Transformation of NIH3T3 mouse fibroblast cells by MUC16 mucin (CA125) is driven by its cytoplasmic tail

PANAGIOTA GIANNAKOYROS, ISABELLE MATTE, CLAUDINE RANCOURT and ALAIN PICHÉ

Département de Microbiologie et Infectiologie, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Québec J1H 5N4, Canada

Received May 8, 2014; Accepted June 26, 2014

DOI: 10.3892/ijo.2014.2707

Abstract. MUC16 (CA125) is a transmembrane mucin that contributes to the progression of epithelial ovarian cancer (EOC). Expression of MUC16 is not detectable in the epithelial surface of normal ovaries. MUC16 expression is, however, common in serous EOC as well as in metastatic and recurrent tumors. Despite these observations, its contribution to the development of EOC is unknown. We stably expressed either empty vector, MUC16 C-terminal domain (MUC16 CTD) or MUC16 TMU (a construct that lacks the cytoplasmic tail) in NIH3T3 mouse fibroblast cells. In this study, we provide evidence for the role of MUC16 CTD in oncogenic transformation. We show that ectopic expression of MUC16 CTD enhances the growth of NIH3T3 cells under normal and low serum conditions, and promotes anchorage-dependent colony formation. The deletion of the cytoplasmic tail abrogated these effects. MUC16 CTD expression in NIH3T3 cells also enhances the formation of colony in soft agar as compared to MUC16 TMU. MUC16 CTD expression enhances tumor formation in nude mice. Our findings provide the first evidence that MUC16 induces the transformation of NIH3T3 cells and indicate that MUC16 functions as an oncogene. Furthermore, our data suggest that the cytoplasmic tail is critical for MUC16 oncogenic properties.

Introduction

MUC16 mucin (CA125) is overexpressed in the majority of high grade serous ovarian carcinomas (HGSOC) but not by normal ovarian surface epithelial (OSE) cells (1-7). In addition, the transformation of OSE cells has been associated with expression of MUC16 at high levels (8,9). Rising and falling levels of serum MUC16 correlate with ovarian cancer (OC) progression and regression, making MUC16 the single most important OC clinical biomarker (10,11). MUC16 mucin is a high molecular weight glycoprotein (200-2000 kDa) that shares many characteristics of the membrane-bound mucin proteins (12-20). MUC16 is type I transmembrane mucin with a large N-terminal domain (>12000 amino acid residues) that is heavily glycosylated. The MUC16 extracellular central domain contains up to 60 glycosylated tandem repeated sequences (156 amino acids), a characteristic of mucins. The extracellular portion of MUC16 is shed after proteolytic cleavage and found in serum of women with HGSOC. The C-terminal domain (CTD) is composed of an extracellular region with potential proteolytic cleavage sites, a transmembrane domain and a short 31 amino acid cytoplasmic tail (CT) (13-15). The phosphorylation of MUC16 CT stimulates its cleavage and release into the serum (21). MUC16 CT, which shares no homology with other transmembrane mucins CT, contains a highly conserved RRRKK amino acid polybasic sequence acting as a ezrin/radixin/moesin (ERM) family binding site (12-15). Despite its clinical importance as a serum biomarker for OC, its role in the pathogenesis of OC has not been well characterized. The large extracellular domain appears to play a significant role in determining the adhesive properties of tumor cells. MUC16 binds to galectin-1 protein (22). Although the biological significance of this interaction remains unclear, the cell-surface recruitment of galectin-1 has been associated with processes such as regulation of cell adhesion (23). MUC16 facilitates heterotypic cell adhesion through its binding with mesothelin (24,25). Mesothelin-MUC16 interaction could facilitate heterotypic cell-cell adhesion and peritoneal metastasis of ovarian tumors through the adhesion with mesothelial cells. Patankar et al reported that natural killer (NK) cells incubated with soluble MUC16 exhibited a 50-70% decrease in the lysis of tumor cells (26). MUC16-expressing EOC cells are also protected from lysis by primary NK cells (27). MUC16 downregulates CD16 expression in NK cells, B cells and monocytes via Siglec-9, a receptor found on immune cells that inhibits the NK cell response (28). Secreted MUC16 binds to NK cells, B cells and monocytes via Siglec-9, a receptor found on immune cells that inhibits the NK cell response (28). The high levels of secreted MUC16 found in ascites may be one of the factors contributing to the immunosuppressive properties of ascites. The function of the MUC16 CT is not known. Ectopic expression of MUC16 CTD has been associated with the attenuation of drug-induced apoptosis in EOC cells (30,31). Moreover, MUC16 CTD expression increases the tumorigenicity of SKOV3 cell line in a xenograft mouse model when compared to a similar construct lacking the CT...
domain (32). These data suggest a potential role of MUC16 CT in tumorigenesis.

