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MUC1 activates JNK1 and inhibits apoptosis under genotoxic stress

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ABSTRACT

The MUC1 transmembrane glycoprotein is aberrantly overexpressed in diverse human carcinomas and has been shown to inhibit apoptosis induced by genotoxic agents. In the present work, we report that MUC1 binds to and activates JNK1, an important member of the mitogen-activated protein kinases (MAPK) superfamily. The physical interaction between MUC1 cytoplasmic domain (MUC1-CD) and JNK1 was established by GST-pull-down assay *in vitro* and co-immunoprecipitation assay *in vivo*. We show that MUC1 activates JNK1 and inhibits cisplatin-induced apoptosis in human colon cancer HCT116 cells. Pharmacological inhibition of JNK or knockdown of JNK significantly reduces the ability of MUC1 to inhibit cisplatin-induced apoptosis. Together, our data indicate that MUC1 can inhibit apoptosis via activating JNK1 pathway in response to genotoxic anticancer agents.

1. Introduction

MUC1 (mucin 1), also known as DF3, CA15-3 or episialin, is a heterodimeric transmembrane glycoprotein that is normally expressed on the apical borders of secretory epithelial cells but often overexpressed over the entire surface of carcinoma cells [1]. After translation, MUC1 proteins undergo a cleavage into an N-terminal (N-ter) and a C-terminal (C-ter) subunit, which then form a stable complex [2,3]. The MUC1 N-ter subunit is a large extracellular subunit (>250 kDa) consisting of variable numbers of 20-amino acid tandem repeats that are extensively linked hundreds of O-glycans [4]. The C-ter transmembrane subunit consists of a 58 amino acid extracellular domain, a 28 amino acid transmembrane domain, and a 72 amino acid cytoplasmic domain (CD), which anchors the N-ter extracellular subunit as a heterodimer to the cell surface [5,6]. MUC1 is overexpressed in most human carcinomas and functions to inhibit cellular apoptotic response to DNA damage [7–10], the mechanism of which, however, is not entirely clear.

The stress-activated protein kinase (SAPK), also known as c-Jun N-terminal kinase (JNK), belongs to the Mitogen Activated Protein Kinase (MAPK) super-family [11]. JNK has three isoforms (JNK1, JNK2 and JNK3) [12]. JNK1 and JNK2 are extensively expressed while JNK3 is mainly expressed in neuronal and heart tissues [13]. In response to stimuli, including DNA damage [14,15], heat shock [16], tumor necrosis factor α (TNF-α) [17], and interleukin-1 [18], MKK4 or MKK7 activates JNK1 by phosphorylating Thr-183 and Tyr-185 of JNK1 [19]. Activated JNK1 translocates from cytoplasm to nucleus where it phosphorylates the major downstream effector c-Jun on Ser-63 and Ser-73 [20], which is in turn to impact transcription of a subset of downstream target genes. Activation of JNK1/c-Jun pathway has been shown to display pro-apoptotic [21] or anti-apoptotic [22,23] effects under different circumstances, and also promotes cell proliferation [24].

In the present study, we showed that MUC1 directly binds to JNK1, and enhances the activity of JNK1 and c-Jun, resulting in reduced cellular apoptotic response to DNA damage. Our results suggest that MUC1 can inhibit apoptosis in response to genotoxic anticancer agents via activating JNK1 pathway.

2. Materials and methods

2.1. Cell culture and drug treatment

HCT116/vector, HCT116/MUC1 colon cancer [25] and 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone) with 10% heat-inactivated fetal bovine serum (FBS, Hyclone) and 1% penicillin G/streptomycin (Hyclone) at 37 °C in a humidified 5% CO_2 incubator. Cells were treated with 2 μM doxorubicin (Dox, Sigma), 100 μM cisplatin (CDDP, Sigma) or 50 μM JNK inhibitor II (420119, Calbiochem).

2.2. Cell transfections

HCT116 cells were transfected with pIRESpuro2 or pIRESpuro2-MUC1 using LipofectAMINE 2000 (Invitrogen) and selected in the

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presence of puromycin (InvivoGen). 293 cells were transiently transfected with pIRESpuro2, pIRESpuro2-MUC1, GFP, GFP-MUC1-CD, GFP-MUC1-CD(1-45), GFP-MUC1-CD(46-72), JNK1, Flag-MUC1-CD, c-Jun, using LipofectAMINE 2000. For downregulation of JNK, HCT116/vector and HCT116/MUC1 cells were transfected with siRNA (GenePharma) directed against JNK or negative control (GenePharma) using LipofectAMINE 2000. After 48 h, cells were harvested for analysis.

