Aromatase Inhibitor Exemestane has Antiproliferative Effects on Human Mesothelioma Cells

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Purpose: The aim of this study was to investigate the expression and biological activity of aromatase (CYP19A1) in malignant mesothelioma (MM).

Experimental Design: We found CYP19A1 in five human MM cell lines using reverse transcription polymerase chain reaction and Western immunoblots and in a group of samples from patients with MM by immunohistochemistry. Aromatization activity was determined in MM cells by enzyme-linked immunosorbent assay as a measure of estradiol (E2) product, in basal condition and after addition of cytokine, prostaglandin-E2, and epidermal growth factor to MM cells. Treatment of MM cells with exemestane, a CYP19A1 inhibitor, was assessed by cell proliferation kit, cell cycle analysis, and Western blot for caspase, poly(ADP-ribose)polymerase, Bcl-xL, and v-akt murin thymoma viral oncogene homolog (Akt).

Results: Biological activity of CYP19A1, already present in basal condition, was increased in MPP89 and Ist-Mes1 cells after treatment with cytokine, in all MM cells on prostaglandin-E2 treatment, and in MPP89, Ist-Mes2, and Ist-Mes1 after addition of epidermal growth factor. Treatment of MM cells with exemestane led to significant reduction of tumor cell growth, perturbation of cell cycle, caspase activation, poly(ADP-ribose)polymerase cleavage, and down-regulation of phosphorylation of Akt and Bcl-xL. In tumor tissues, we found a cytoplasmic localization of CYP19A1. By univariate analysis, overall survival resulted to be strongly influenced by high CYP19A1 expression ($p = 0.001$).

Conclusion: These findings show that CYP19A1 is present in MM and that cell growth can be down-regulated by exemestane. As *Akt* pathway and Bcl-xL are implicated in conferring resistance to conventional chemotherapy, exemestane could open new treatment strategies to be associated with standard therapy for patients afflicted with MM.

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Aromatase (CYP19A1) is the cytochrome P450 enzyme complex that converts C19 androgens to C18 estrogens. The human *CYP19A1* gene, located in the 21.2 region on the long arm of chromosome 15 (15q21.2), spans a region that consists of a 30 kb coding region and a 93 kb regulatory region. Its regulatory region contains at least 10 distinct promoters regulated in a tissue- or signaling pathway-specific manner. Each promoter is regulated by a distinct set of regulatory sequences in DNA and transcription factors that bind to these specific sequences. These partially tissue-specific promoters are used in the gonads, bone, brain, vascular tissue, adipose tissue, skin, fetal liver, and placenta for estrogen biosynthesis necessary for human physiology.[1] Estrogens contribute to differentiation and maturation in normal lung[2] and also stimulate growth and progression of lung tumors.[3,4] These biological effects are mediated by estrogen receptors (ERs), with ER transcripts and proteins reported in most non-small cell lung cancers.[3,4] As in breast, CYP19A1 mediates synthesis of estrogens in lung tissues,[4,5] and local production of estrogens in women and in men could affect lung tumor progression in ER-expressing malignancies.[4,6] Cytokines, such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α, have an important role in regulating estrogen synthesis in peripheral tissues. The activity of the CYP19A1 is increased by IL-6 and TNF-α.[7] Multiple factors, including epidermal growth factor (EGF) and prostaglandin-E2 (PGE2), would synergistically up-regulate CYP19A1 expression.[8–10]

Pleural mesothelioma is not just a limiting protective layer for lung but a dynamic cellular structure regulating serial responses to injury, infection, and disease. Mesothelial cells are biologically active because they can sense and respond to signals within their microenvironment. The development of malignant mesothelioma (MM) is associated in most patients with a history of asbestos exposure.[11] Research has demonstrated that asbestos exposure generates reactive oxygen species and activates macrophages and other cell types to produce these compounds, and cytokines and growth factors.[12] Furthermore, the deposition of insoluble amphibole fibers results in a chronic inflammatory state in exposed individuals.[13] The existence of inflammation has been asso-

ciated with up-regulation of the inducible cyclooxygenase-2, leading to an increase in its product PGE2,[14] and is associated with an increased risk of cancer.[15] Even with widespread asbestos abatement efforts, the increase in MM incidence is likely to continue in Western Europe and the United States well into the next decade, and projections suggest that the incidence will peak around 2020.[16] Other factors, such as chemical carcinogens, ionizing radiation, chronic inflammation, and SV40 viral exposure, may contribute to the development of MM.[17] Pleural MM is 10- to 30-fold more common than their peritoneal counterparts.[18,19] Regardless of site of origin, the prognosis is usually poor with a median survival of 4 to 12 months for pleural tumors[20] and less than 1 year for peritoneal tumors.[19] Systemic chemotherapy and radiation do little to improve the outcome. The resistance to conventional chemotherapy and most chemotherapeutic agents have been attributed to activation of v-akt murin thymoma viral oncogene homolog (Akt) and overexpression of prosurvival *Bcl*2 family members, such as Bcl-xL and *Bcl*2.[21,22] The phosphatidylinositol 3-kinase/Akt signaling pathway regulates fundamental cellular processes linked to tumorigenesis, including cell cycle progression; cell survival, adhesion, motility, and spreading; angiogenesis; glucose homeostasis; and cell and organ size control.[23,24] The *Bcl*2 family proteins are pivotal regulators of apoptotic cell death that are considered as attractive targets for drug design.[25]

Thus, it clearly seems that, to improve the clinical outcome in the pharmacological treatment of this refractory tumor, drugs directed against novel tumor-specific cellular targets and/or characterized by a more specific mechanism of action are needed.