In the present study, MUC16 CTD or a similar construct lacking the CT domain (MUC16 TMU) were stably expressed in mouse fibroblast NIH3T3 cells by lentiviral infection and their effects were assessed using in vitro and in vivo functional assays. We demonstrate that ectopic expression of MUC16 CTD, when compared to MUC16 TMU, enhances anchorage-dependent and -independent growth and colony formation as well as tumorigenesis in nude mice, the hallmarks of malignant transformation. Together, these observations support the oncogenic potential of MUC16 and suggest that the CT domain is essential.

Materials and methods

Cell culture and reagents. The immortalized non-tumorigenic mouse fibroblast NIH3T3 cell line was obtained from the American Type Culture Collection (Rockville, MD). NIH3T3 cells were grown in DMEM (Wisent, St-Bruno, QC, Canada) supplemented with 10% FBS (Wisent), 2 mM L-glutamine (Wisent), 100 U/ml penicillin and 100 µg/ml streptomycin. 293T cells were cultured in DMEM (Wisent) supplemented with 10% FBS, 0.1 mM MEM non-essential amino acid solution and 10 mM HEPES. Cells were maintained at 37°C in humidified 5% CO₂ incubators. MUC16 C-terminal domain (MUC16 CTD) (containing last 284 C-terminal residues) was amplified by PCR from pME18S-FLJ14303 (Helix Research Institute, Japan). The 5’ primer integrated an EcoRV restriction site while the 3’ primer integrated a Xhol restriction site. The digested amplicon was inserted in a pLenti6V5 vector containing at the 5' end an IgK leader sequence, a myc-tag and a small linker (23 residues) and at the 3’ end a linker sequence and a Flag-tag (21 residues) (33,34). The control vector lacked the amplified MUC16 CTD sequence. MUC16 TMU contains the extracellular unique region and the transmembrane domain. To ensure anchorage of the construction at the cytoplasmic membrane, three residues adjacent to the transmembrane domain in the cytoplasmic tail were conserved in MUC16 TMU. After verification by sequencing, 293T cells were transfected with equal amount of pLenti vectors Plp1, Plp2, PlpVSVG and either the pLenti6V5-MUC16-CTD, -EV or -MUC16 TMU using Lipofectamine 2000 (Life Technologies, Burlington, ON, Canada). Lentivirus produced by 293T cells -MUC16 TMU using Lipofectamine 2000 (Life Technologies, Burlington, ON, Canada). Anti-Flag M2 antibody (1:500) and anti-α-tubulin (1:2500) antibodies were purchased from Sigma. Anti-c-myc sc-789 antibody (1:1000) and anti-ERK 1: sc-93 (1:250) were from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-phospho-p44/42 MAPK (1:1000), anti-AKT (1:1000), anti-Bcl-xL (1:1000), anti-cyclin A2 (1:1000), anti-cyclin D1 (1:1000), anti-cyclin D2 (1:1000), anti-cyclin E2 (1:1000) and HRP-conjugated rabbit anti-mouse antibody and anti-rabbit antibody were all from Cell Signaling Technology (Danvers, MA, USA). Anti-Mcl1 (1:500) was purchased from BD Bioscience (Mississauga, ON, Canada). Anti-Bcl-2 (1:1000) antibody was from Dako (Burlington, ON, Canada). Anti-Phospho-Akt (Ser 473) was from Cell Signaling Technology (Danvers, MA, USA). Anti-Mcl1 (1:500) and anti-Flag M2 antibody (1:500) were from Sigma and proteins were quantified by Bradford assay (Bio-Rad, Mississauga, ON, Canada).