2.3. Immunoprecipitation and immunoblot analysis

Cells were lysed in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% Nonidet P-40, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) for 30 min on ice. Lysates were cleared by centrifugation at 14,000*g* for 20 min. Protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad). For the immunoprecipitation experiment, equal amounts of total proteins from cell lysates were incubated with anti-JNK (Cell Signaling Technology), and normal rabbit IgG. After incubation for 2 h at 4 °C, the immune complexes were precipitated with protein A-agarose. The immunoprecipitates were washed with lysis buffer, separated by SDS–PAGE, separated by SDS–PAGE, and transferred to polyvinylidene difluoride membrane (Bio-Rad). The immunoblots were probed with appropriate primary antibody and horseradish peroxidase-conjugated secondary antibody for subsequent detection by ECL (Millipore). Immunoblot analysis was performed with anti-MUC1 (Thermo Scientific), anti-JNK (Cell Signaling Technology), anti-pho-JNK (Cell Signaling Technology, pThr183/pTyr185), anti-c-Jun (Cell Signaling Technology), anti-pho-c-Jun (Cell Signaling Technology, pSer63), anti-FLAG (Sigma), anti-GFP (Santa Cruz Biotechnology), anti-β-actin (Santa Cruz Biotechnology) or anti-PARP (Zen bioscience).

2.4. GST pull down assays

500 μg of protein derived from whole-cell lysates of 293 cells transfected with JNK1 was subjected to a GST pull down assay [26]. *In vitro* translated JNK1 was added into 800 μl of binding buffer (50 mM Tris–Cl, pH 8.0, 120 mM NaCl, 1 mM EDTA, pH 8.0, 0.5% Nonidet P-40) with GST or GST-MUC1-CD immobilized on Sepharose-glutathione beads. After incubation for 3 h at 4 °C, the beads were washed for three times with the binding buffer. The proteins were subjected to immunoblot analysis.

2.5. Luciferase assays

HCT116/Vector and HCT116/MUC1 cells were transfected with a mixture of AP1-Luc and pRL-TK-Renilla Luc. Cells were harvested at 36 h after transfection and lysed in Passive Lysis Buffer (Promega). Lysates were analyzed for firefly and Renilla luciferase activities using the Dual Luciferase Reagent Assay Kit (Promega). Luciferase assays were performed as described (Luciferase assay system; Promega). Luminescence was measured in a luminometer. Relative luciferase activity was determined by normalizing luciferase activity with Renilla.

2.6. Flow cytometry analysis (FACS)

Cells were fixed in 70% ethanol at 4 °C overnight and stained with 50 μg/ml propidium iodide (Sigma) supplemented with 100 μg/ml RNase A (Sigma) for 40 min at 37 °C in the dark. Then, cells were subjected to FACS analysis by FACScan flow cytometer (Becton Dickson). Data were analyzed using the Cell Quest program.

3. Results

3.1. MUC1 directly binds to JNK1

Previous studies revealed that MUC1 cytoplasmic domain (MUC1-CD), which is a part of MUC1 C-ter subunit, can bind to multiple kinases [9,25,27,28]. To investigate whether MUC1-CD can form a stable complex with JNK1, we used purified GST or GST fusion protein containing MUC1-CD (GST-MUC1-CD) for interaction with *in vitro* translated JNK1. The results demonstrated that JNK1 was pulled down by GST-MUC1-CD but not GST alone, indicating an *in vitro* interaction between JNK1 and MUC1-CD (Fig. 1A). To assess whether MUC1-CD associates with JNK1 *in vivo*, we transiently expressed JNK1 and Flag-MUC1-CD in 293 cells, which are negative for MUC1 expression [29]. Cell lysates were subjected to immunoprecipitation with a JNK1-specific antibody, followed by immunoblotting using an antibody specific for Flag epitope. The results showed that JNK1 and Flag-MUC1-CD forms a stable complex in 293 cells (Fig. 1B). To map the region of MUC1-CD involved in JNK1 binding, we used constructs expressing fusion protein with GFP fused to the full-length MUC1-CD (amino acids 1-72), MUC1-CD (amino acids 1-45) and MUC1-CD (amino acids 46-72). Co-transfection and co-immunoprecipitation experiments showed that JNK1 formed a complex with MUC1-CD(1-45), but not with MUC1-CD(46-72) (Fig. 1C). These data indicate that MUC1 directly binds to JNK1 through the MUC1-CD (amino acids 1-45) region.

