TNF-alpha Activates MUC2 Transcription via NF-kappaB but Inhibits via JNK Activation

¹Dae-Ho Ahn, ^{1,2}Suzanne C. Crawley, ¹Ryota Hokari, ¹Shingo Kato, ¹Stacey C. Yang, ³Jian-Dong Li and ^{1,2}Young S. Kim

¹Gastrointestinal Research Laboratory, Veterans' Affairs Medical Center, San Francisco, ²Department of Medicine, University of California, San Francisco, ³Gonda Department of Cell and Molecular Biology, House Ear Institute, University of Southern California, Los Angeles

Key Words

Colon • NF-kappaB • PI3K • NIK • JNK • MUC2 • Mucin
• Inflammatory bowel disease • Akt • TNF-alpha

Abstract

The molecular mechanisms responsible for TNF- α -mediated MUC2 intestinal mucin up-regulation in HM3 colon adenocarcinoma cells were analyzed using promoter-reporter assays of the 5'-flanking region of the MUC2 gene. Chemical inhibitors, mutant reporter constructs, and EMSA confirmed I-kappaB/NF-kappaB pathway involvement. Wortmannin, LY294002 and dominant negative Akt, as well as dominant negative NF-kappaB-inducing kinase (NIK) inhibited MUC2 reporter transcription, indicating that both phosphatidylinositol-3-OH kinase (PI3K)/Akt signaling pathway and NIK pathways mediate the effects of TNF-alpha. Wortmannin inhibited NF-kappaB binding and transcriptional activity without inhibiting NF-kappaB translocation to the nucleus, indicating that PI3K/Akt signaling activates NF-kappaB transcriptional activity directly. Our results demonstrate that TNF-alpha up-regulates MUC2 in human colon epithelial cells via several signaling pathways, involving both

NIK and PI3K/Akt, which converge at the common IKK/I-kappaB/NF-kappaB pathway. TNF-alpha activated JNK, but JNK inhibitor SP600125 and dominant negative cJun consistently activated transcription, revealing a negative role for this signaling pathway. Thus TNF-alpha causes a net up-regulation of MUC2 gene expression in cultured colon cancer cells because NF-kappaB transcriptional activation of this gene is able to counter-balance the suppressive effects of the JNK pathway. However, the existence of this inhibitory JNK pathways suggests a mechanism whereby - in the absence of NF-kappaB activation - TNF-alpha production during inflammation in vivo could actually inhibit MUC2 production, giving rise to the defective mucosal protection which characterizes inflammatory bowel disease.

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Introduction

Mucins are very large and heavily glycosylated proteins synthesized by the epithelial cells lining the gastrointestinal, respiratory, and genitourinary tracts,

where they protect the epithelium from mechanical, chemical, enzymatic and microbial damage [1-3]. Secretory and membrane-associated mucins are expressed in a tissue and cell type specific manner (recently reviewed in [1]). MUC2, a major secretory mucin, is highly expressed in goblet cells but not in absorptive cells of the intestine. The MUC2 gene is approximately 27 kb in size, producing a message that is 16 kb and a polypeptide that has more than 5000 amino acids, including a variable number of (usually more than 100) tandem repeat units, which are extensively *O*-glycosylated [4]. A small but significant number of intestinal tumors developed in Muc2 knockout mice within one year of age, indicating that Muc2 plays an indirect role in suppression of colon cancer in mice [5]. Perhaps more significantly, these mice have since been reported to be very susceptible to dextran sulfate-induced colitis [2]. The mucin layer of the colon is thinner in ulcerative colitis, and MUC2 secretion has been shown to be reduced in active ulcerative colitis and in the IL10 (-/-) mouse model of inflammatory bowel disease [2, 3, 6-10].

A variety of molecules have been shown to up-regulate MUC2 expression, including lipopolysaccharide [10], inflammatory cytokines (TNF- α , interleukins-1, -4, -6, -9, -13) [11-16], growth factors (epidermal growth factor, transforming growth factors α and β) [17-20], phorbol 12-myristate 13-acetate (PMA) [21], retinoic acid [22], and surface molecules of non-typeable *Haemophilus influenzae* [23]. In addition several cis elements involved in MUC2 transcription have been identified and analyzed within the MUC2 promoter, including Sp1 [24], NF- κ B [23], Cdx-2 [25] and p53 [26] binding sites.