Materials and methods continued...

RT-PCR. Primers for PCR amplification of MUC16 CTD were as follow: 5'-CACTCGAGGTGACATCCTCCAGTGGTCACCAGGAC TAG-3' (sense) and 5'-ACCATACCCCTCTGAGGGAC ATC-3' (antisense). Primers for PCR amplification of MUC16 TMU were as follow: 5'-AAATCTTAAACCGCACCAGCG GTGCTACACCAGGAC-3' (sense) and 5'-ATGCCGGCCAT CTAGGCGCACGACCTCTGGAGGAC-3' (antisense) (Integrated DNA Technologies, Inc., Coralville, IA). PCR was performed as follows: 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1 min and finalized at 72°C for 15 min.

Immunoblot analysis. Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.1% SDS, 150 mM NaCl, 0.5 mM EGTA, 1% Triton X-100) with freshly added protease inhibitors [1 µg/ml 4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride, 2 µg/ml aprotinin, 0.7 µg/ml pepstatin and 0.5 µg/ml leupeptin] (Sigma) and proteins were quantified by Bradford assay (Bio-Rad, Mississauga, ON, Canada).

Soft agar colony formation. Cells (1x10⁵) were suspended in complete medium containing 0.33% of melted bactoagar. The resulting suspension was added to a 60-mm plate covered with a 2-ml layer of solidified 0.66% bacto-agar in complete medium. Grossly visible colonies were stained with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) and counted after 30 days of growth at 37°C in 5% CO₂ incubators. Three experiments were performed in triplicate. The mean ± SD was calculated from three independent experiments.

Immunofluorescence. NIH3T3 clones were grown on glass slides until a 50-70% confluence was reached. Glass slides were then washed in cold PBS and cells fixed in 3% formaldehyde (Fisher) for 10 min. Glass slides were next rinsed for 5 min in PBS and quenched (glycin 0.1 M) during 30 min. Depending on the experiment, cells were permeabilized in PBS containing 0.2% Triton X-100 for 20 min at room temperature. Slides were rinsed twice in PBS and blocked overnight in PBS/2% goat serum at 4°C. Slides were washed 3 times in PBS, incubated with primary anti-c-myc sc-789
(1:200) antibodies in blocking buffer at room temperature for 1 h. Slides were washed 3 times in PBS, incubated for 45 min at room temperature with Alexa FluoR 594 or F(ab’)2 fragment of goat anti-mouse or goat anti-rabbit IgG from Life Technologies. After washing, slides were incubated for 2 min in 4’,6-diamidino-2-phenylindole (DAPI) to visualize nuclei, washed again in PBS and mounted for visualization by fluorescence microscopy with an Olympus IX70 (Olympus, Hamburg, Germany). Expression of MUC16 CTD was detected using anti-c-myc and a His at the C-terminal. (B) Detection of MUC16 CTD by RT-PCR in NIH3T3 cells. Total RNA from parental NIH3T3 cells, empty vector (EV)-, MUC16 CTD clone 1 (CTD1)- and clone 2 (CTD2)-expressing NIH3T3 cells was reverse transcribed and assayed by PCR. C+ is from MUC16 CTD-expressing SKOV3 cells (34). (C) Expression of MUC16 CTD by immunoblot using anti-c-myc and anti-Flag antibodies. (D) Expression of MUC16 CTD and MUC16 TMU by immunofluorescence. Cells were grown on coverslips, fixed and non-permeabilized cells were stained with anti-c-myc antibody.

