Human MUC1 Carcinoma Antigen Regulates Intracellular Oxidant Levels and the Apoptotic Response to Oxidative Stress*

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The DF3/MUC1 transmembrane oncoprotein is aberrantly overexpressed by most human carcinomas. Certain insights are available regarding a role for MUC1 in intracellular signaling; however, no precise function has been ascribed to this molecule. The present results demonstrate that MUC1 expression is up-regulated by oxidative stress and that this response is mediated by activation of MUC1 gene transcription. A role for MUC1 in the oxidative stress response is supported by the demonstration that MUC1 expression is associated with attenuation of endogenous and H2O2-induced intracellular levels of reactive oxygen species (ROS). MUC1-dependent regulation of ROS is mediated at least in part by up-regulation of anti-oxidant enzyme (superoxide dismutase, catalase, and glutathione peroxidase) expression. In concert with these findings, we show that the apoptotic response to oxidative stress is attenuated by a MUC1-dependent mechanism. These results support a model in which activation of MUC1 by oxidative stress provides a protective function against increased intracellular oxidant levels and ROS-induced apoptosis.

The human DF3/MUC1 mucin-like transmembrane is normally expressed on the apical borders of secretory epithelial cells (1). In carcinoma cells, polarization of MUC1 is lost with high levels of expression over the entire cell surface (1). Estimates indicate that over 70% of newly diagnosed cancers aberrantly express MUC1 (2). The MUC1 proteins consist of an N-terminal ectodomain with variable numbers of 20-amino acid tandem repeats that are extensively modified with O-linked glycans (3, 4). The C-terminal region includes a transmembrane domain and a 72-amino acid cytoplasmic tail. Following proteolytic cleavage, the >250-kDa ectodomain remains associated with the ~25-kDa C-terminal subunit at the cell surface. β-Catenin, a component of the adherens junctions of mammalian cells, interacts directly with the MUC1 intracellular region (5). Other studies have shown that phosphorylation of MUC1 by glycosyn thase 3β, c-Src, or the epidermal growth factor receptor contributes to regulation of the interaction between MUC1 and β-catenin (6–8). More recent work has demonstrated that MUC1 colocalizes with β-catenin in the nucleus and that MUC1 induces transformation (9, 10).

Normal cellular metabolism is associated with the production of reactive oxygen species (ROS).† Common forms of ROS include superoxide (O2−), hydrogen peroxide (H2O2), hydroxyl radicals, and nitric oxide. Mitogenic signals induced by certain growth factors and activated Ras are mediated by ROS production (11, 12). Under nonphysiologic conditions, increases in ROS levels above the reducing capacity of the cell can cause damage to DNA, proteins, and lipids (13, 14). To prevent damage associated with increases in ROS, aerobic cells have developed enzymatic (superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx)) and non-enzymatic (glutathione and thioredoxin) defense mechanisms to balance the reduction-oxidation (redox) state (15). In the absence of an adequate defense, cells respond to oxidative stress with the induction of apoptosis (14). Although few insights are available regarding mechanisms responsible for ROS-induced cell death, H2O2 has been shown to activate topoiso merase II-mediated cleavage of chromosomal DNA and thereby apoptosis (16). The p66(Shc) adaptor protein (17, 18) and the p85 subunit of phosphatidyli nositol 3-kinase (19) have also been implicated in the apoptotic response to H2O2.

The present studies demonstrate that MUC1 expression is activated by oxidative stress. The results also demonstrate that MUC1 regulates intracellular oxidant levels and attenuates the apoptotic response to oxidative stress.

MATERIALS AND METHODS

Cell Culture—Human breast (MCF-7, ZR-75-1, colon (HCT116), and cervical (HeLa) carcinoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). MCF-7, HCT116, and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (high glucose; Cellgro) supplemented with 10% heat-inactivated fetal calf serum, and 2 mm l-glutamine. ZR-75-1 cells were cultured in RPMI 1640 medium (Cellgro) supplemented with 10% fetal calf serum and 2 mm l-glutamine. Cells were treated with H2O2 (Sigma).

