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Abstract

Overexpression of MUC1 oncoprotein is frequently observed in cancer and contributes to confer resistance to genotoxic agents. Papillary, follicular, and anaplastic thyroid carcinomas are the three forms of thyroid epithelial cancer. Anaplastic tumors are less differentiated and extremely aggressive, characterized by a poor prognosis. Little is known about the role of MUC1 in thyroid cancer. We recently showed that autocrine production of interleukin (IL)-4 and IL-10 controls thyroid cancer cell survival, growth, and resistance to chemotherapy through activation of Janus-activated kinase/signal transducers and activators of transcription (JAK/STAT) and phosphatidylinositols 3′-OH kinase (PI3K)/Akt pathways. In the present study, we showed that MUC1 COOH-terminal subunit (MUC1-C) is overexpressed in all the histologic variants of thyroid cancer cells and localizes to mitochondria where it interferes with the release of mitochondrial proapoptotic proteins. Moreover, IL-4 and IL-10 promote the increase of MUC1-C expression levels in normal thyroid cells, whereas blockage of both cytokines or neutralization of JAK/STAT and PI3K/Akt pathways through the exogenous expression of SOCS-1 and AktK179M leads to a significant decrease of MUC1-C in primary thyroid cancer cells. Interestingly, down-regulation of MUC1 expression by direct targeting with RNA interference sensitizes anaplastic thyroid cancer cells to chemotherapy-induced apoptosis in vitro. Thus, MUC1 is a main component of the survival network acting in thyroid cancer and could be considered a key molecular target for sensitizing cancer cells to conventional or novel treatments. [Cancer Res 2007;67(11):5522–30]

Introduction

Three main types of malignant tumors originate from thyroid epithelium: the more differentiated papillary and follicular thyroid carcinomas (PTC and FTC) and undifferentiated/anaplastic thyroid carcinomas (UTC; ref. 1). Total thyroidectomy is accepted as elective treatment for most patients with differentiated thyroid carcinomas, whereas UTC is associated with an extremely high disease-specific mortality and the treatment is generally palliative for a disease that is rarely cured and almost always fatal (2, 3).

Clinical trials with chemotherapeutic drugs have produced only rare positive responses in thyroid cancers as a result of activation of effective antiapoptotic pathways (4, 5). Antineoplastic drugs are a class of cytotoxic compounds that activate the intrinsic apoptotic pathway involving the release from mitochondria of death factors, such as cytochrome c, Apaf-1, apoptosis-inducing factor (AIF), and second mitochondria-derived activator of caspases/direct inhibitor of apoptosis–binding protein with low isoelectric point (Smac/DIABLO; ref. 6). In the cytosol, cytochrome c forms a complex with Apaf-1 and activates caspase-9, which can directly activate caspase-3 and the subsequent execution pathway (7). The relative levels and competing dimerization between Bcl-2 family members regulate cytochrome c release from mitochondria, thus determining cell susceptibility to apoptotic signals (8, 9). In this regard, as we previously reported, interleukin (IL)-4 and IL-10 act as autocrine growth factors in thyroid cancer microenvironment, inducing up-regulation of antiapoptotic proteins, such as Bcl-2, Bcl-xl, FLIP, and PED, which protect thyroid cancer cells from death induced by CD95 and chemotherapeutic drugs (10, 11). Moreover, treatment of thyroid cancer cells with neutralizing antibodies against IL-4 and IL-10 results in down-regulation of antiapoptotic proteins and sensitization to CD95-induced and cytotoxic drug–induced apoptosis (10, 11).

IL-4 binding to its receptor results in activation of Janus-activated kinase (JAK) 1/signal transducers and activators of transcription (STAT) 6 transduction pathway, inducing the expression of several genes, including antiapoptotic ped and flip genes, in FTC cells (10). IL-4 induces phosphorylation of insulin-like receptor substrate (IRS) molecules, which are essential for IL-4–stimulated mitogenesis (12). IRS molecule activation allows the recruitment of phosphatidylinositols 3′-OH kinase (PI3K) to the inner surface of plasma membrane, resulting in PI3K-generated phospholipids, which act as docking sites for Akt/protein kinase B serine/threonine protein kinase, leading to Akt cascade activation (13). Increasing evidences have shown that PI3K/Akt signaling is deregulated in several human malignant diseases (14, 15). In thyroid cancer cells, PI3K/Akt signaling pathway is aberrantly activated (16, 17).