**Cell proliferation assay.** Cell growth was determined by XTT assay. Briefly, cells were resuspended in complete medium and plated into 96-well plates at a density of 1x10⁴ cells/well. Cells were cultured for various times in complete and low serum concentration before adding XTT reagents. After incubating for 1 h at room temperature, the absorbance was read at 450 nm using a microplate reader at 450 nm (Tecan Sunrise, Research Triangle Park, NC, USA). All assays were performed in triplicate and mean ± SEM was calculated from three independent experiments.

**Anchorage-dependent clonogenic assay.** Cells were seeded in 6-well plates in complete medium. Fourteen days post-confluence, the cells were fixed and stained with crystal violet (Sigma) and the number of colonies composed of >50 cells was determined. All assays were performed three times. The mean ± SEM was calculated from three independent experiments.

**Spheroid formation.** A drop of 15 µl medium containing 5x10⁵ cells was pipetted onto the inner surface of a Petri dish lid. The lid was then placed on the Petri dish so that the drops were hanging from the lid with the cells suspended within them. To eliminate evaporation, 10 ml PBS was placed in the bottom of the Petri dish. During 5 days of incubation at 37˚C in 5% CO₂ incubators, the lid of the Petri dish was inverted and photographed using a microscope at x100 magnification.

**Tumorigenicity assay.** Mice (athymic nu/nu) were purchased from Charles River (St-Constant, QC, Canada) and housed in pathogen-free animal facilities. Mice were treated according to the Institution Animal Care guidelines. To assess tumorigenic capacity of EV-, MUC16 CTD- and MUC16 TMU-expressing...
NIH3T3 cells, 5×10⁶ cells were suspended in serum-free medium and injected subcutaneously into 6-8 weeks old female athymic CD-1 nude mice. Each mouse received 4 injections in the flanks and each group contained 2 mice. Tumors were excised and weighted after 23 days. The mean tumor weight ±SEM was calculated from eight independent site injections.

Statistical analyses. Statistical analysis was done using the BMDP statistical analysis software (UCLA, Los Angeles, CA). The threshold for statistical significance is a probability of 0.05. In the nude mouse model, data were expressed as mean ± SEM of tumor weight. The two-sided Student’s t-test was used to assess statistical differences between groups for the in vivo mouse models.

Results

Expression of MUC16 constructs in NIH3T3 mouse fibroblast cells. To assess the potential of MUC16 in mediating oncogenic transformation, we infected the MUC16 negative immortalized non-tumorigenic NIH3T3 mouse fibroblast cell line with lentiviruses to generate NIH3T3 cell lines expressing empty vector (EV), MUC16 TMU or MUC16 CTD. MUC16 CTD consists of the last C-terminal 283 residues which include the transmembrane domain (TM) and cytoplasmic tail (CT) (Fig. 1A). Because of the lack of available antibody against MUC16 CTD, the construct was tagged with c-myc epitope at the N-terminus and a Flag epitope at the C-terminus (Fig. 1A). MUC16 TMU is similar to MUC16 CTD except that the CT domain was deleted (Fig. 1A). Stable clones were screened for MUC16 CTD expression and two independent clones that exhibited MUC16 CTD expression were selected (CTD1 and CTD2). Empty vector and MUC16 TMU consisted of pooled populations. The expression of MUC16 CTD in NIH3T3 cells was validated by RT-PCR (Fig. 1B) and immunoblotting with anti-c-myc and anti-Flag antibodies (Fig. 1C). The expression of MUC16 CTD and MUC16 TMU was further characterized by immunofluorescence with anti-c-myc antibody on non-permeabilized cells (Fig. 1D).

MUC16 CTD enhances anchorage-dependent cell growth and promotes growth in low serum dependence. One of the
The hallmarks of transformed cells is their ability to grow in the absence of exogenous factors, which is shown by the ability to grow in low serum conditions (35). To assess the effects of MUC16 expression on cell growth, EV-, MUC16 TMU and MUC16 CTD-expressing NIH3T3 cells were seeded at low density and the number of viable cells was assessed at different time intervals for up to 6 days in normal (10%) and low (1%) serum concentration (Fig. 2A). Expression of MUC16 CTD in both MUC16 CTD1 and MUC16 CTD2 led to significantly accelerated cell growth under both normal (10%) and low (1%) serum conditions when compared to cells expressing the empty vector or MUC16 TMU.