Immunoblot Analysis—Cells were lysed in ice-cold lysis buffer (20 mm Tris-HCl, pH 8.0, 150 mm NaCl, 1% Triton X-100, 1 mm phenylmethylsulfonyl fluoride, 1 mm dithiothreitol, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) for 30 min. Lysates were cleared by centrifugation for 20 min at 4 °C as described (20). Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-DF3/MUC1 (1), anti-SOD1 (Santa Cruz Biotechnology), anti-SOD2 (Upstate Biotechnology, Inc.), anti-catalase (Sigma), anti-GPx (MBL Medical and Biological Laboratories) or anti-β-actin (Sigma). The antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL, Amersham Life Sciences).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)—Total cellular RNA was extracted in Trizol, dissolved in RNAse-free water, and incubated for 10 min at 55 °C. MUC1-specific primers (5’-TCTAAC-TCTGTGGCAACAACGG-3’ and 5’-TTATATCGAGAGGCTGCTTCC-5’) were designed to span a region within genomic DNA that contains two introns, resulting in the amplification of a 489-bp fragment from RNA and a 738-bp fragment from genomic DNA. RNA-specific primers for human β-actin were used as a control. The RNA was reverse transcribed and amplified using SuperScript One-Step RT-PCR with Plat-GPx, glutathione peroxidase; HE, hydroethidine; DCF, dichlorodi-hydrofluorescein; DCFH-AM, 5-(and -6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate.

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‡ The abbreviations used are: ROS, reactive oxygen species; RT-PCR, reverse transcription PCR; Luc, luciferase; SOD, superoxide dismutase.
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Human MUC1 Regulates Response to Oxidative Stress

RESULTS

Up-regulation of MUC1 Protein by Oxidative Stress—To assess the effects of oxidative stress on MUC1 expression, human MUC1-positive MCF-7 cells were exposed to 0.4 mM \( \text{H}_2\text{O}_2 \) as a source of ROS. Lysates of the \( \text{H}_2\text{O}_2 \)-treated cells were analyzed by immunoblotting with anti-MUC1 (DF3 antibody). The results demonstrate that MUC1 levels increase 3-fold at 15 min (Fig. 2A). MUC1 expression was up-regulated through 45 min and then declined at 60 min of \( \text{H}_2\text{O}_2 \) treatment (Fig. 1A). As a control, immunoblot analysis of the lysates with anti-\( \beta \)-actin demonstrated equal loading of the lanes (Fig. 1A). To extend these findings, MCF-7 cells were treated with different concentrations of \( \text{H}_2\text{O}_2 \) for 30 min. The results show that over a range of 0.1 to 1.0 mM, increases in MUC1 expression were apparent at 0.1–0.2 mM and maximal at 0.4–0.6 mM \( \text{H}_2\text{O}_2 \) (Fig. 1B). Treatment of human MUC1-positive ZR-75-1 cells with \( \text{H}_2\text{O}_2 \) was also associated with increases in MUC1 expression (Fig. 1C). The kinetics, however, differed somewhat from that found in MCF-7 cells with maximal increases at 2 h and down-regulation to below base-line levels at 6 h (Fig. 1C). By contrast, similar studies with MUC1-negative HCT116 cells demonstrated no detectable induction of MUC1 expression in response to \( \text{H}_2\text{O}_2 \) treatment (data not shown). These findings indicate that MUC1-positive cells respond to oxidative stress with increases in MUC1 expression.