In normal secretory epithelial cells, MUC1 is expressed as a transmembrane glycoprotein that provides protection against pathogens and shows cell signaling ability (18). Following synthesis as a single polypeptide and cleavage in the endoplasmic reticulum, MUC1 is expressed on cell membrane as a heterodimer (19). The MUC1 NH2-terminal subunit (MUC1-N) consists of variable numbers of 20–amino acid tandem repeats that are modified by O-glycans (20, 21). MUC1-N is tethered to the cell membrane through dimerization with the approximately 20- to 25-kDa COOH-terminal subunit (MUC1-C), which consists of a 58–amino acid extracellular domain, a 28–amino acid transmembrane, and a...
72-amino acid cytoplasmic domain (MUC1-CD; ref. 22). With transformation and loss of polarity, MUC1 is found at high levels in the cytosol and throughout the cell membrane of carcinoma cells (23). MUC1 oncoprotein overexpression is sufficient to attenuate oxidative-induced and genotoxic stress–induced apoptosis in most cancers (24, 25). In addition, recent findings have revealed that diverse carcinoma cells express the MUC1-C in mitochondria or in the nucleus in association with the Wnt effector β-catenin (25–29). MUC1 interacts with members of the ErbB family of receptor tyrosine kinases and with the fibroblast growth factor receptor 3 (26, 30, 31). Stimulation of such receptors induces c-Src–dependent tyrosine phosphorylation of the MUC1-CD on a YEKV motif and thereby results in nuclear localization of MUC1 and β-catenin or heat shock protein (Hsp) 90–mediated targeting of MUC1 to mitochondria (25, 31, 32).

Recently, it has been reported that MUC1-induced transformation of fibroblasts is due to activation of the antiapoptotic PI3K/Akt and Bcl-xL pathways (33). By contrast, in colon and breast carcinoma cells, MUC1 cytoplasmic domain activates the FOXO3a transcription factor that induces oxidant scavenging and DNA repair in a survival response to oxidative stress; this observation is due to the reduced activation of PI3K/Akt pathway and thereby to the decreased FOXO3a phosphorylation (34). These findings collectively suggest that the close cross-talk occurring between MUC1 and Akt signal transduction pathway depends on the cell context.

MUC1 oncoprotein overexpression has been proposed as a key molecular event in the pathogenesis of aggressive PTC, thus designating it as a prognostic marker and potential therapeutic target for this disease (35–37). In the present study, we examined MUC1-C expression and intracellular targeting in tumor cells of all the histologic variants of thyroid carcinoma. We speculated that both IL-4 and IL-10 cytokines may contribute to the regulation of muci1 transcription and/or MUC1-C protein expression by activating both JAK/STAT and PI3K/Akt signal transduction pathways, eventually controlling the sensitivity of thyroid cancer cells to conventional chemotherapeutic agents.

Materials and Methods

**Human tissues.** Thyroid cancer specimens were obtained at the time of surgical treatment. Thyroid normal tissues were obtained from the uninvolved, contralateral lobes of tumor glands or from nontoxic goiter glands in accordance with the ethical standards of the institutional committee responsible for human experimentation. Histologic diagnosis was based on the behavioral microscopic features of carcinoma cells determining the histologic type and grade.

**Human primary cell purification.** Normal and cancer thyroid tissues were digested for 2 h with collagenase (1.5 mg/ml; Life Technologies) and hyaluronidase (20 μg/ml; Sigma Chemical Co.) in DMEM as described previously (11). Once digested, cells were maintained on plastic in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), antibiotics, and 1-glutamine at 37°C in a humidified atmosphere of 5% CO2 for 12 h, which allowed the removal of other cells. Fibroblasts were depleted, exposing cell cultures to trypsin/EDTA for 1 min.

**Cell culture.** Human NPA and BC-PAP PTC cells were cultured in DMEM and RPMI 1640, respectively. Human WRO and FTC 133 FTC cells were grown in DMEM/F12, human ARO UTC cells were grown in RPMI 1640. MCF-7 breast carcinoma cells were cultured in DMEM/F12. Ten percent of heat-inactivated FBS, antibiotics, and 1-glutamine were added to each culture medium. Cells were detached with trypsin/EDTA for gene transcript and protein expression analyses.

Freshly purified normal thyroid cells and UTC cells were cultured, respectively, in the presence or absence of human recombinant IL-4 (20 ng/ml) or IL-10 (40 ng/ml; Euroclone) for 24 h and neutralizing antibodies against human IL-4 (15 μg/ml) or IL-10 (15 μg/ml; R&D Systems) for 72 h; after exposures, cells were detached with trypsin/EDTA for mRNA and protein expression analyses. Untransfected UTC cells (control), UTC cells transfected with a nonspecific small interfering RNA (siRNA; scrambled), or MUC1siRNA was treated with Cisplatin (300 ng/ml), Doxorubicin (5 μmol/L), or Taxol (5 μmol/L) for 24 h and assessed for apoptotic events.