To further determine growth-related features, we assessed the capacity of cells to form three-dimensional structures using the hanging droplets technique (36). Larger spheroids were observed for MUC16 CTD-expressing NIH3T3 at Days 1 and 5 when compared to EV-expressing cells (Fig. 2B). Expression of MUC16 CTD in both MUC16 CTD1 and MUC16 CTD2 led to significantly accelerated cell growth under both normal (10%) and low (1%) serum conditions when compared to cells expressing the empty vector or MUC16 TMU.

To further determine growth-related features, we assessed the capacity of cells to form three-dimensional structures using the hanging droplets technique (36). Larger spheroids were observed for MUC16 CTD-expressing NIH3T3 at Days 1 and 5 when compared to EV-expressing cells (Fig. 2B). Expression of MUC16 CTD in both MUC16 CTD1 and MUC16 CTD2 led to significantly accelerated cell growth under both normal (10%) and low (1%) serum conditions when compared to cells expressing the empty vector or MUC16 TMU.

MUC16 CTD induces colony formation under anchorage-dependent and -independent conditions. The effects of MUC16 expression on colony formation was assessed under anchorage-dependent and anchorage-independent conditions. Cells were seeded at low density and after 2 weeks (post-confluence), they were fixed and stained with crystal violet (Fig. 3A). MUC16 CTD-expressing NIH3T3 cells were characterized by loss of contact inhibition, overlapping growth and showed significantly (P<0.001) enhanced foci formation when compared to MUC16 TMU-transfected cells. Anchorage-independent growth was assessed by the formation of colony in soft agar. Under these conditions, there were few colonies with NIH3T3 cells expressing the empty vector or MUC16 TMU (Fig. 3B). NIH3T3 cells have been reported to occasionally spontaneously transform (37). More importantly, the number of soft agar colonies was markedly (P<0.001) increased with NIH3T3 cells expressing MUC16 CTD when compared to MUC16 TMU or EV-expressing cells. These data suggest that MUC16 CTD expression induces the transformation of NIH3T3 cells.

MUC16 CTD induces tumorigenicity. To further assess the oncogenic potential of MUC16 CTD expression, we examined the formation of subcutaneous tumors in mice injected with MUC16 CTD and MUC16 TMU. Because MUC16 CTD1 and MUC16 CTD2 expression resulted in similar phenotype in vitro, only MUC16 CTD1-expressing cells were further selected for in vivo experiments. Mice injected with MUC16 CTD1-expressing NIH3T3 cells grew much larger tumors over time when compared to MUC16 TMU-expressing cells (Fig. 4A and B). The average tumor volume in NIH3T3

![Figure 3. MUC16 CTD promotes colony formation under anchorage-dependent and -independent conditions. (A) Colony formation assay under anchorage-dependent conditions was performed by seeding 1x10^4 cells in 6-well plates and incubating cells for 2 weeks. Representative phase-contrast pictures of colonies after 2 weeks (magnification, x100). Colonies were counted and expressed as the number of colonies (mean ± SD) per 6-well plates from three independent experiments. (B) NIH3T3 cells expressing either EV, MUC16 TMU, MUC16 CTD1 and MUC16 CTD2 were suspended in soft agar and incubated for 3 weeks. Representative photomicrographs at x100 magnification are included. Colonies were counted and the results were expressed as the number of colonies (mean ± SD) from three independent experiments.](image-url)
MUC16 CTD1 cell-injected mice exceeded 0.25 g at 23 days. In contrast, tumors from NIH3T3 MUC16 TMU-injected mice were 0.067 g at 23 days. The expression of MUC16 CTD and MUC16 TMU in the subcutaneous tumors was confirmed by RT-PCR (Fig. 4C).