3.2. MUC1 induces the phosphorylation/activation of JNK1 and c-Jun under genotoxic stress

JNK1 is phosphorylated/activated and exerts its kinase function on substrates in response to DNA damage [14,30]. Transcription factor c-Jun is the most important substrate of JNK1. JNK1 can specifically phosphorylate c-Jun at its N-terminal regions (serines 63 and 73) [31]. To test whether MUC1 influences JNK1 kinase activitiy, in a way similar to its effects on c-Abl and GSK3β [9,27], we treated HCT116 cells stably expressing MUC1 (HCT116/MUC1), or its vector control (HCT116/vector), with cisplatin (CDDP) or doxorubicin (Dox). The activation of JNK1 was measured by immunoblot analysis for phosphorylated JNK1 (pho-JNK1) and phosphorylated c-Jun (pho-c-Jun). As shown in Fig. 2A and 2B, CDDP and doxorubicin induced phosphorylation of JNK1 and c-Jun, which was further enhanced by ectopic expression of MUC1 at 12 h after treatment. While total JNK1 protein levels were comparable, total c-Jun protein levels were increased, likely because phosphorylation can stabilize c-Jun [32]. Since the JNK1/c-Jun signaling pathway can regulate AP-1 transcription activity [33], we then tested the effect of MUC1 on transcription activity of AP-1 using luciferase reporter assays. As shown in Fig. 2C and 2D, expression of MUC1 increased AP-1 transcription activity in the presence of CDDP or doxorubicin in a time-dependent manner. Together, these data demonstrate that MUC1 enhances the activation of JNK1/c-Jun pathway and AP-1 transcription activity under genotoxic stress.

3.3. MUC1 inhibits DNA damage-induced apoptosis via JNK1 pathway

It has been reported that MUC1 blocks DNA damage-induced apoptosis [7–9]. To investigate whether JNK1 is involved in anti-apoptotic roles of MUC1, we treated HCT16/MUC1 cells with CDDP in combination with JNK inhibitor II, and analysed apoptosis by means of immunoblotting for cleaved PARP, a hallmark of apoptosis, and flow cytometry. As shown in Fig. 3A, CDDP clearly induced PARP cleavage. MUC1 expression significantly reduced PARP cleavage,

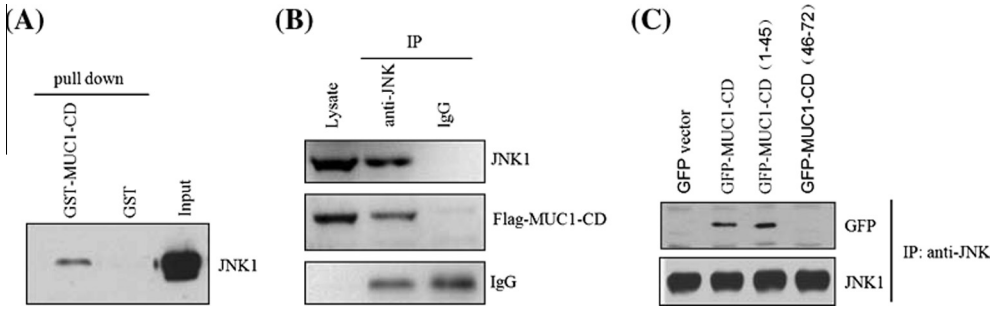

Fig. 1. MUC1 interacts with JNK1. (A). GST or GST-MUC1-CD bound to glutathione beads was incubated with recombinant JNK1. GST-MUC1-CD not incubated with JNK1 was used as a control. The adsorbates were analyzed by immunoblotting with anti-JNK1. (B). 293 cells were co-transfected to express JNK1 and Flag-MUC1-CD. Lysates were subjected to immunoprecipitation with anti-JNK1. Rabbit IgG was used as a control. The immunoprecipitates and lysates not subjected to immunoprecipitation were analyzed by Immunoblotting with anti-JNK and anti-Flag. (C). 293 cells were transfected to express GFP, GFP-MUC1-CD, GFP-MUC1-CD(1-45), GFP-MUC1-CD(46-72). Lysates were subjected to immunoprecipitation with anti-JNK. The immunoprecipitates were analyzed by Immunoblotting with anti-GFP (upper panel) and anti-JNK (lower panel).