**Immunoblot analysis.** Cell pellets were resuspended in ice-cold NP40 lysis buffer [50 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 1 mmol/l EDTA, 1% NP40 containing proteases and phosphatase inhibitors as described previously (38)]. Equal amounts of proteins (30 μg) were separated by SDS-PAGE and transferred to Hybond-C nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were blocked for 1 h with 5% nonfat dry milk in TBS containing 0.05% Tween 20 and successively incubated overnight with antibodies specific to MUC1-C (clone Ab5, hamster monoclonal IgG; NeoMarkers), Hsp60 (clone LK2, mouse IgG1; Sigma-Aldrich), SOCS-1 (clone N-18, goat polyclonal IgG; Santa Cruz Biotechnology), HA (clone 16B12, mouse IgG1; Babco), phosphorylated Akt (Ser473, rabbit polyclonal; Cell Signaling Technology), Akt (rabbit polyclonal; Cell Signaling Technology), phosphorylated JAK1 (Tyr1022/1023, rabbit polyclonal; Cell Signaling Technology), JAK1 (rabbit polyclonal; Cell Signaling Technology), STAT6 (rabbit polyclonal; Cell Signaling Technology), STAT1α (rabbit polyclonal; Cell Signaling Technology), and β-actin (clone Ab-1, mouse IgG; Calbiochem). After washing, blots were incubated for 1 h with horseradish peroxidase–conjugated anti-mouse, anti-rabbit (Amersham Biosciences UK Ltd.), anti-goat (Chemicon International), or anti-Armenian hamster antibodies (Jackson ImmunoResearch Laboratories) and visualized with enhanced chemiluminescence detection systems (SuperSignal West Pico/Dura Extended Duration Substrate, Pierce Biotechnology).

**Reverse-transcription and real-time PCR analysis.** Total RNA from cell pellets was obtained using the RNeasy Mini kit (Qiagen GmbH) according to the manufacturer's instructions. Reverse transcription and PCR amplification of 250 ng of total RNA were done using One-Step Reverse Transcription-PCR (RT-PCR) kit (Qiagen) according to the manufacturer's instructions. Oligonucleotide primers for RT-PCR were designed according to the published sequences [MUC1, Genbank accession no. NM_002046.4; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Genbank accession no. NM_002046.4]. The primers used for reverse transcription and amplification were the following: MUC1, 5'-CGACCTGAGAGATCGTTCACTA-3' (forward; 465–488) and 5'-TAGGCTGCACTGGAACATTCA-3' (reverse; 732–755); GAPDH, 5'-TGACATCAAGAGGGTGTGA-3' (forward; 870–889) and 5'-TCCACCACTTGTGCTGTA-3' (reverse; 1060–1079). RT-PCR was done using the following conditions: 50°C for 30 min, 95°C for 15 min, 1 cycle; 94°C for 45 s, 72°C for 45 s, 25 cycles. PCR products were analyzed by electrophoresis on 4% agarose gels.

For real-time PCR analysis, RNA was reverse transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems). Quantitative Taqman PCR analysis was done with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystem) in a reaction volume of 25 μl containing 1x Taqman Universal Master Mix (Applied Biosystems) and 1x probes and primer sets Hs00159357_m1 (MUC1; Taqman Gene Expression Assays, Applied Biosystems) or 1x Hu GAPDH (Pre-Developed Taqman Assay Reagents, Applied Biosystems). Reactions were done using the following thermal cycler variables: incubation at 50°C for 2 min and denaturation at 95°C for 10 min and then 40 cycles of the amplification step (denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min).

All amplification reactions were done in triplicate, and the relative quantitation of MUC1 gene expression was calculated using the comparative Ct method (ΔΔCt). Levels of mRNA expression were expressed after normalization with endogenous control, GAPDH. Data processing and statistical analysis were done using the ABI PRISM SDS, software version 2.1 (Applied Biosystems).
**Immunofluorescence.** Cells were cultured on coverslips and incubated in DMEM containing 100 nmol/L MitoTracker Red Mitochondrion-Selective Probe CMXRos for 30 min at 37°C in the dark. After staining, cells were washed with fresh growth medium, fixed in 2% paraformaldehyde/PBS, permeabilized in PBS containing Triton X-100 for 3 min at room temperature, and stained with anti-MUC1-C (clone Ab5, hamster monoclonal IgG) or anti-cytokeratin c (clone 66H2B, mouse IgG1; BD PharMingen) antibodies for 1 h at 37°C in the dark. Cells were then incubated with FITC-conjugated anti-Armenian hamster or anti-mouse (Molecular Probes) antibodies for 1 h at 37°C in the dark and finally incubated with Hoechst (Molecular Probes) for 2 min at room temperature. After mounting the coverslips, images were captured with a fluorescence microscope.

