Induction of E-Cadherin in Lung Cancer and Interaction with Growth Suppression by Histone Deacetylase Inhibition

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Introduction: Loss of E-cadherin confers a poor prognosis in lung cancer patients and is associated with in vitro resistance to endothelial growth factor receptor inhibitors. Zinc finger E box-binding homeobox (ZEB)-1, the predominant transcriptional suppressor of E-cadherin in lung tumor lines, recruits histone deacetylases (HDACs) as co-repressors.

Methods: NSCLC cell lines were treated with HDAC inhibitors and analyzed for E-cadherin induction, growth inhibition and apoptosis. National Cancer Institute-H157 cells expressing ectopic E-cadherin were tested for tumorigenicity in murine xenografts.

Results: We found that treatment with MS-275, compared to vorinostat (SAHA), valproic acid or trichostatin A, was most effective in E-cadherin up-regulation and persistence in non-small cell lung cancers. As with other tumor types and HDAC inhibitors, MS-275 inhibited growth and induced apoptosis. Importantly, blocking E-cadherin induction by short hairpin RNA resulted in less inhibition by MS-275, implicating the epithelial to mesenchymal phenotype process as a contributing factor. In contrast to H460 and H661, H157 cells were resistant to E-cadherin up-regulation by HDAC inhibitors. However, E-cadherin was restored, in a synergistic manner, by combined knockdown of ZEB-1 and ZEB-2. In addition, H157 cells stably transfected with E-cadherin were markedly attenuated in their tumor forming ability. Lastly, combining MS-275 with the microtubule stabilizing agent, paclitaxel, or 17-(allylamino)-17-demethoxygeldanamycin, a heat shock protein 90 inhibitor, resulted in synergistic growth inhibition. Since MS-275 has no reported activity against HDAC6, which regulates both microtubule and heat shock protein 90 functions, other mechanisms of synergy are anticipated.

Conclusions: These results support the role of ZEB proteins and HDAC inhibitors in the pathogenesis and treatment of lung cancer.

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Lung cancer is the leading cause of cancer deaths in the United States.[1,2] Although early detection and prevention have the potential to make a significant impact on lung cancer mortality, current approaches, e.g., helical-computerized tomography, are still controversial.[3–8] In addition, only modest progress has been made in the treatment of locally progressed or metastatic disease. One important advance has been achieved with endothelial growth factor receptor (EGFR) tyrosine kinase inhibitors, although responses are limited to a small subset of tumors with EGFR mutations or copy number increases.[9,10] This responsive subset may also be further restricted to tumors with amplifications of eIF3H and avian myelocytomatosis viral oncogene homolog.[11]

Identification of biologic factors responsible for disease pathogenesis or progression is a key strategy for development of new interventional therapeutics. We and others have shown that loss of E-cadherin in patients with non-small cell lung cancer (NSCLC) is associated with a substantially increased risk of early death.[12,13] E-cadherin loss plays a pivotal role in the transition of cells from an epithelial to mesenchymal phenotype, which is associated with increased invasion and metastases.[14] In addition, we reported that E-cadherin loss is associated with resistance to EGFR inhibitors[15] and that zinc finger E box binding homeobox (ZEB)-1 is the predominant transcriptional suppressor of E-cadherin in NSCLC cell lines.[16] Of note, ZEB-1 also seems responsible for inhibiting the tumor suppressor, SEMA3F.[17] As ZEB factors recruit histone deacetylase (HDAC) corepressors, treatment with a class I/II HDAC inhibitor resulted in up-regulation of both E-cadherin[16] and SEMA3F.[17] Moreover, HDAC inhibitors decreased binding of ZEB-1 to the SEMA3F promoter, at least in National Cancer Institute (NCI)-H661 cells.

HDAC inhibitors block cancer cell growth by multiple mechanisms including sensitizing cells to apoptosis, cell-cycle inhibition, downregulation of angiogenic factors, and, as recently described, proteasome inhibition.[18–21] HDAC inhibitors have been combined with multiple agents in human cancer cells with promising preliminary results. These agents include methylation and multityrosine kinase inhibitors,[22,23] peroxisome proliferator-activated receptor γ agonists,[24] tu-

mor necrosis factor-related apoptosis-inducing ligand,[25] and single-agent (e.g., etoposide)[26] and combination chemotherapy (e.g., carboplatin plus paclitaxel).[27,28]

Heat shock protein (HSP) 90 is a chaperone that regulates the folding and stability of many proteins involved in cancer (e.g., avian erthroblastic leukemia viral oncogene homolog-B2, v-raf-1 murine leukemia viral oncogene homolog, and hypoxia inducible factor-1α). In hematologic neoplasms, HDAC and HSP90 inhibitors have been successfully combined.[29,30] However, there is little published information regarding this combination in lung cancer cells. Of note, the function of HSP90 is regulated by acetylation. Both mutations and HDAC inhibitors that affect HSP90 acetylation have been reported to inhibit the binding of client proteins and other chaperones.[31] Although HDAC6 has a particular role in deacetylating HSP90, other HDACs also seem to be involved.