Inappropriate production of TNF- α and persistent activation of TNF- α signaling has been implicated in the pathogenesis of a wide spectrum of human diseases, including inflammatory bowel disease [27]. Since TNF- α activates NF- κ B, and since MUC2 was shown to be up-regulated by both lipopolysaccharide and PMA via NF- κ B [23, 21], we hypothesized that TNF- α would up-regulate MUC2 via NF- κ B activation. We analyzed the involvement of NF- κ B and the various roles of upstream signaling pathways including PI3K/Akt, NIK/IKK/I- κ B, MEK1/2 and JNK/cJun.

Materials and Methods

Reagents

TNF- α was purchased from R&D Systems Inc. (Minneapolis, MN). Caffeic acid phenyl ester (CAPE), wortmannin, LY294002, SP600125, UO126, SB203580, SB202190, and PD98059 were obtained from EMD Biosciences (La Jolla, CA). Antibodies against I- κ B- α (#9242), Akt (#9272), phospho-Akt-Ser473 (#9271S), phospho-ERKp44/42 Thr202/Tyr204 (#9101), phospho-p38Thr180/Tyr204 (#9211) and phospho-SAPK-JNKThr183/Tyr185 (#9251) were obtained from Cell Signaling Technology (Beverly, MA). Antibodies for p50 (sc-114X), p65 (sc-109X) and c-Rel (sc-70X) subunits of NF- κ B were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). NF- κ B inhibitors BA 11-7082, parthenolide and SN50 were obtained from Biomol. GAPDH monoclonal antibody was from Ambion.

Cell lines and plasmids

HM3 cells (derived from LS174T cell line) were maintained at 37°C in 5% CO₂ atmosphere in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, penicillin, and streptomycin. The cell line HM3M2 is an HM3 cell line stably transfected with the MUC2 promoter region from -2864 to +19 subcloned into pGL2-Basic luciferase vector (Promega Corp., Madison, WI), along with pcDNA3 carrying the *neo* gene.

Plasmids were prepared using the EndoFree Plasmid MAXI prep kit (Qiagen). MUC2 promoter-reporter deletion constructs [24], and MUC2 constructs with promoter elements subcloned upstream of a minimal thymidylate kinase (TK) promoter luciferase vector (-1.5/-1.3-TK containing basepairs -1528/-1307 and -1.5/-1.4-TK containing the region -1528 to -1430), were described previously [23]. Sequences of these constructs are illustrated in the figure.

Mutated versions of the -1628/+19 MUC2 reporter were made by PCR using the megaprimer method [28]. Forward primer annealing at -1459 to -1433 (5'-CCG TCC TGT AGT TTC CCC AGG GCT CAG-3' for -1628/+19MUC2-LUC-MT1 or 5'-CCG TCCTTG GAG CTC CCC AGG GCT CAG-3' for -1628/+19MUC2-LUC-MT2; NF- κ B consensus sequence underlined, mutated bases in bold) were used with reverse primer from -1277 to -1296 (5'-CAG GGT CTG CGC TCC CTG C-3'). The resulting PCR fragment was used as the reverse primer in touch-down PCR, with pGL1 (Promega) as the forward primer, to give a PCR fragment which was digested with BglII (cut at -1624) and PmlI (cut at -1311) and ligated into the -1628/+19 MUC2 promoter vector cut with the same enzymes.

Dominant negative NIK (K429-430A) [29] and I- κ B- α (S32/36A) [30] were described previously. Plasmid encoding dominant negative Akt was obtained from David Stokoe, University of California, San Francisco.

RNA isolation and RT-PCR

Cells were serum-starved overnight before treatment with TNF- α (1 nM) for 4 hours. Total RNA was harvested using

TriReagent (Molecular Research Center Inc., Cincinnati, OH), and RT-PCR was carried out for MUC2 as previously described [21].

Transient transfection and inhibitor assays

Cells were transfected with reporter vectors, treated and assayed as described previously [21]. All transient transfections were carried in triplicate, with standard deviations indicated by error bars, and statistical differences analyzed by Student's *t* test.