Oxidative Stress Induces MUC1 Transcription—To determine whether activation of MUC1 transcription contributes to up-regulation of MUC1 protein in the oxidative stress response, MUC1 mRNA levels were quantitated by RT-PCR. Treatment of MCF-7 cells with \( \text{H}_2\text{O}_2 \) was associated with increases in MUC1 transcripts at 15 min (Fig. 2A). Moreover, in concert with regulation at the protein level, MUC1 mRNA levels were increased through 45 min and then declined at 60 min (Fig. 2A). As a control, there was little effect of \( \text{H}_2\text{O}_2 \) on \( \beta \)-actin mRNA levels (Fig. 2A). Treatment of ZR-75-1 cells with \( \text{H}_2\text{O}_2 \) was also associated with increases in MUC1 transcripts (Fig. 2B). The increase in MUC1 transcripts was maximal at 1 h of \( \text{H}_2\text{O}_2 \) exposure and was detectable in the absence of changes in \( \beta \)-actin mRNA levels (Fig. 2B). To assess the effects of \( \text{H}_2\text{O}_2 \) on MUC1 gene transcription, MCF-7 cells were transfected to express a MUC1 promoter-Luc reporter and SV40-\( \text{Renilla} \) Luc constructs. Treatment with \( \text{H}_2\text{O}_2 \) was associated with an increase in firefly, and not \( \text{Renilla} \), luciferase activity, which was maximal at 45 min (Fig. 2C). In ZR-75-1 cells transfected with pMUC1-Luc and treated with \( \text{H}_2\text{O}_2 \), induction of firefly luciferase activity was maximal at 1 h (Fig. 2D). These findings demonstrate that \( \text{H}_2\text{O}_2 \) activates MUC1 gene transcription and thereby increases MUC1 mRNA and protein levels.

MUC1 Regulates ROS Levels—To assess the role of MUC1 in response to oxidative stress, MUC1-negative HCT116 cells were transfected to stably express the empty vector or MUC1 (Fig. 3A). Expression of MUC1 in two separate isolates of stable
HCT116 transfectants was somewhat lower than that found in MCF-7 cells (Fig. 3A and data not shown). HeLa cells, which constitutively express MUC1 (6), were stably transfected to express MUC1 at relatively higher levels (Fig. 3A). Analysis of the HCT116 transfectants by flow cytometry demonstrated that MUC1 is expressed on the cell surface (Fig. 3B). The HeLa cells stably transfected with the MUC1 vector also demonstrated an increase in cell surface MUC1 expression (Fig. 3B). These findings indicate that, like endogenous MUC1, transfected MUC1 is expressed as a transmembrane glycoprotein.

To determine whether MUC1 affects ROS levels, cells were incubated with DCFH-AM, and H2O2-mediated oxidation of the fluorochrome was assayed by flow cytometry. The results demonstrate that, compared with HCT116 cells expressing the empty vector, MUC1-positive HCT116 cells exhibited substantially lower H2O2 levels (Fig. 4A). Moreover, increased expression of MUC1 in HeLa cells resulted in marked decreases in H2O2 levels (Fig. 4B). To extend this analysis, HCT116 cells were exposed to H2O2, and then assayed for oxidation of DCFH-AM. Compared with HCT116/vector cells, which exhibited substantial increases in H2O2 levels, expression of MUC1 was associated with attenuation of this response (Fig. 4C). The

electrophoresis. C and D, MCF-7 (C) and ZR-75-1 (D) cells were transfected with pMUC1-Luc and SV40–Renilla luciferase and then exposed to 0.4 mM H2O2 for the indicated times. Lysates were analyzed for firefly and Renilla luciferase activities. The results are expressed as the mean ± S.D. of three separate experiments, each performed in triplicate, in which the ratio of firefly to Renilla luciferase activities is relative to that of control cells.
HeLa/vector cells, which express endogenous MUC1, exhibited a less pronounced increase in H$_2$O$_2$ levels compared with HCT116/vector cells (Fig. 4D). Moreover, HeLa cells transfected to express increased MUC1 levels showed an attenuated response to H$_2$O$_2$ treatment (Fig. 4D). These findings demonstrate that MUC1 expression is associated with down-regulation of endogenous and induced intracellular H$_2$O$_2$ levels.