In this study, we observed that E-cadherin was induced in NSCLC cell lines by multiple HDAC inhibitors, with the class I inhibitor, MS-275, yielding the strongest response and persistence of expression after agent removal. HDAC inhibition also reduced growth rates and induced apoptosis, as evidenced by flow cytometry and poly ADP ribose polymerase (PARP) cleavage. Although HDAC inhibitors have been reported to affect the expression of many hundreds of genes,[32] we found that blocking the upregulation of E-cadherin by short interfering RNA (siRNA) made cells significantly more resistant to MS-275. This suggests that the observed negative effects on growth/proliferation by HDAC inhibitors stems, in part, from the epithelial to mesenchymal phenotype and especially E-cadherin. The role of ZEB-1/2 in these processes was supported by the finding that combined ZEB1/2 knockdown was necessary to restore E-cadherin in NCI-H157 cells, which are refractory to HDAC inhibitors. Moreover, E-cadherin transfectants of H157 were significantly impaired in their ability to form tumors in nude mice. Combinations of MS-275 with taxotere, or the HSP90 inhibitor, 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) resulted in synergistic growth inhibition in most NSCLC cell lines examined.

METHODS

Cell Lines

Fourteen cell lines derived from NSCLC lung cancers (NCI-H157, -H290, -H292, -H322, -H460, -H513, -H647, -H661, -H1648, -H1703, -H1793, -H1299, -H2122, and A549) and two additional lines, NCI-H28 (mesothelioma) and NCI-1334 (origin unclear) were used. Cells were routinely cultured in RPMI 1640 (InVitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2.5% glutamine, 50,000 units penicillin, and 80-μM streptomycin in a humidified 5% CO_2 atmosphere. E-cadherin-expressing variants of NCI-H157 were generated by $CaPO_4$-mediated cotransfection with pEGFP-C1 and the β-actin promoter-based expression vector pBATEM containing human wild-type E-cadherin.[33] G418 resistant colonies were expanded and subcloned by single cell sorting using the flow cytometry core facility of the University of Colorado Cancer Center.

Reagents

MS-275 (batch number: 81300002; high performance liquid chemotherapy purity, 99.82%) was supplied as a crystalline white powder by Schering AG (Berlin, Germany), and vorinostat (VOR, suberoylanilide hydroxamic acid) was obtained from Aton Pharma (Lot no. AP-390-1, Tarrytown, NY). Both HDAC inhibitors were dissolved in dimethyl sulfoxide at 10 mM, and final dimethyl sulfoxide concentrations in medium did not exceed 0.01%. Valproic acid (VPA), trichostatin A (TSA), and 17-AAG were purchased from Sigma (St. Louis, MO). HDAC inhibitors were used at micromolar concentrations, generally in the range of 1 to 10 μM. Treatment times were as indicated in the figure legends.

Gene Knockdown

Oligonucleotides targeting E-cadherin (CDH1), designed for expression from pSuperior.puro (OligoEngine, Inc.), were prepared from messenger RNA (mRNA) position 299 to 317 (NM_004360). Oligos were (forward) 5′-GATCCGATTGCACCGGTCGACAAATTCAAGAGATTTGTCGACCGGTGCAATCTTTTTTGGAAC and (reverse) 5′-TCGAGTTCCAAAAAAGATTGCACCGGTCGACAAATCTCTTGAATTTGTCGACCGGTGCAATCG. Mutant control oligos were (forward) 5′-GATCCGATTGCAC*A*GGTCGACAAATTCAAGAGATTTGTCGACC*T*GTGCAATCTTTTTTGGAAC and (reverse) 5′-TCGAGTTCCAAAAAAGATTGCAC*A*GGTCGACAAATCTCTTGAATTTGTCGACC*T*GTGCAATCG. Oligos were annealed and ligated to *Bgl*II/*Xho*I cleaved vector. Clones were sequence verified. NCI-H661 cells were electroporated with 1 μg of each short hairpin RNA (shRNA) construct and colonies selected with 3 μg/ml puromycin. Effective knockdown of E-cadherin was verified by Western blot after induction by MS-275 (Figure 3*B*). For ZEB-1 and -2, prevalidated siRNAs were acquired from Invitrogen, and one with the highest knockdown efficiency and specificity for each target gene was chosen for further analysis (cat no. HSS110549 for ZEB-1; no. HSS190654 for ZEB-2). No significant advantage was observed with the use of more than one siRNA against the same target. Transfections were performed using HiPerFect according to manufacturer's recommendations (Qiagen, Inc.). Cells were plated at 40 to 60% confluency and transfected 15 hours later with final concentrations of 5nM. Media were replaced at 24 hours and harvested for analysis at 96 hours posttransfection.

Total RNA was isolated using the RNAeasy kit (Qiagen, Inc.) and quantitated spectrophotometrically. Equimolar amounts were reverse-transcribed using SuperScript III First-Strand Synthesis SuperMix (Invitrogen) according to manufacturer's instructions. Real-time detection of target cDNA was performed using 2 μL of 1:5 diluted cDNA in 20 μL total reaction volumes in 96-well plates. Amplifications were analyzed on an ABI 7500 real-time PCR machine, using AmpliTaq Gold with UNG Amperase (Applied Biosystems) according to manufacturer's instructions. Primer sequences were ZEB-1_Forward, 5′-AGCAGTGAAAGAGAAGGGAATGC-3′, ZEB-1_Reverse 5′-GGTCCTCTTCAGGTGCCTCAG-3′ Reverse; ZEB-2_Forward, 5′-AACCCAAGGAGCAGGTAATCG-3′, and ZEB-2_Reverse, 5′-GGAACCAGAATGGGAGAAACG-3′. Normalization primers have been described.[34]