Cell staining

Cells grown on 1cm glass coverslips in 24-well plates were serum-starved and then treated with TNF- α for 14 hours. Cells were fixed in wells with 4:1 methanol/glacial acetic acid, rinsed with methanol, and treated with 3% H₂O₂ in 90 % methanol for 1 h. Monolayers were then blocked and stained using the Zymed Broad Spectrum Histostain kit, with 1:300 dilution of Ccp58 anti-MUC2 antibody (Biomedica) in 1% BSA/TBST for 1 h. AEC chromogen (red; Zymed) was used for the final color development step, nuclei were counterstained using Meyer's hematoxylin, and coverslips were mounted with aqueous mounting media onto glass slides. Microscopic fields were randomly selected for counting and MUC2 protein expression in the different cell lines was scored and expressed as percentage of positive cells per field (*n*=12 fields, typically containing 100-200 cells each).

Western blotting

Cells were treated with TNF- α for indicated times. Total cell lysates were prepared and equivalent amounts of protein analyzed by western blotting as described previously [21] using antibodies indicated in the figures.

Electrophoretic mobility shift assay and supershift assay

Cells were treated with TNF- α and nuclear extracts were prepared according to Andrews and Faller [31], with protein concentration determined using the Bradford Assay method (Biorad, Hercules, CA). A double-stranded oligonucleotide probe corresponding to the human MUC2 promoter region from base pairs -1458/-1430 was prepared by annealing forward primer 5'-CGTCCTTGGGTTTCCCCAGGGCTCAGTGC-3' with reverse-complementary deoxynucleotide 5'-GCACTAGCCCTGGGGAAAC-3' and filling in the resulting overhang using Klenow fragment with [α -³²P]dCTP. NF- κ B consensus probe was made by annealing 5'-GAGTTGAGGGGACTTTCCAGG-3' and 5'-GGCCTGGGAAAGTCCCT-3', labeled in the same way. Nuclear extracts (5 μ g protein) were incubated with radiolabeled probes at room temperature for 20 minutes in a solution containing 1 μ g of poly (dI-dC) in 10 mM Tris-HCl at pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 0.5 mM dithiothreitol, 0.05% NP-40 and 10 % glycerol. For competition experiments, a 100-fold excess of unlabeled oligonucleotide was added. For supershift assays,

antibodies specific for p50, p65 or c-Rel subunits of NF- κ B were added to the reaction for 30 minutes prior to addition of probe. Samples were applied to 4% polyacrylamide gels in Tris-acetate-EDTA buffer at 130 volts for 3 hours; the gel was dried and exposed to X-ray film.

Results and Discussion

TNF- α increases MUC2 transcription

Figure 1 shows that treatment of HM3 cells with TNF- α causes an increase in the level of MUC2 mRNA over time, as measured by RT-PCR (Fig. 1A). Densitometry analysis revealed that TNF- α induced a 3.5-fold increase of mRNA level after 7 hours. The luciferase reporter activities of HM3M2 cells were also determined after treatment with TNF- α : Transcriptional activity was found to be up-regulated in a time-dependent manner up to 7 hours (Fig. 1B). In addition, TNF- α up-regulated MUC2 mRNA levels in two other colon cancer cell lines, HCT8 and HT29 (Fig. 1C). MUC2 protein synthesis was confirmed by cell staining of TNF- α -treated cells with Ccp58 antibody, which recognizes the tandem repeat sequence of the non-glycosylated - i.e. newly synthesized - polypeptide (Figs. 1D and 1E).

NIK and Akt are involved in MUC2 up-regulation by TNF- α

According to the current model, TNF- α binds to its receptor TNF- α -R1, inducing assembly and activation of a signaling complex or complexes containing TRAFs, RIP, and IKK's, recruiting and activating several possible IKK kinases, which activate the IKK's that phosphorylate I- κ B. This phosphorylation triggers I- κ B ubiquitination and degradation, allowing NF- κ B translocation into the nucleus [32-34]. Several IKK kinases have been described, with different ones apparently responding to activation of different receptors in different cell types. NIK and PKB/Akt are two upstream activating kinases that have been implicated in TNF- α signaling [32-34]. Western blot showed that TNF- α treatment caused a perceptible increase in the level of Akt phosphorylation (Fig 2A). Wortmannin, an inhibitor of Akt activator PI3K, inhibited Akt phosphorylation, as expected (Fig. 2A). PI3K inhibitors wortmannin and the structurally unrelated synthetic quercetin derivative LY294002 were both shown to inhibit

Fig. 1. TNF- α up-regulates MUC2 transcription. A) RT-PCR analysis of RNA from TNF- α treated and untreated HM3 cells, using primers for MUC2 and 18S rRNA (internal control). B) HM3M2 cells were treated with or without TNF- α (1 nM) for indicated times and luciferase activity was measured (n=3). C) MUC2 mRNA was analyzed by RT-PCR after TNF- α treatment of HCT 8 and HT29 cell lines for the indicated times. D) Cell lines were treated with TNF- α for 14 hours and then stained (red) for newly-synthesized MUC2 using tandem repeat antibody Ccp58, and E) MUC2 protein expression calculated as a percentage of the total number of cells which are MUC2 positive (250X magnification).