Treatment of cells with H$_2$O$_2$ is associated with mitochondrial dysfunction and thereby the generation of superoxide radicals (O$_2^-$) (25). To assess the effects of MUC1 on O$_2^-$ levels, the HCT116 cell transfectants were incubated with HE and then assayed by flow cytometry. The results demonstrate that O$_2^-$ levels increase substantially after treatment of HCT116/vector cells with H$_2$O$_2$ (Fig. 5A). By contrast, this response to H$_2$O$_2$ treatment was attenuated in HCT116/MUC1-A cells (Fig. 5A). Analysis of HE oxidation at different time points confirmed that MUC1 expression in HCT116/MUC1-A and HCT116/MUC1-B cells is associated with decreased O$_2^-$ levels as compared with that in HCT116/vector cells (Fig. 5B). Treatment of HeLa/vector cells with H$_2$O$_2$ also resulted in increased HE oxidation; this response was attenuated in HeLa/MUC1-A cells (Fig. 5C). These findings were confirmed at different time points in the HeLa/MUC1-B cells (Fig. 5D). Taken together with the DCF data, the results indicate that MUC1 expression attenuates H$_2$O$_2$-induced increases in intracellular oxidant levels.

MUC1 Increases Expression of Anti-oxidant Enzymes—The predominant enzymatic mechanisms that regulate intracellular oxidant levels are mediated by SOD, catalase, and GPx (26). To determine whether MUC1 affects expression of these antioxidant enzymes, lysates from the HCT116 transfectants were subjected to immunoblot analysis with anti-SOD1 and -SOD2, anti-catalase, and anti-GPx. The results of a representative

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**Fig. 4.** MUC1 regulates intracellular H$_2$O$_2$ levels. A and B, HCT116 (A) and HeLa (B) cells expressing empty vector or MUC1 were incubated with DCFH-AM for 30 min. Fluorescence of oxidized DCF was measured by flow cytometry. C and D, HCT116 (C) and HeLa (D) cell transfectants were left untreated (solid bars) or treated with 0.3 mM H$_2$O$_2$ for 10 min (hatched bars) or 30 min (shaded bars). DCFH-AM was then added for an additional 30 min. The results are expressed as the relative H$_2$O$_2$ level (mean ± S.D. for three separate determinations) compared with the untreated vector transfectants.

**Fig. 5.** MUC1 attenuates superoxide levels. HCT116 (A and B) and HeLa (C and D) cell transfectants were left untreated (solid profiles and bars) or treated with 0.3 mM H$_2$O$_2$ for 10 min (hatched bars), 30 min (C and shaded bars), 120 min (A and open bars). HE was then added for an additional 20 min. The fluorescence of oxidized HE was measured by flow cytometry. The results are expressed as the mean O$_2^-$ level (mean ± S.D. for three separate determinations) compared with the untreated vector transfectants.
experiment show that, compared with HCT116/vector cells, SOD1 and SOD2 levels were increased up to 2.7-fold in the MUC1 transfectants (Fig. 6A). MUC1 expression was also associated with a 1.6–2.2-fold increase in catalase levels (Fig. 6A). Notably, GPx levels were increased 6–8-fold in the HCT116/MUC1 as compared with HCT116/vector cells (Fig. 6A). Immunoblotting for β-actin demonstrated equal loading of the lanes (Fig. 6A). Increased expression of MUC1 in HeLa cells was also associated with similar increases in SOD1, SOD2, catalase, and GPx levels (Fig. 6B). These findings demonstrate that MUC1 expression is associated with increases in antioxidant enzyme levels.

**MUC1 Inhibits the Apoptotic Response to Oxidative Stress**—To determine whether MUC1 regulates the response to oxidative stress, H₂O₂-treated HCT116/vector and HCT116/MUC1 cells were assayed for induction of apoptotic cells with sub-G₁ DNA. The results demonstrate that H₂O₂-induced apoptosis is significantly attenuated in MUC1-positive as compared with MUC1-negative HCT116 cells (Fig. 7, A and B). The apoptotic response to H₂O₂ was also attenuated by increased expression of MUC1 in HeLa cells (Fig. 7, C and D). As confirmation of the induction of apoptosis, ethidium bromide staining of H₂O₂-treated HCT116/vector (Fig. 8, A and B) and HeLa/vector cells (Fig. 8, C and D) further demonstrated bright orange areas of condensed chromatin in nuclei, which distinguishes late apoptotic from necrotic cells. Notably, there was little if any detectable ethidium bromide staining of untreated control cells or H₂O₂-treated MUC1 expressing cells (Fig. 8). These findings