**Mitochondria isolation.** Cells were homogenized with a glass-Teflon directly into an ice-cold isolation buffer, TKV, containing 0.1 mol/L Tris-HCl (pH 7.4), 0.423 mol/L KC1, 0.001 mol/L EDTA, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 mmol/L phenylmethylsulfonyl fluoride. The homogenates were collected and centrifuged at 3,200 × g for 10 min at 4°C. Supernatants were transferred to clean tubes and centrifuged at 14,000 × g for 10 min at 4°C. Following centrifugation, mitochondrial pellets were washed twice with PBS and resuspended, on ice, in the same lysis buffer used for protein extraction for 30 min. The samples were then subjected to SDS-PAGE and immunoblot analysis.

**Production of lentiviral particles and infection of thyocytes, SOCS-1.** (kindly provided by Dr. A. Yoshimura, Department of Infectious Diseases, University of Miyazaki, Miyazaki, Japan; ref 39) and AktK179M (HA-tagged Akt, kindly provided by Prof. G.L. Condorelli, University of Rome “La Sapienza,” Rome, Italy) were subcloned in the Tween vector, generated by engineering p8RlNSin.PPT.ICMV.GFP.Wpre. In this vector, the ICMV.GFP cassette was substituted with the ICMV.lPBGK.GFP. Lentiviral supernatants were produced by calcium phosphate transient transfection in the packaging human embryonic kidney cell line 293T (40). UTC cells (1 × 10⁵) were plated in 25-cm² flask in the presence of viral supernatants containing 40 μg/mL of polybrene to improve infection efficiency. Cells were allowed to grow for 2 h at 37°C in humidified atmosphere of 5% CO2. After two cycles of infection, cells were replated, and allowed to grow overnight before treatment. Knockdown efficiency was evaluated by RT-PCR and Western blot.

**Cell death quantitation.** Cells were plated in 96-well plates in triplicate at 15,000 per well. The percentage of viable cells was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenoxophenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corp.) following the manufacturer’s instructions. Death of control, scrambled, and MUC1siRNA-transfected UTC cells exposed to chemotherapy agents was also analyzed by orange acridine/ethidium bromide staining.

**Caspase-3 activity detection.** Caspase-3 activity was detected by ApoAlert Caspase-3 Colorimetric Assay kit (Clontech) according to the instruction manual. Briefly, lysates of 2 × 10⁵ cells per well were transferred into each well of a Caspase Profiling Assay Plate containing a specific caspase-3 substrate (Asp-Glu-Val-Asp or DEVD) linked to chromophore p-nitroanilide (pNA) and then incubated at 37°C for 2 h. On cleavage of DEVD-pNA by caspase-3, released pNA was monitored colorimetrically by absorbance at 405 nm.

**Statistical analysis.** Data were expressed as mean ± SD. ANOVA (one way) with Bonferroni adjustment was used to analyze the statistical significance of the results, and the analysis was done using GraphPad Prism version 4.00 for Windows (GraphPad Software). P values of <0.05 were considered significant.

**Results**

**Thyroid cancer cells express high levels of MUC1-C.** To evaluate MUC1-C expression levels in thyroid carcinoma cells, immunoblot analyses on cell lysates obtained from freshly purified normal, goiter, FTC, FTC-133, and UTC cells and from five thyroid cancer cell lines were done. Interestingly, MUC1-C was found overexpressed in primary thyroid cancer cells derived from all the histologic variants of thyroid carcinoma and basically not detectable in primary thyroid normal cells, whereas freshly purified...
One representative of three independent experiments. and UTC cells were subjected to SDS-PAGE and immunoblot analysis for positive control.


A

B

Figure 2. MUC1-C intracellular localization in thyroid cancer cells.

A, immunofluorescence analysis of goiter, PTC, FTC, and UTC cells stained for anti-MUC1-C (green). Mitochondria were stained with MitoTracker Red probe (red) and nuclei with Hoechst (blue). MCF-7 cells were used as positive control. B, lysates from the mitochondrial fractions of goiter, PTC, FTC, and UTC cells were subjected to SDS-PAGE and immunoblot analysis for MUC1-C. Hsp60 detection in the same membrane was used as loading control. One representative of three independent experiments.

goiter cells resulted strongly positive (Fig. 1A). MUC1-C overexpression in goiter cells was probably due to the high proliferative rate of these cells, resulting from activation of mitogenic pathways, in which MUC1-C could be strictly involved. MUC1-C expression pattern in thyroid cancer cell lines showed that MUC1-C levels increased in the histologic variants with low grade of differentiation and high malignancy extent (Fig. 1B). The observation that mRNAs for MUC1 were homogeneously expressed in all thyroid cells analyzed suggests that the considerable MUC1-C formation in thyroid tumor cells could be considered a post-translational event in which MUC1-C could be strictly involved. MUC1-C expression mainly active in highly aggressive tumor cells (Fig. 1C and D).