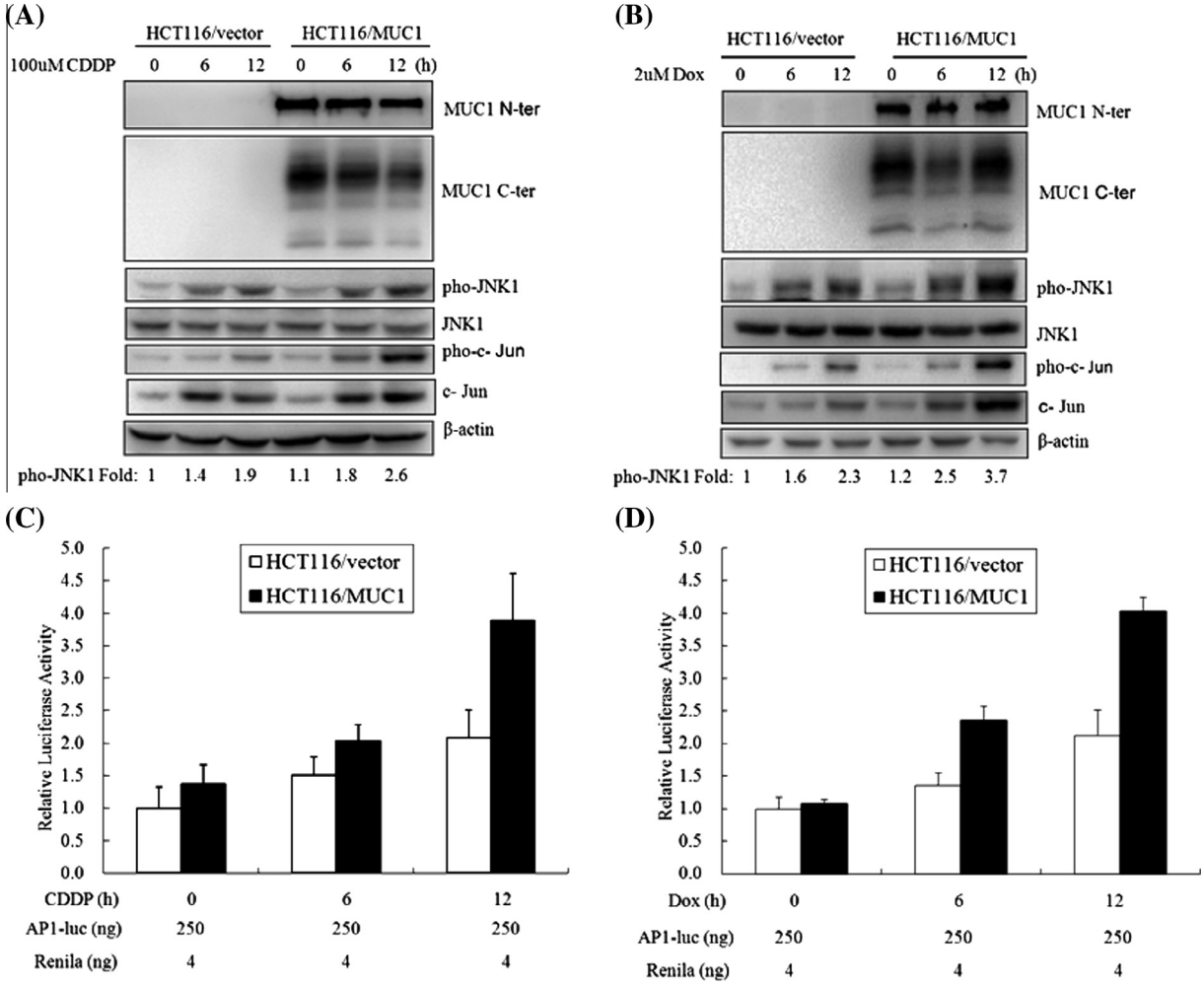

Fig. 2. MUC1 increases the phosphorylation and activation of JNK1 and c-Jun. A and B. HCT116/vector and HCT116/MUC1 cells were treated with 100 μM CDDP (A) or 2 μM doxorubicin (B) for the indicated times. Immunoblot analysis of the lysates was performed with anti-MUC1, anti-pho-JNK, anti-JNK, anti-c-Jun, anti-pho-c-Jun and anti-actin. C and D. HCT116/vector and HCT116/MUC1 cells were transfected with AP1-Luc and renila. 24 h after transfection, cells were treated with 100 μM CDDP (C) or 2 μM doxorubicin (D) for the indicated times and was performed luciferase assay. The luciferase activity was normalized to Renilla control, and expressed as fold increase (mean ± S.D). Experiments were performed two times in triplicate.

which was reversed in the presence of JNK inhibitor II, suggesting that JNK activation is critical for MUC1-mediated anti-apoptotic effect. Same conclusions were obtained by FACS analysis (Fig. 3B).