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**Fig. 6.** MUC1 increases expression of antioxidant enzymes. HCT116 (A) and HeLa (B) cells expressing empty vector or MUC1 were subjected to immunoblotting with the indicated antibodies. The intensity of the signals as determined by densitometric scanning is expressed as -fold change relative to that in cells expressing the empty vector. Similar results were obtained in four independent experiments.

**Fig. 7.** MUC1 attenuates induction of cells with sub-G₁ DNA by oxidative stress. HCT116 (A and B) and HeLa (C and D) cells expressing the empty vector or MUC1 were treated with 0.3 mM H₂O₂ for 18 h. A and C, the percentage of cells with sub-G₁ DNA was determined by flow cytometry. B and D, the results are expressed as the percentage (mean ± S.D. of three separate experiments each performed in duplicate) of control (solid bars) and H₂O₂-treated (hatched bars) cells with sub-G₁ DNA.
collectively demonstrate that MUC1 expression is associated with an attenuated apoptotic response to oxidative stress.

**DISCUSSION**

**Activation of MUC1 in Response to Oxidative Stress**—The heavily glycosylated mucins are believed to function in the protection of epithelial surfaces. Secreted mucins and the transmembrane mucins that are tethered at the cell surface form a protective mucous barrier. The transmembrane mucins may also function in signaling the presence of adverse conditions in the extracellular environment. MUC1 is expressed at the cell surface as a heterodimer of the 250-kDa glycosylated N-terminal ectodomain and the 25-kDa transmembrane C-terminal subunit. The extensive O-glycosylation of the MUC1 ectodomain and the resulting rod-like structure that extends beyond the glycocalyx probably contributes to the mucous barrier. Shedding of the ectodomain may also contribute to mucous formation. The available information, however, provides few if any insights into the function of MUC1 in stress-induced signaling mechanisms.

The present results indicate that MUC1 is involved in the response of cells to oxidative stress. As a consequence of pro-oxidant conditions in the extracellular milieu, ROS can damage DNA, RNA, proteins, and lipids (13, 14). Moreover, the presence of excessive ROS-induced damage can result in the activation of cell death mechanisms (14). Our results demonstrate that MUC1 expression is activated by exposure of cells to H$_2$O$_2$ as a form of ROS. As evidenced by use of a luciferase reporter construct under control of the MUC1 promoter, ROS increase transcription of the MUC1 gene. In concert with this finding, ROS exposure was also associated with increases in MUC1 transcripts and MUC1 protein. Activation of MUC1 expression was transient in response to ROS exposure and returned to constitutive levels within 1–3 h depending on the cell type. These findings indicate that carcinoma cells respond to oxidative stress with a transient activation of MUC1 expression.

**MUC1 Regulates Oxidant Levels**—Expression of MUC1 in the oxidative stress response could reflect the activation of pathways to increase protection of the mucous barrier and/or function of MUC1 as an intracellular signaling molecule. To define the potential role of MUC1, we stably expressed MUC1 in carcinoma cells that otherwise exhibit undetectable (HCT116) or low (HeLa) MUC1 levels. Analysis of the oxidation of DCFH-AM led to the unexpected finding that MUC1 decreases endogenous intracellular H$_2$O$_2$ levels. Moreover, increases in H$_2$O$_2$ levels in response to H$_2$O$_2$ treatment were attenuated in independent MUC1 transfectants of both the HCT116 and HeLa cells. In concert with the demonstration that increases in H$_2$O$_2$ levels cause mitochondrial dysfunction and the generation of O$_2^-$ (25), we found that oxidation of HE is increased in H$_2$O$_2$-treated cells. Importantly, MUC1 expression was associated with the attenuation of H$_2$O$_2$-induced O$_2^-$ levels. Whereas H$_2$O$_2$ is readily diffusible across cell membranes, expression of the glycosylated MUC1 ectodomain seemed unlikely to decrease intracellular ROS levels. An alternative explana-

