has been demonstrated that MUC1 knock out mice exhibit increased levels of TNF-α following *H. pylori* infection.[9] This anti-inflammatory effect is not only exhibited against bacterial but also viral infections. A previous in vitro study has revealed that Muc1 knockdown resulted in greater TNF-α production following respiratory syncytial virus (RSV) infection. It also showed that the increase in TNF-α upregulates MUC1 which in turns suppresses further increase in TNF-α induced by RSV. Hence, MUC1 controls the level of TNF-α during RSV infection, thus preventing the potential harmful effects of excessive TNF-α.[17]

Regarding the variations in IL-8 results in this study, it has been demonstrated that possibly the siRNA transfection used can exert an effect on the cells that might influence the adherence of bacteria to the cell surface which eventually will have an impact on IL-8 production.

CONCLUSION

This study has shown that knockdown of MUC1 expression in MKN7 human gastric epithelial cells suppressed IL-8 production at the early phase of incubation, but promoted an increase in IL-8 production at the late phase, in response to *C. jejuni*.Furthermore, with respect to the dynamic nature of bacterial/cell culture, the actual growth of bacteria in relation with MOI number needs to be worked out more accurately.

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REFERENCES

1. Linden SK, Sutton P, Karlsson NG, Korolik V, McGuckin MA. Mucins in the mucosal barrier to infection. Mucosal Immunol 2008;1:183-97.
2. Hattrup CL, Gendler SJ. Structure and function of the cell surface (tethered) mucins. Annu Rev Physiol 2008;70:431-57.

3. Kobayashi M, Lee H, Nakayama J, Fukuda, M. Roles of gastric mucin-type O-glycans in the pathogenesis of Helicobacter pylori infection. Glycobiology 2009;19:453-61.
4. McAuley JL, Linden SK, Ping CW, King RM, Pennington HL, Gendler SJ, et al. MUC1 cell surface mucin is a critical element of the mucosal barrier to infection. J Clin Invest 2007;117:2313-24.
5. McGuckin MA, Every AL, Skene CD, Linden SK, Chionh YT, Swierczak A, et al. MUC1 mucin limits both Helicobacter pylori colonization of the murine gastric mucosa and associated gastritis. Gastroenterology 2007;133: 1210-8.
6. Linden SK, Sheng YH, Every AL, Miles KM, Skoog EC, Florin TH, et al. MUC1 limits Helicobacter pylori infection both by steric hindrance and by acting as a releasable decoy. PLoS Pathog 2009;5:e1000617.
7. Lu W, Hisatsune A, Koga T, Kato K, Kuwahara I, Lillehoj EP, et al. Cutting edge: enhanced pulmonary clearance of Pseudomonas aeruginosa by MUC1 knockout mice. J Immunol 2006;176: 3890-4.
8. Ueno K, Koga T, Kato K, Golenbock DT, Gendler SJ, Kai H, et al. MUC1 mucin is a negative regulator of toll-like receptor signaling. Am J Respir Cell Mol Biol 2008;38:263-8.
9. Guang W, Ding H, Czinn SJ, Kim KC, Blanchard TG, Lillehoj EP. MUC1 cell surface mucin attenuates epithelial inflammation in response to a common mucosal pathogen. J Biol Chem 2010;285:20547-57.
10. Lillehoj EP, Kim H, Chun EY, Kim KC. Pseudomonas aeruginosa stimulates phosphorylation of the airway epithelial membrane glycoprotein MUC1 and activates MAP kinase. Am J Physiol Lung Cell Mol Physiol 2004;287:L809-15.
11. Thompson EJ, Shanmugam K, Hattrup CL, Kotlarczyk KL, Gutierrez A, Bradley JM, et al. Tyrosines in the MUC1 cytoplasmic tail modulate transcription via the extracellular signal-regulated kinase 1/2 and nuclear factor-kappa B pathways. Mol Cancer Res 2006;4:489-97.
12. Ahmad R, Raina D, Trivedi V, Ren J, Rajabi H, Kharbanda S, et al. MUC1 oncoprotein activates the I kappa B kinase beta complex and constitutive NF-kappa B signalling. Nat Cell Biol 2007;9:1419-27.
13. Hickey TE, McVeigh AL, Scott DA, Michielutti RE, Bixby A, Carroll SA, et al. Campylobacter jejuni cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells. Infect Immun 2000;68:6535-41.
14. Zheng J, Meng J, Zhao S, Singh R, Song W. Campylobacter-induced interleukin-8 secretion in polarized human intestinal epithelial cells requires Campylobacter-secreted cytolethal distending toxin- and Toll-like receptor-mediated activation of NF-kappa B. Infect Immun 2008; 76:4498-508.
15. Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. Cell Death Differ 2003; 10:45-65.
16. Bradley JR. TNF-mediated inflammatory disease. J Pathol 2008;214:149-60.
17. Li Y, Dinwiddie DL, Harrod KS, Jiang Y, Kim KC. Anti-inflammatory effect of MUC1 during respiratory syncytial virus infection of lung epithelial cells in vitro. Am J Physiol Lung Cell Mol Physiol 2010;298:L558-63.