MUC1-C localizes to mitochondria of thyroid cancer cells. An aberrant intracellular targeting of the COOH-terminal subunit of MUC1 oncprotein has been reported in several epithelial tumors, such as colon, breast, and lung cancers (25). This event seems to play a key role in modulating mitochondrial release of proapoptotic molecules (25).

To analyze the expression pattern and the intracellular localization of MUC1-C in goiter and thyroid cancer cells, an immunofluorescence staining was done with an antibody specific to MUC1-C (Fig. 2A, green). Mitochondria were stained using MitoTracker Red probe (Fig. 2A, red), whereas nuclei were counterstained with Hoechst (Fig. 2A, blue).

The results showed that MUC1-C was expressed on the cell membrane of both goiter and tumor cells, whereas mitochondrial localization was revealed only in cancer cells (Fig. 2A).

To confirm these findings, mitochondrial lysates from freshly purified goiter and thyroid cancer cells were subjected to immunoblot analysis for MUC1-C detection. As expected, MUC1-C was detectable in the mitochondrial fractions from cancer but not from goiter cells (Fig. 2B). Of note, mitochondrial localization of MUC1-C increased in FTC and UTC cells, suggesting a potential correlation between MUC1-C peculiar intracellular localization and aggressiveness of thyroid tumor cells (Fig. 2B). Equal loading of mitochondrial lysates was proved by detecting Hsp60 protein.

**IL-4 and IL-10 modulate MUC1-C expression.** Thyroid cancer cell refractoriness to death ligand– and chemotherapeutic drug–induced apoptosis is due to the autocrine production of IL-4 and IL-10, resulting in the overexpression of several antiapoptotic factors (10, 11). To investigate the possible involvement of these cytokines in the regulation of MUC1-C overexpression, freshly purified normal thyrocytes were exposed to recombinant IL-4, IL-10, or both cytokines for 24 h. Immunoblot analysis of MUC1-C expression showed that exposure to both cytokines resulted in a prominent increase of MUC1-C levels, whereas exposure to a single cytokine exerted a lower effect (Fig. 3A). To confirm that IL-4 and IL-10 presence in thyroid cancer microenvironment is responsible for increased MUC1-C levels, purified UTC cells were treated with neutralizing antibodies against both cytokines for 72 h. Single blockage of IL-4 or IL-10 induced a partial reduction of MUC1-C expression, which was considerably enhanced following the combined treatment with both antagonist antibodies (Fig. 3B). Furthermore, real-time PCR analysis of MUC1 mRNA levels in the same samples revealed that muc1 gene transcription was significantly influenced by the presence of IL-4 or IL-10, whose neutralization dramatically reduced the levels of MUC1 mRNA (Fig. 3C and D). These results show that IL-4 and IL-10 affect MUC1 transcription and MUC1-C expression in normal and tumor thyroid cells.

**SOCS-1 and AktK179M exogenous expression decreases MUC1-C levels.** To evaluate the role of the induction of JAK/STAT and PI3K/Akt pathways in the regulation of muc1 transcription and MUC1-C expression, we produced lentiviral vectors that allowed the stable delivery of SOCS-1 and AktK179M in purified UTC cells (Fig. 4A). SOCS-1 hampers JAK1/STAT6 pathway by direct binding to JAK1 phosphorylation site, whereas AktK179M is a kinase defective mutant form of Akt. The effective block of JAK1/STAT6 or PI3K/Akt pathways was verified by evaluating the phosphorylation of JAK1, STAT6, and Akt (Fig. 4B). Western blot analysis revealed that SOCS-1 or AktK179M exogenous expression yielded a significant down-regulation of MUC1-C expression levels, which was even more pronounced when both transduction pathways were hindered (Fig. 4C). Remarkably, as shown by real-time PCR analysis, SOCS-1 alone or in combination with AktK179M was particularly effective in attenuating muc1 transcription, whereas AktK179M exerted a weaker effect (Fig. 4D). Taken together, these data show that both JAK1/STAT6 and PI3K/Akt pathways differentially affect MUC1 expression at mRNA and protein level. JAK1/STAT6 pathway seems to be mainly involved in the modulation of muc1 transcription, whereas PI3K/Akt pathway seems to play a role also in regulating MUC1-C protein stability within the cell.
MUC1 down-regulation sensitizes thyroid cancer cells to chemotherapy-induced apoptosis. Tumor cell resistance to anticancer agents is often due to activation of antiapoptotic pathways. To investigate whether MUC1 contributes to the resistance of thyroid cancer cells to chemotherapeutic drug-induced apoptosis, we knocked down MUC1 with siRNA duplexes. Exposure of freshly purified UTC cells to MUC1siRNA was associated with a marked down-regulation of MUC1 expression (Fig. 5A and B). In contrast, there was not detectable down-regulation of MUC1 in UTC cells exposed to unspecific siRNA (scrambled; Fig. 5A and B). Control, scrambled, and MUC1siRNA-transfected UTC cells were then treated with Cisplatin, Doxorubicin, or Taxol, conventionally used in thyroid cancer treatment. UTC cells were resistant to chemotherapeutic drugs even at dosages higher than those measurable in vivo in the clinical setting (Fig. 5C). As determined by MTS assay, the rate of death in scrambled and MUC1siRNA-transfected UTC cells was not significantly increased compared with untransfected (control) UTC cells (Fig. 5D). Importantly, MUC1siRNA-transfected UTC cells exposed to Cisplatin, Doxorubicin, or Taxol showed a 2-fold increase in the percentage of cell death (Fig. 5D). Similar results were obtained by orange acridine/ethidium bromide staining and fluorescence microscopy analysis (Fig. 5D; data not shown). Thus, MUC1 controls the resistance of thyroid cancer cells exposed to chemotherapeutic drugs.