Fig. 2. NIK and Akt are involved in TNF- α signaling. A) Western blot shows that Akt is phosphorylated in response to TNF- α treatment, and this phosphorylation is reduced by wortmannin. B) HM3M2 cells were pre-treated with varying concentrations of PI3K inhibitors wortmannin or LY294002 before stimulation with TNF- α (* P <0.05, compared to no inhibitor). C) Dominant-negative forms of NIK and Akt reduced TNF- α -induced MUC2 up-regulation. HM3 cells were transiently co-transfected with 1.6 μ g of -1.5/-1.3-TK, 0.4 μ g of pRL0 and the indicated amounts of dominant-negative forms of NIK (DN-NIK) or Akt (DN-Akt), with pcDNA3 vector added to ensure equal amounts of DNA. Asterisks indicate values which are significantly different from control assays lacking

dominant negative vector (P <0.05). D) The combination of PI3K inhibitors plus dominant negative NIK effectively inhibited MUC2 -1.5/-1.3-TK reporter transcription. HM3 cells were transfected with -1.5/-1.3-TK plasmid, plus either pCDNA3 (control) or DN-NIK. Cells were pre-treated with or without wortmannin ("wort.", 100 nM) before addition of TNF- α for 4 hours. Luciferase activities were measured and normalized with respect to pRL0 activity. Asterisks indicate values which are significantly different from corresponding control assays without added inhibitor (P <0.05). Daggers ($\dagger$) indicate that DN-NIK combined with wortmannin significantly lowers activity compared with assays which include only a single inhibitor wortmannin or DN-NIK (P <0.05).

Fig. 3. An NF-kappaB binding site mediates responsiveness to TNF-alpha. A) Transcriptional activities of deletion constructs were measured after 4 hour treatment with TNF-alpha or PMA (positive control). Firefly luciferase activities were normalized to renilla luciferase internal control (*P<0.05, **P<.001). B) Mutations within the NF-kappaB consensus sequence of -1.5/-1.4-TK reporters (MT2 and MT3) abrogated responsiveness to TNF-alpha but mutation outside of this region (MT4) did not impair the response to TNF-alpha. Mutation of the NF-kappaB site within the intact -1628/+19-MUC2-LUC (MT1 and MT2) promoter also abolished response to TNF-alpha. Activities were normalized to response of the respective wild-type reporters (*P<0.05, relative to wild-type). C) NF-kappaB pathway inhibitors MG132 (30 μM), parthenolide (20 μM) and CAPE (26 μM) reduced TNF-alpha-mediated up-regulation of MUC2 promoter-reporter activity in HM3M2 cells. Activities are expressed relative to untreated control (*P<0.05 relative to control). RT-PCR analysis of RNA from HM3M2 cells pre-treated with the same inhibitors ± TNF-alpha as indicated (lower panel, 5 μM MG132, 20 μM parthenolide and 26 μM CAPE). The ratios of band intensities for MUC2/18SrRNA are provided below the gel. D) TNF-alpha increased transcription factor binding to MUC2 NF-kappaB element in EMSA. Oligonucleotide probe containing the human MUC2 NF-kappaB binding site (-1458/-1430) was incubated with nuclear proteins (5 μg) from HM3 cells treated with (lane 2) or without (lane 1) TNF-alpha. The shifted band is indicated with an arrow. Pre-treatment with wortmannin (200 nM) reduced band shift intensity. Unlabeled probe (cold) blocked nuclear protein binding (lane 4). Antibody to p50 reduced band intensity and gave a faint supershifted band (ss, arrow; lane 5), and antibody to p65 gave a strong supershift (lane 7), whereas antibody to c-Rel had little effect (lanes 6). NF-kappaB consensus probe, used as a positive control, produced a more intense band than the MUC2 NF-kappaB probe (lane 8), and was supershifted by p65 (lane 9) and p50 (lane 10). E) Nuclear proteins used for EMSA (40 μg) were analyzed by western blot, using antibodies to p65 and p50. F) Co-transfection with dominant-negative I-kappaB-alpha (S32/36A) reduced TNF-alpha-induced MUC2 up-regulation. HM3 cells were transiently co-transfected with 1.6μg of -1.5/-1.3-TK, 0.4μg of pRLO and indicated amounts of plasmid encoding I-kappaB-alpha S32/36A (DN-IkB), with pcDNA3 vector used as negative control (*P<0.05 relative to control). G) Western blot shows that I-kappaB-alpha is degraded in response to TNF-alpha treatment of HM3 cells, and this response is not inhibited by 200 nM wortmannin. Probing with antibody to GAPDH demonstrates equal loading of wells.