**MUC16 CTD expression and cell proliferation markers.** MUC16 CTD expression increases NIH3T3 cell proliferation in vitro (Fig. 2) and in vivo (Fig. 4). We used immunoblot analysis to investigate the association between MUC16 CTD expression and the corresponding changes in the expression of proteins that are involved in cell cycle regulation, including ERK1/2, Akt, cyclin D1, cyclin D2, cyclin E and cyclin A. NIH3T3 cells expressing MUC16 CTD showed increase phosphorylation of the cell cycle regulatory protein ERK1/2 and had higher levels of G1-S cell cycle transition regulatory cyclin D2 and the short form of S-phase regulatory protein cyclin E that usually increases cell proliferation (Fig. 5). The presence of the shorter form of cyclin E is usually found in tumors of ovarian cancer cells, but not in normal cells (38). The levels of G2-M phase regulatory protein cyclin A1 was decreased whereas cyclin B1, another G2-M phase regulatory protein, was unchanged. The expression of proteins usually associated with cell survival such as Akt, Mcl-1, Bcl-2 and Bcl-XL were unaffected by MUC16 CTD expression. Thus, MUC16 CTD expression is associated with increased expression of cell cycle regulatory proteins.

**Discussion**

MUC16 is emerging as a critical factor involved in different steps of OC oncogenesis. Ectopic expression of its C-terminal domain has been shown to confer resistance to genotoxic drugs such as cisplatin (30) and to death receptor-induced
apoptosis (31). MUC16 CTD expression also enhances tumor cell growth, migration, invasion and metastasis of ovarian cancer cells (32). Furthermore, the epithelial-mesenchymal transition (EMT) of OC cells was shown to be regulated by MUC16 (39). Thus, MUC16 mucin is not only a useful clinical marker of disease progression and regression; it is also an important factor for the pathogenesis of OC. In this study, we further expand the functions of MUC16 by demonstrating that it induces oncogenic transformation of NIH3T3 cells. MUC16 CTD expression induces loss of contact inhibition, increases cell proliferation under normal and low serum conditions, enhances anchorage-independent growth and promotes tumorigenesis in vivo. These data suggest that, in addition to being important for ovarian cancer progression, MUC16 appears to play a role in early steps of cancer development. Interestingly, the acquired phenotypes of transformation induced by MUC16 CTD are quite similar to those reported with the expression of the C-terminal domain of other membrane-bound mucins such as MUC1 and MUC4 (40,41). MUC1 and MUC4 mucins have well established growth-promoting activity (42-44). Although the oncogenic function of MUC16, MUC1 and MUC4 appears to be located in their CTD, MUC16 CT shares no homology with MUC1 CT and MUC4 CT, suggesting that the underlying mechanisms for transformation differs between MUC16 and MUC1 and MUC4.

The detailed molecular mechanisms by which MUC16 CTD promotes oncogenic transformation of NIH3T3 remain unknown. The findings that expression of MUC16 CTD, but not MUC16 TMU, is sufficient for cell transformation indicate that intracellular signaling function of MUC16 CT is responsible for the transformed phenotype. We found that MUC16 CTD expression promoted cell proliferation which was associated with increased levels of proteins that controlled the cell cycle such as cyclin D2 and short cyclin E isoform. MUC16 CTD expression also resulted in the activation ERK1/2 signaling in NIH3T3 cells. In contrast, proteins associated with cell survival such Akt and anti-apoptotic proteins Bcl-2, Bcl-XL and Mcl-1 were not altered by MUC16 CTD expression. The activation of ERK1/2 signaling and the upregulation of cyclin D2 and cyclin E might be responsible for MUC16 CTD-induced NIH3T3 cell proliferation.

In this study, the oncogenic potential of MUC16 CTD was tested with a classical model; the NIH3T3 cells. However, it will be interesting to further determine whether MUC16 CTD expression can induce the transformation of ovarian surface epithelial (OSE) cells. Carcinomas arising from the ovarian surface epithelium or the related distal part of the fimbriae are by far the most common subtypes. The transformation of human OSE cells have been generally achieved using a set of combined genetic elements such as introducing SV40 N-terminal domain doubles the size of this extracellular superstructure. Endoc Rev 22: 255-288, 2001.