MUC1 expression reduces the activation of the intrinsic apoptotic pathway in thyroid cancer cells treated with chemotherapeutic drugs. We next investigated whether the sensitization of thyroid cancer cells to chemotherapeutic drugs was due to an enhanced activation of the intrinsic apoptotic pathway following MUC1siRNA transfection. Control, scrambled, and MUC1siRNA-transfected UTC cells treated with Doxorubicin were therefore analyzed for cytochrome c release and caspase-3 activation. Immunofluorescence analysis showed increased levels of cytosolic cytochrome c following Doxorubicin treatment in MUC1siRNA-transfected UTC cells compared with control cells or cells treated with a scrambled sequence (Fig. 6A). The increased release of cytochrome c from mitochondria may promote a more intense apoptosome formation and activation of executioner caspsases. Accordingly, immunoblot analysis showed that Doxorubicin promoted an increased formation of caspase-3 cleavage products in thyroid cancer cells transfected with MUC1siRNA (Fig. 6B). Similarly, MUC1-C down-regulation allowed a significant increase in DEVDase activity after Doxorubicin exposure (Fig. 6C), confirming that MUC1 expression prevents the amplification of the apoptotic cascade activated by mitochondria. Thus, knockdown of

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**Figure 3.** IL-4 and IL-10 modulate MUC1-C expression. A, immunoblot analysis of MUC1-C expression in normal thyrocytes treated with 20 ng/mL IL-4, 40 ng/mL IL-10, or in combination IL-4 plus IL-10 for 24 h. Detection of β-actin was used as loading control. One representative of three independent experiments. B, immunoblot analysis of MUC1-C in UTC cells exposed to IgG, anti-IL-4 (15 μg/mL), or anti-IL-10 (15 μg/mL) or to a combination of both neutralizing antibodies for 72 h. Loading controls were done by detecting β-actin in the same membranes. One representative of three independent experiments. C, real-time PCR analysis of MUC1 mRNA levels in normal thyrocytes treated as in (A). Columns, mean of three independent experiments; bars, SD. *, P < 0.05; **, P < 0.01. D, real-time PCR analysis of MUC1 mRNA levels in UTC cells treated as in (B). Columns, mean of three independent experiments; bars, SD. ***, P < 0.001.
MUC1 expression increases the sensitivity of thyroid cancer cells to chemotherapeutic drugs through activation of the intrinsic apoptotic pathway.

Discussion

The ability of tumor cells to escape the cytotoxic activity of chemotherapeutic drugs may result from several molecular alterations involving cell cycle and apoptosis (41). In thyroid cancer, the scarce efficacy of chemotherapy led to the use of radioiodine therapy, which is capable of eradicating well-differentiated thyroid cancers through the induction of a localized cytotoxicity. However, such treatment yields very limited response in patients affected by UTC, for whom life expectancy does not exceed 6 months (2, 3, 5).

MUC1 oncoprotein has been found overexpressed in most human carcinomas and has been involved in tumor progression (25). MUC1-C is indeed able to cross-talk with several signal transduction pathways involved in tumor transformation (18, 42). Recent findings showed that MUC1 overexpression in human cancers results in the inhibition of oxidative stress–induced and genotoxic drug–induced apoptosis. In colon, breast, and lung tumor cells, Hsp70/Hsp90-mediated mitochondrial targeting of MUC1-C seemed to occur and is thought to be responsible for the reduced release from mitochondria of proapoptotic molecules, such as cytochrome c, AIF, and Smac/DIABLO, contributing to cancer cell refractoriness to genotoxic agent–induced apoptosis (25, 32, 34).