the TNF-alpha-induced MUC2 up-regulation in a dose-dependent manner (Fig. 2B). Dominant negative forms of NIK (K429-430A) [27] and Akt were both found to reduce the responsiveness of the MUC2 -1.5/-1.3-TK reporter (Fig. 2C). The incomplete inhibition observed in Figures 2B and 2C suggested that both NIK and Akt are involved in TNF-alpha signaling, thus the effectiveness of combined treatment using both DN-NIK and wortmannin was evaluated (Fig. 2D). The two treatments inhibited reporter transcription more effectively when combined than either treatment did alone, indicating that these two separate pathways responded independently and additively to TNF-alpha.

An NF-kappaB cis element is involved in TNF-alpha-induced transcription

In order to deduce the location of the TNF-alpha-responsive element(s), MUC2 promoter-reporter deletion constructs were tested for their ability to respond to TNF-alpha (Fig. 3A). As shown, -343/+19 and -1308/+19 constructs do not produce higher luciferase activity in response to TNF-alpha, whereas the larger constructs exhibit higher activity, indicating that a TNF-alpha-responsive element lies upstream of -1308. In contrast, all constructs were responsive to PMA, used as a positive control. In general it should be noted that the effect of

TNF-alpha on transient transfectants was low relative to that of stably transfected cell line HM3M2 containing the same -2864/+19 promoter region. Cell staining indicated that basal expression of MUC2 in the parental HM3 cell line is higher than in HM3M2 cells, which may reduce the apparent effects of transcriptional activators. There are also likely differences in the way that stably incorporated DNA is transcriptionally activated compared with transiently transfected plasmid DNA [35]. For example, transcriptional activation involving chromatin remodeling may not be discernible using transiently transfected reporter vectors. Since the response of the stably transfected cells closely followed the behavior of the endogenous gene, we do not believe that the local environment of the stably incorporated gene is responsible for its greater responsiveness.

A functional NF-kappaB site was previously identified at basepairs -1441 to -1452 of the MUC2 promoter [24]. Comparison of the activities of wild type and mutated versions of the heterologous -1.5/-1.4-TK promoter reporter showed that cells transfected with mutant vectors MT2 and MT3 were unresponsive to TNF-alpha, whereas wild type reporter (WT) and mutant MT4 (containing mutations outside of the NF-kappaB consensus site) retained the ability to respond to TNF-alpha stimulation (Fig. 3B, left panel). In order to confirm

Fig. 3. An NF-kappaB binding site mediates responsiveness to TNF-alpha. (for legend see previous page)

Fig. 4. JNK, ERK and p38 signaling pathways are activated by TNF- α . Western blots of lysates from cells treated with TNF- α were probed with antibodies to phosphorylated forms of JNK, p38, and ERK.

Fig. 5. MEK and p38 inhibitors reduce transcriptional activation, whereas JNK pathway inhibitors activate. A) HM3M2 cells were pre-treated with MEK1/2 inhibitor U0126 (2 μ M), MEK inhibitor PD98059 (50 μ M), p38 inhibitors SB203580 (2 μ M) and SB202190 (20 μ M) or JNK inhibitor SP600125 (20 μ M) before addition of TNF- α for 4 hours (* P <0.05, compared to control assays without added inhibitor). B) Co-transfection with dominant negative cJun (TAM67) activated MUC2 reporter transcription (* P <0.05, ** P <.001). Relative luciferase activities were normalized to control values.

these results, mutations were also introduced into this NF- κ B site within the intact -1628/+19 MUC2 promoter reporter. Fig. 3B (right panel) shows that both of these mutations were able to abrogate responsiveness to TNF- α . The -1628/+19 MUC2 promoter reporter is less responsive than the heterologous-1.5/-1.4-TK promoter reporter; this may be due to the presence of repressive cis elements within the -1628/+19 sequence, discussed later.