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**Fig. 8.** MUC1 attenuates the apoptotic response to oxidative stress. HCT116 (A and B) and HeLa (C and D) cells expressing the empty vector or MUC1 were treated with 0.3 mM H$_2$O$_2$ for 18 h. The cells were stained with ethidium bromide to assess nuclear morphology. A and C, chromatin condensation as detected by the presence of bright orange areas in the nuclei distinguishes late apoptotic from necrotic cells. B and D, the results are expressed as the percentage of apoptotic cells (mean ± S.D. of three determinations) for control (solid bars) and H$_2$O$_2$-treated (hatched bars) cells.
tion is that MUC1, particularly the transmembrane subunit, contributes to a signaling cascade that regulates levels of ROS. **MUC1 Increases Expression of Anti-oxidant Enzymes**—The present results demonstrate that MUC1 increases expression of the three predominant anti-oxidant enzymes that regulate intracellular H$_2$O$_2$ levels. In mammalian cells, O$_2^-$ is metabolized to H$_2$O$_2$ by the 32-kDa dimeric Cu,Zn-SOD (SOD1) in the cytosol and the 80-kDa tetrameric Mn-SOD (SOD2) in mitochondria. Increases in both SOD1 and SOD2 were found in the MUC1 transfectants. H$_2$O$_2$ is converted to H$_2$O and O$_2$ in a reaction that oxidizes glutathione to its disulfide form. The present results demonstrate that MUC1 expression is also associated with increases in catalase and GPx levels. Thus, it is likely that increased expression of these anti-oxidant enzymes in MUC1-positive cells contributes, at least in part, to the attenuation of endogenously generated and H$_2$O$_2$-induced oxidant levels.

The present studies do not exclude the possibility that MUC1 regulates other enzymes or the non-enzymatic mechanisms involved in maintaining redox balance. Moreover, the specific MUC1-mediated signals that regulate expression of SOD, catalase, and GPx as major enzymatic effectors of the ROS response are presently not known. Indeed, little is known about the signaling mechanisms that control intracellular ROS levels. Recent studies have shown that p66$^{shc}$ regulates oxidant levels in mammalian cells (18, 28). In addition, the forkhead FKHR1 protein increases H$_2$O$_2$ scavenging and resistance to oxidative stress by increasing catalase expression (18). Other work has demonstrated that the c-Abl and Arg tyrosine kinases are activated by oxidative stress and that these proteins regulate intracellular oxidant levels (29–31). Further experiments will be needed to determine whether MUC1 signaling interacts with the p66$^{shc}$, FKHR1, or c-Abl.Arg pathways.

**Does MUC1 Expression by Human Carcinomas Confer a Survival Advantage?**—MUC1 is normally expressed at the apical borders of glandular epithelial cells (1). By contrast, the polarization of MUC1 expression is lost in carcinoma cells that aberrantly overexpress the protein in the cytoplasm and overexpress the protein in the cytoplasm and cytosol, and thus attenuate the apoptotic response to oxidative stress. Alternatively, MUC1 may attenuate ROS-induced apoptosis by a mechanism in addition to or independent of its effects on oxidant levels. MUC1 may also contribute to survival by blocking the necrotic response to oxidative and other types of stress. In this regard, overexpression of MUC1 is sufficient to confer transformation as assessed by anchorage-independent growth and tumorigenicity (10). Whether the survival advantage attributable to MUC1 expression by carcinoma cells in vitro applies to human MUC1-positive tumors is not yet clear. The present findings, however, provide the first evidence that links a protective function of a mucin to regulation of intracellular oxidant levels and the apoptotic stress response.

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**REFERENCES**