In this study, we showed that MUC1-C is expressed at high levels in all the histologic variants of thyroid carcinoma, with a pattern that suggests an aberrant mitochondrial localization. Although MUC1-C was undetectable in normal thyroid samples, it is overexpressed on the cell membrane of goiter cells but did not show mitochondrial localization, suggesting a possible contribution in cell proliferation but not on the control of cell death. Such mitochondrial localization of MUC1-C is associated with a decreased release of cytochrome c with a consequent reduction of caspase activation and apoptosis in thyroid cancer cells treated with chemotherapeutic drugs. These data collectively indicate that

Figure 4. Exogenous expression of SOCS-1 and Akt\textsubscript {K179M} decreases MUC1-C levels. A, Western blot analysis of SOCS-1 or HA in UTC cells transduced with empty vector (Vector), SOCS-1, or Akt\textsubscript {K179M}. Loading control was done by \(\beta\)-actin detection. B and C, lysates from UTC cells transduced with empty vector (Vector), SOCS-1, Akt\textsubscript {K179M}, or both lentiviral constructs were subjected to immunoblot analysis with the indicated antibodies. Loading control was done by \(\beta\)-actin staining. One representative of three independent experiments. p-Akt, phosphorylated Akt; p-JAK1, phosphorylated JAK1; p-STAT6, phosphorylated STAT6. D, real-time PCR analysis of MUC1 transcript levels in UTC cells transduced as in (B) and (C). Columns, mean of three independent experiments; bars, SD. ***, \(P<0.001\).
MUC1-C overexpression and aberrant intracellular targeting contribute together with antiapoptotic Bcl-2 family members to prevent the activation of the intrinsic apoptotic pathway in response to the exposure to chemotherapeutic drugs.

We have previously shown that the autocrine production of IL-4 and IL-10 by thyroid cancer cells promotes tumor cell growth and resistance to chemotherapy through the up-regulation of antiapoptotic proteins (10, 11, 43). Accordingly, IL-4 and IL-10 blockage increased the effectiveness of anticancer agents and proapoptotic stimuli occurring from death receptor activation (10, 11). Here, we provide evidence that MUC1-C expression is strictly associated with activation of the IL-4– and IL-10–dependent signal transduction pathways in thyroid cancer cells. Indeed, MUC1-C expression and muc1 transcription significantly increased in normal thyroid cells following the exposure to IL-4 and IL-10, whereas neutralization of both IL-4 and IL-10 in UTC cells promoted a dramatic decrease of MUC1-C along with MUC1 mRNA levels.

Organization and transcriptional regulation of the MUC1 promoter have been recently investigated (44, 45). The presence of candidate binding sites for transcription factors of the STAT and κB families upstream of the transcription start site has been identified, suggesting the involvement of such transcription factors in overexpression of MUC1 in tumor cells (44, 45). STATs are activated by phosphorylation when associated with or recruited to JAK/receptor complexes at the cell membrane in response to