In this study, we have investigated the possibility that the growth of MM cells may be influenced by CYP19A1. NCI-H2452, MPP89, Ist-Mes1, Ist-Mes2, and MSTO-211H cell lines were chosen as a model. Our experiments show that MM cell lines, as well as human MM tissues, express ERs and CYP19A1. The data produced suggest a specific role of the CYP19A1 in the growth of MM. Furthermore, the antiproliferative action of exemestane provides strong evidence that anti-CYP19A1 drugs inhibit tumor growth and may be a novel strategy to sensitize the MM cells to standard therapy.

MATERIALS AND METHODS

Cell Lines

The human MM cell lines MSTO-211H and NCI-H2452 were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Cells were cultured as monolayers in flasks using American Type Culture Collection complete growth medium in a humidified atmosphere containing 5% CO_2 at 37°C. The human MM cell lines Ist-Mes1, Ist-Mes2, and MPP89 were from Genova Institute Culture Collection. Ist-Mes1 and Ist-Mes2 were replaced by Dulbecco modified eagle medium with piruvate supplemented with 10% fetal bovine serum, glutamine (2 mM) 1% nonessential amino acids, and antibiotics (0.02 IU/ml penicillin and 0.02 mg/ml streptomycin), whereas the established cell line, MPP89, was maintained in Ham's F10 with 15% fetal bovine serum and supplemented with glutamine (2 mM) and antibiotics (0.02 IU/ml penicillin and 0.02 mg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C).

Protein Extraction and Western Blot Analysis

Cell lysates were prepared by treating cells with ice-cold lysis buffer (20mM Tris pH 8, 1% NP40, 10% glycerol, 137 mM NaCl, 10 mM ethylenediaminetetraacetic acid, and inhibitor of protease and phosphatase) for 20 minutes followed by centrifugation at 4°C for 15 minutes. Proteins (80 μg) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and then transferred on polyvinylidenedifluoride membrane. Membranes were incubated with antibodies of interest. Goat anti-mouse/rabbit immunoglobulin-G horseradish peroxidase-conjugated secondary antibodies (1:3000) (Bio-Rad Laboratories; Hercules, CA) were used. Antibody reaction was visualized using ECL Western blotting detection reagents (Amersham-Pharmacia, Uppsala, Sweden). Membranes were stripped by incubation in 1 M Tris-HCl (pH 6.8), 10% sodium dodecyl sulfate, and 10 mM dithiothreitol for 30 minutes at 55°C, and reprobed with different antibodies. The blots were, then, reacted with ECL Western blotting detection reagents. Actin was used as a loading control. The experiments were performed in triplicate. Proteins were probed with antibodies against CYP19A1(Santa Cruz Biotechnology, Santa Cruz, CA), anti-ERα (F-10) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-ERβ1 (Serotec), anti-Akt, and phosphorylation of Akt (p-Akt) (Cell Signaling Technology, Danvers, MA) anti-Bcl-xL (Cell Signaling Technology) anti-caspase-3 (E-8) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-poly-(ADP-ribose)polymerase (PARP) (Cell Signaling Technology), and with antiactin antibody (Sigma, St. Louis, MO) to normalize the sample loading and then detected with horseradish peroxidase-conjugated secondary antibodies from Santa Cruz Biotechnology, Santa Cruz, CA. The experiments were done in triplicate.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction Assay of CYP19A1

Total RNA was prepared from cultured MPP89, Ist-Mes2, NCI-H2452, MSTO-211H, and Ist-Mes2 using TRIzol Reagent (Invitrogen Life Technologies, Paisley, UK) according to the manufacturer's protocols as described.[26] Briefly, reverse transcription of RNA, for first-strand complementary DNA (cDNA) synthesis was performed using 4 μg total RNA and 0.5 μg oligo (dT) 12 to 18 primer (Invitrogen Life Technologies, Paisley, UK), 10 mM deoxynucleotidetriphosphate mix (Invitrogen Life Technologies, Paisley, UK) in a final volume of 12 μl. The reaction was incubated at 70°C for 10 minutes and immediately chilled on ice. Primer extension was then performed 10 minutes at temperature room and 42°C for 2 minutes after addition of first-strand buffer, 10 mM dithiothreitol, and 40 U RNase OUT Recombinant Ribonuclease Inhibitor (Invitrogen Life Technologies, Paisley, UK) in a final volume of 19 μl. Then 1 μl (200U) SuperScript II Reverse Transcriptase (Invitrogen Life Technologies, Paisley, UK) was added and incubated at 42°C for 50 minutes. The reaction was inactivated by heating at 70°C for10 minutes. cDNA was stored at −20°C.