To further determine the specific role of JNK1 or JNK2 in DNA damage-induced apoptosis, we used a siRNA directed against a common sequence of JNK1 and JNK2 [34]. As shown in Fig. 3C, knockdown of JNK1 and JNK2 by siRNA (siJNK) led to efficient down-regulation of phoshorylated JNK1 and abrogated MUC1-mediated anti-apoptotic effects, as evidenced by the appearance of PARP cleavage and sub-G1 population by FACS analysis. By

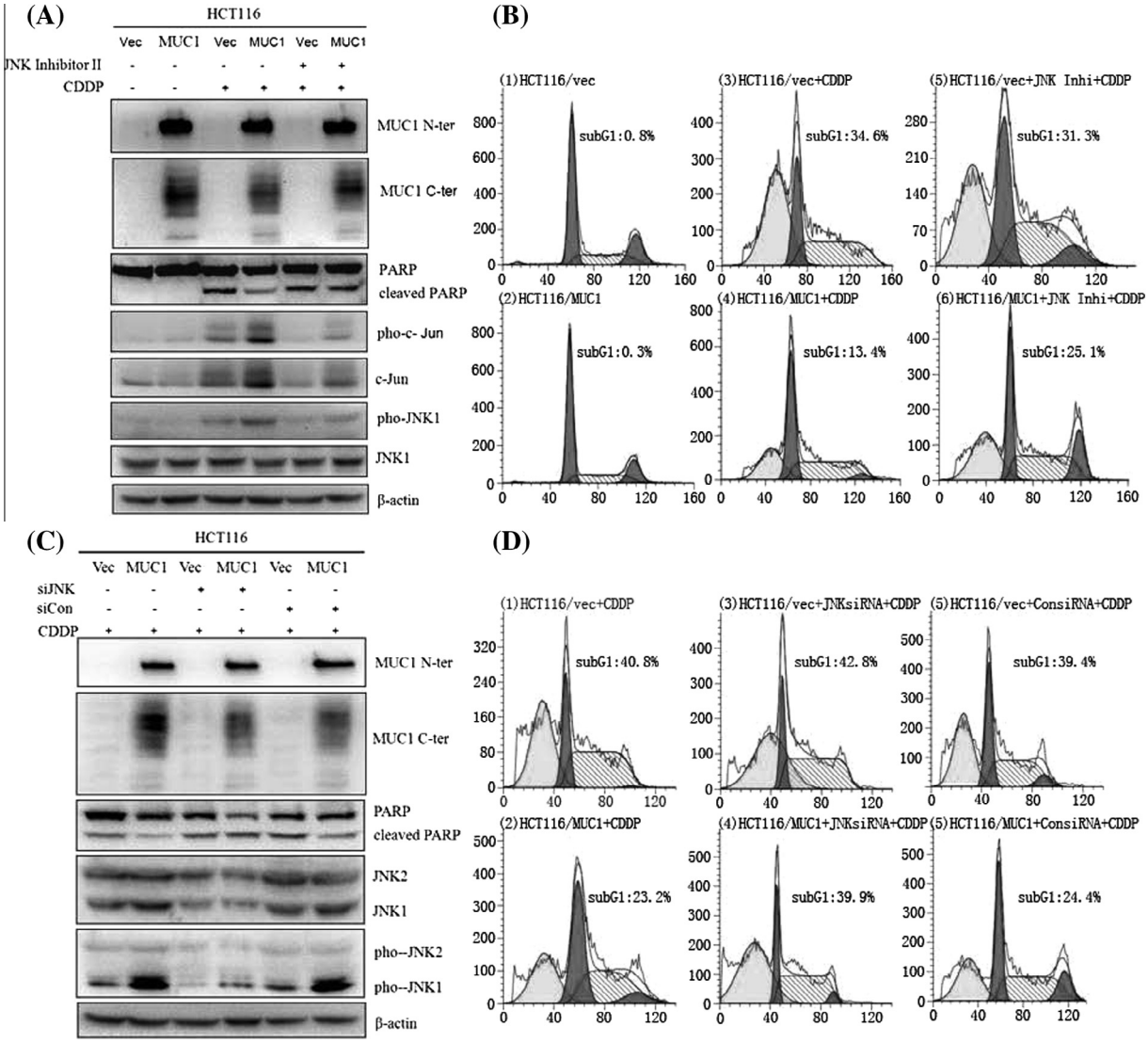

Fig. 3. MUC1 blocks the apoptotic response of HCT116 cells to DNA damage through JNK1 pathway. A and B. HCT116/vector and HCT116/MUC1 cells were treated with 50 μM JNK inhibitor II for 1 h prior to treatment with CDDP (100 μM) for 24 h. (A) Cell lysates were subjected to Western blot analysis with anti-MUC1, anti-parp, anti-pho-c-Jun, anti-c-Jun, anti-pho-JNK, anti-JNK, anti-β-actin. (B) Cells were subjected to flow cytometry for sub-G1 analysis. C and D. HCT116/vector and HCT116/MUC1 cells were transiently transfected with siRNA against JNK or control siRNA. After transfection 24 h, cells were treated with 100 μM CDDP for 24 h. (C) Cell lysates were subjected to Western blot analysis with anti-MUC1, anti-PARP, anti-JNK, anti-pho-JNK, anti-β-actin. (D) Cells were subjected to flow cytometry for sub-G1 analysis.

contrast, the scrambled siRNA control (siCon) exhibited little effects under similar experimental conditions.

4. Discussion

Many genotoxic anticancer agents often induce apoptosis depending on activation of some intrinsic pathways [35,36]. The cellular apoptotic process is regulated by various intracellular signaling pathways, including the JNK pathway [12,37]. It has been reported that JNK1 pathway is activated by diverse genotoxic agents and plays important roles in apoptotic response of cells to stresses and stimuli [37]. Once activated, JNK1 phosphorylates c-Jun, which is a component of the AP-1 family of leucine zipper transcription factors, and increases protein stability and transcriptional activity of c-Jun [32].

In most human carcinomas, overexpression of MUC1 blocks apoptosis induced by genotoxic anticancer drugs [7–9]. MUC1 can binds to c-Abl to attenuate phosphorylation of c-Abl and the interaction between c-Abl and cytosolic 14-3-3 [9]. MUC1 also binds directly to GSK3β and blocks GSK3β-mediated phosphorylation and degradation of β-catenin [28]. The present study demonstrates that MUC1 directly binds to JNK1 through MUC1-CD (amino acids 1-45) region. Further investigation reveals that activation of JNK1 (but not JNK2) and its downstream target c-Jun are increased by MUC1 overexpression when cells are irritated with chemotherapeutic agents, including doxorubicin and cisplatin.