NF- κ B involvement was further demonstrated using chemical inhibitors MG132, parthenolide and CAPE,

which all reduced MUC2 reporter transcription to varying degrees, as shown in Fig. 3C. Basal MUC2 promoter reporter transcription was also suppressed by proteasome inhibitor MG132, suggesting that it profoundly inhibited MUC2 transcription via other, non-specific pathways. These results were confirmed by RT-PCR. Significantly, SN50, a peptide inhibitor of NF- κ B translocation, and IKK inhibitor BAY 11-7082 (20 μ M) both failed to reduce MUC2 transcription (not shown).

EMSA and supershift assays were performed using an oligonucleotide containing the MUC2 -1458/-1430 NF-

Fig. 6. Scheme illustrating pathways proposed to be involved in TNF-alpha-mediated regulation of MUC2 reporter transcription. Solid arrows indicate pathways demonstrated in this study, dotted arrows show possible but unproven pathways, blocked lines (--|) indicate inhibitory agents or pathways. Staggered lines indicate indirect effects.

kappaB binding site (Fig. 3D). Nuclear extracts from untreated HM3 cells exhibited little binding of transcription factors to this probe (lane 1), whereas nuclear extracts from TNF-alpha-treated cells contained a protein which bound to the probe (arrow; lane 2). This binding was reduced for nuclear extracts from wortmannin-treated cells (lane 3). Binding of nuclear protein to radiolabeled probe was effectively blocked by addition of unlabeled oligonucleotide (lane 4). The addition of p50 antibody caused a reduction in band intensity and the appearance of a very faint supershifted band ("ss" arrow; lane 5); this (non-ideal) behavior is typical for this particular antibody. Antibody to cRel had little effect (lane 6) whereas the p65 antibody produced a super-shifted protein-oligonucleotide complex (lane 7). The positive control NF-kappaB consensus oligonucleotide produced an intense band (lane 8) which, upon addition of antibodies, exhibited behavior similar to the MUC2 NF-kappaB probe (lanes 9 and 10). Thus, EMSA indicated that TNF-alpha induces NF-kappaB (p50p65) binding to the MUC2 NF-kappaB site.

The same nuclear extracts used for EMSA were further analyzed by western blotting. Nuclear extracts

from TNF-alpha-treated cells contained much more p65 protein than nuclei from untreated cells (Fig. 3E). Unexpectedly, this apparent translocation was not inhibited by wortmannin (Fig. 3E). On the other hand, western blots showed the same amount of p50 in all three nuclear extracts, indicating abundant, constitutive expression of p50 in the nucleus (Fig. 3E). These results suggest that inhibition of p50 translocation by SN50 failed to inhibit MUC2 reporter transcription simply because p50 translocation is not a rate-limiting step. This may also explain why IKK inhibitor BAY 11-7082, expected to impair I-kappaB phosphorylation and consequent p50 translocation, failed to inhibit transcription. On the other hand, CAPE and parthenolide are thought to inhibit NF-kappaB via a mechanism involving direct, irreversible modification of p65 protein [36, 37]. This fact, combined with the observations that wortmannin inhibited Akt phosphorylation and MUC2 transcription, as well as p65 binding to the MUC2 NF-kappaB, all without reducing p65 import into the nucleus in response to TNF-alpha, suggested that direct p65 activation – likely via a PI3K mediated phosphorylation – is a rate-limiting step in TNF-alpha induction of MUC2 up-regulation [38, 39].

As shown in Fig. 3F, over-expression of stable, dominant-negative I-kappaB-alpha (S32/36A) rendered the MUC2 -1.5/-1.3-TK reporter unresponsive to TNF-alpha. Western blot analysis of I-kappaB-alpha showed that its degradation began after 15 minutes of TNF-alpha treatment, with a pronounced reduction within thirty minutes (Fig. 3G). However, simultaneous treatment with PI3K inhibitor wortmannin did not prevent the degradation of I-kappaB-alpha (Fig. 3G), supporting the hypothesis that, in this cell line, PI3K/Akt signaling acts either downstream from, or independent of, the classic IKK/I-kappaB/NF-kappaB pathway.