![Figure 5. MUC1 down-regulation sensizes thyroid cancer cells to chemotherapy. A, RT-PCR analysis of MUC1 mRNA levels in freshly purified untransfected (control) or transfected with scrambled or MUC1siRNA UTC cells, harvested 72 h after transfection. B, Western blotting analysis of MUC1-C in UTC cells treated and harvested as in (A). Loading control was done on the same membrane blot detected for β-actin. MUC1 knockdown was observed for 9 to 10 d after transfection. C, dose response curves for Cisplatin, Doxorubicin, and Taxol after 24-h treatment of UTC cells. Points, mean of three independent experiments. D, control, scrambled, and MUC1siRNA-transfected UTC cells were collected 72 h after transfection, plated in a 96-well plates at 15,000 cells per well, and allowed to grow overnight before 24-h exposure to Cisplatin, Doxorubicin, or Taxol. Percentage cell death in control (white columns), scrambled (black columns), and MUC1siRNA-transfected (hatched columns) UTC cells was quantified by MTS assay. Columns, mean of four independent experiments; bars, SD. ***, P < 0.001. Two representative images of three independent experiments of orange acridine/ethidium bromide staining of scrambled or MUC1siRNA-transfected UTC cells treated with Doxorubicin. Viable cells show a bright green nucleus with intact structure, whereas apoptotic cells exhibit condensed or fragmented chromatin (green or orange); necrotic cells display an orange nucleus with intact structure.](image)
activation of a variety of different types of cytokine receptors, including IL-4 receptor (46, 47). IL-4 receptor stimulation results in activation of JAK1/STAT6 pathway and in the phosphorylation of IRS-1, which activates Akt pathway through the recruitment of PI3K to the plasma membrane (48). PI3K/Akt pathway modulates transcription factor nuclear factor-κB (NF-κB) activation, which has been related to tumorigenesis and apoptosis inhibition (49). In this regard, it is likely that the continuous presence of IL-4 and IL-10 cytokines in the thyroid cancer microenvironment leads to the persistent activation of JAK1/STAT6 and PI3K/Akt pathways, which are in turn responsible for the up-regulation of several antiapoptotic molecules and refractoriness to CD95-induced and chemotherapeutic drug–induced apoptosis (10). We showed that the blockage of JAK1/STAT6 and PI3K/Akt pathways results in a considerable reduction of both MUC1 mRNA and MUC1-C protein levels. Interestingly, when PI3K/Akt pathway is hindered by Akt K179M, muc1 transcription seems to be mainly under the control of JAK/STAT pathway. Accordingly, we found higher MUC1 mRNA levels in UTC cells expressing AktK179M than in UTC cells expressing SOCS-1. Of note, the pattern of expression of MUC1-C in these cells does not reproduce the levels of MUC1 mRNA observed, suggesting that inhibition of PI3K/Akt pathway could result in activation of molecular mechanisms affecting MUC1-C protein half-life. Indeed, in SOCS-1–infected UTC cells, muc1 transcription is strongly reduced whereas the constant activation of PI3K/Akt pathway associates with higher MUC1-C protein levels. Thus, in thyroid cancer cells, MUC1 overexpression results from the combined action of JAK1/STAT6 pathway, mainly at transcriptional level, and PI3K/Akt pathway, which acts at transcriptional level, most likely through NF-κB, and in the regulation of MUC1 protein half-life. MUC1 interacts directly with the Wnt pathway effector β-catenin and glycogen synthase kinase 3β (GSK3β; refs. 27, 28). GSK3β phosphorylates MUC1 cytoplasmic domain on serine in a SPY site, decreasing the interaction with β-catenin (28). Conversely, tyrosine phosphorylation of the SPY site increases the formation of complexes MUC1–β-catenin (26, 29). MUC1–β-catenin complexes localize in the nucleus of several human carcinoma cells and function as coactivators of Tcf/LEF-1 target gene transcription (42). In thyroid cancer, β-catenin plays a direct role in the dedifferentiation commonly observed in late-stage disease (50). Activating mutations in β-catenin have been shown in late-stage thyroid tumors and lead to β-catenin nuclear localization and poor prognosis (50). The considerable activation of the PI3K/Akt pathway in thyroid cancer cells results in GSK3β phosphorylation and deactivation and subsequent β-catenin up-regulation (50). Based on these remarks, MUC1–β-catenin complexes might form and localize in the nucleus of thyroid cancer cells, contributing to activation of a variety of different types of cytokine receptors, including IL-4 receptor (46, 47). IL-4 receptor stimulation results in activation of JAK1/STAT6 pathway and in the phosphorylation of IRS-1, which activates Akt pathway through the recruitment of PI3K to the plasma membrane (48). PI3K/Akt pathway modulates transcription factor nuclear factor-κB (NF-κB) activation, which has been related to tumorigenesis and apoptosis inhibition (49). In this regard, it is likely that the continuous presence of IL-4 and IL-10 cytokines in the thyroid cancer microenvironment leads to the persistent activation of JAK1/STAT6 and PI3K/Akt pathways, which are in turn responsible for the up-regulation of several antiapoptotic molecules and refractoriness to CD95-induced and chemotherapeutic drug–induced apoptosis (10). 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the malignant phenotype. In addition, overexpression of MUC1 in the absence of GSK3β activity might inhibit the formation of the E-cadherin-β-catenin complexes and favor cell migration and metastasis formation by weakening adherent junctions.

Recent in vitro and in vivo findings have shown that MUC1 down-regulation resulted in an increased sensitivity to chemotherapy-induced apoptosis in breast and lung cancers (25). Importantly, we observed that MUC1 knockdown sensitized UTC cells to apoptosis induced by cytotoxic agents. Chemotherapy is often the only possible treatment for highly aggressive tumors, such as undifferentiated thyroid carcinoma (2, 3). Therefore, sensitization of cancer cells to chemotherapeutic treatment represents one of the most important goals for an effective approach to such tumors.

Taken together, our results provide new insights into mechanisms responsible for up-regulation of MUC1 in thyroid cancer and show that MUC1-C overexpression and mitochondrial localization in thyroid cancer cells interfere with the induction of the intrinsic pathway of apoptosis following exposure to anticancer agents. Thus, MUC1-C down-regulation could be exploited to increase the effectiveness of conventional chemotherapy in the treatment of thyroid cancer.

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References

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