It has been reported that JNK can function as a pro-apoptotic kinase or an anti-apoptotic kinase depending on cell types and stimulus types: in the absence of NF-κB activation, prolonged JNK activation contributes to TNF-α induced apoptosis through production of jBID, which is a proteolytic fragment of BID and an activator of caspase 8; on the other hand, JNK can suppress apoptosis in IL-3-dependent hematopoietic cells via phosphorylation of the proapoptotic Bcl-2 family protein BAD [38]. Our results demonstrate that in HCT116 cells, MUC1 overexpression significantly blocks CDDP-induced apoptosis in the presence of intrinsic JNK activities, while inhibition or knockdown of endogenous JNKs abrogates this blockage. Our data suggest that JNK1 displays anti-apoptotic activity, which can be augmented by MUC1 via promoting phosphorylation/activation of JNK1, in HCT116 cells. Though we have not

tested effects of endogenous MUC1 on JNK1, we suppose that physiological and functional interaction between MUC1 and JNK1 may exist in some carcinoma cells, such as some breast cancer cells, which simultaneously have high levels of endogenous JNK1 and MUC1 C-ter subunit in cytoplasm. Since MUC1 lacks kinase activity, we speculate that binding to MUC1-CD may facilitate phosphorylation of JNK1 mediated by MKK7 or other kinases. Further studies could be directed to investigate the mechanism of MUC1-mediated phosphorylation/activation of JNK1 and the effects of endogenous MUC1 on JNK1. Previous studies show that MUC1 blocks DNA damage-induced apoptosis by the following mechanisms: (1) MUC1 C-terminal subunit localizes to the mitochondrial outer membrane for attenuating release of mitochondrial apoptogenic factors [8], and binds directly to the Bax to block Bax function in activating the mitochondrial death pathway [39]; (2) MUC1 sequesters c-Abl in the cytoplasm and thereby inhibits apoptosis in the response to genotoxic anticancer agents [9]. This study provides us a different mechanism to understand the anti-apoptotic function of MUC1 in response to DNA damage.

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