Involvement of MAP kinase signaling pathways

MAP kinase pathways are also activated in response to TNF-alpha, but the connections between the TNF-alpha receptor complex and downstream MAP kinase kinases (MKKs; such as MKK3 which activates p38; MKK7 which activates JNK; and MEK-1 which activates ERK) have not been precisely defined [32, 33]. Western blot was used to determine which MAP kinase signaling pathways were activated by TNF-alpha in HM3 cells (Fig. 4). Both p38 and ERK exhibited pronounced phosphorylation under basal conditions and, in both cases, this phosphorylation was slightly enhanced by TNF-alpha treatment. JNK, which is slightly phosphorylated in untreated cells, is distinctly phosphorylated upon TNF-alpha treatment (Fig. 4). The functional significance of these phosphorylation events was explored using inhibitors specific for each pathway (Fig. 5A). MEK1/2 inhibitor U0126 and p38 inhibitor SB203580 gave 60% and 40% inhibition of TNF-alpha-mediated induction, respectively. MEK inhibitor PD98059 and p38 inhibitor SB202190 were also inhibitory, by 50 % and 41 %, respectively. Previous work in this lab [21] demonstrated a role for ERK in MUC2 transcription, so inhibition of transcription by MEK inhibitor PD98059 was not unexpected. This previous work also showed that p38 pathway inhibitor SB203580 had no effect on MUC2 promoter reporter transcription induced by PMA, whereas the present study shows that two different p38 inhibitors were able to reduce MUC2 transcription induced by TNF-alpha. Surprisingly, JNK inhibitor SP600125 not only failed to inhibit TNF-alpha-mediated induction, but actually stimulated reporter activity to an extent comparable with the effect of TNF-alpha itself. In fact, this stimulation by SP600125 was considerably enhanced by TNF-alpha, giving a further 2.8-fold induction (Fig. 5A). Consistent with this

unexpected result, we found that co-transfection of -1628/+19 MUC2 promoter reporter vector with TAM67 (dominant-negative cJun) expression vector also activated transcription, independent of TNF-alpha (Fig. 5B). p38 has been reported to negatively regulate JNK [40]. Thus it is possible that transcriptional inhibition by p38 inhibitors is an indirect effect, arising from relief of TNF-alpha-activated JNK's suppressive on MUC2 transcription.

Summary

Signaling pathways responsible for MUC2 reporter transcriptional activation by TNF-alpha are summarized and illustrated in Figure 6. We have demonstrated that treatment of HM3 cells with TNF-alpha results in degradation of I-kappaB via NIK and PI3K pathways, causing translocation of p65 into the nucleus, where it effects up-regulation of MUC2. Our results indicate that nuclear translocation of NF-kappaB is not the rate-limiting step in this process and that further wortmannin-inhibitable activation is required for DNA binding and maximal reporter transcription. JNK is also activated but was shown to have a negative effect on MUC2 transcription. The NF-kappaB-mediated up-regulation of MUC2 reporter transcription by TNF-alpha is substantially lower than NF-kappaB-mediated up-regulation by PMA, likely due in part to the concomitant activation of the negatively acting JNK signaling pathway. It has been shown that normal gut microflora suppress NF-kappaB activation by blocking ubiquitination of I-kappaB (41), and that several NF-kappaB target genes are required to prevent sustained JNK activation (33). Thus, although we have demonstrated here that TNF-alpha gives net up-regulation of MUC2 gene transcription in cultured colon cancer cells, the existence of an inhibitory JNK pathway suggests a mechanism whereby production of TNF-alpha within the digestive tract during inflammation may be able to reduce expression of MUC2, thereby effecting a loss of the protective mucin layer and damage to the underlying epithelium.

Abbreviations

CAPE (caffeic acid phenethyl ester); EMSA, (electrophoretic mobility shift assay); ERK (extracellular signal-regulated kinase); I-kappaB (inhibitor of κ B); IKK (I-kappaB-kinase); JNK (c-jun N-terminal kinase); MEKK (mitogen-activated protein kinase/ERK kinase

kinase); NF-kappaB (nuclear transcription factor- κ B); NIK (NF-kappaB-inducing kinase); PI3K (phosphatidylinositol-3-OH kinase); PMA (phorbol 12-myristate 13-acetate); RT-PCR (reverse transcriptase polymerase chain reaction); TNF (tumor necrosis factor).

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