Glucocorticoid Receptor Status Is a Principal Determinant of Variability in the Sensitivity of Non–Small-Cell Lung Cancer Cells to Pemetrexed

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Introduction: Pemetrexed is an S-phase targeted drug in front-line or maintenance therapy of advanced nonsquamous non–small-cell lung cancer (NSCLC) but methods are needed for predicting the drug response. Dexamethasone is typically administered the day before, the day of, and the day after pemetrexed. As dexamethasone strongly regulates many genes including p53 through the glucocorticoid receptor (GR), we hypothesized that dexamethasone influences tumor response to pemetrexed.

Methods: Eight nonsquamous NSCLC cell line models with varied p53 and GRα/GRβ status were used for gene expression and cell-cycle analyses and for loss- or gain-of-function experiments.

Results: In three cell lines dexamethasone profoundly, but reversibly, suppressed the fraction of S-phase cells. Dexamethasone also reversibly repressed expression of thymidylate synthase and dihydrofolate reductase, which are primary targets of pemetrexed but are also quintessential S-phase enzymes as well as the S-phase–dependent expression of thymidine kinase 1. Dexamethasone also decreased expression of the major pemetrexed transporters, the reduced folate carrier and the proton coupled folate transporter. Only cells expressing relatively high GRα showed these dexamethasone effects, regardless of p53 status. In cells expressing low GRα, the dexamethasone response was rescued by ectopic GRα. Further, depletion of p53 did not attenuate the dexamethasone effects. The presence of dexamethasone during pemetrexed treatment protected against pemetrexed cytotoxicity in only the dexamethasone responsive cells.

Conclusions: The results predict that in nonsquamous NSCLC tumors, reversible S-phase suppression by dexamethasone, possibly combined with a reduction in the drug transporters, attenuates responsiveness to pemetrexed and that GR status is a principal determinant of tumor variability of this response.

Key Words: Non–small-cell lung cancer, Pemetrexed, p53, Glucocorticoid receptor.

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Non–small-cell lung cancer (NSCLC) comprises 85% of all lung cancer cases and the majority of those patients have regional or systemic metastases. Therefore systemic chemotherapy forms an important component of the management of NSCLC. The efficacy of various chemotherapeutic agents used for the treatment of NSCLC was, for many years, similar among the different histologic subtypes. Randomized clinical trials have now shown that the efficacy of pemetrexed is superior to that of other chemotherapy drugs in nonsquamous NSCLC and inferior to that of other drugs in squamous cell lung cancer when given as monotherapy or in combination with a platinum compound.[1–4] Pemetrexed has now received approval by regulatory agencies in the United States and in Europe for nonsquamous NSCLC patients for front-line therapy in advanced-stage disease in combination with cisplatin, as maintenance therapy after front-line therapy, and in patients with recurrent disease. On the basis of these data and in consideration of its limited toxicity profile, pemetrexed has become the preferred chemotherapy drug in the management of nonsquamous NSCLC.

Despite its widespread use, the efficacy of pemetrexed in advanced-stage NSCLC is modest, with a median progression-free survival of 5.5 months in the front-line setting when combined with cisplatin and only 3.5 months as single agent in patients with recurrent nonsquamous NSCLC. These data suggest that the clinical benefit from pemetrexed is quite variable even among patients with nonsquamous NSCLC, highlighting the need to identify predictors of clinical benefit from pemetrexed. Such predictors would enable treatment decisions that would greatly benefit individual patients while avoiding extended chemotherapies that are ineffective.

Pemetrexed is transported into the cell through two pharmacologically relevant principal membrane transporters, the reduced folate carrier (RFC) and the proton coupled folate transporter (PCFT).[5] Polyglutamation of pemetrexed by folyl-polyglutamate synthase (FPGS) results in increased cellular retention of the drug and increases its affinity for some of its target enzymes.[6] Pemetrexed inhibits nucleotide biosynthesis principally by inhibiting thymidylate synthase (TS) but at pharmacologic doses it is also an inhibitor of dihydrofolate reductase (DHFR), glycinamide ribonucleotide formyltransferase (GARFT), and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (AICARFT).[7] It is generally accepted that TS inhibition causes cytotoxicity

via deprivation of deoxythymidine monophosphate (dTMP) needed for DNA synthesis and the associated "thymineless death"[8] and also via elevation of deoxyuridine monophosphate (dUMP) and its misincorporation into DNA.[9] Clearance of pemetrexed through the kidneys is rapid, resulting in a systemic half-life of only 3.5 hours, with up to 90% of the drug being eliminated in the urine within 24 hours.[10] The multitargeted action of pemetrexed, together with its prolonged cellular retention because of its relatively higher affinity for FPGS results in greater antitumor activity compared with older-generation antifolate drugs such as methotrexate.

The synthetic glucocorticoid dexamethasone (Dex) is used in standard practice as a concomitant medication during treatment with pemetrexed. Dex is typically dosed at 4 mg twice daily the day before, the day of, and the day after therapy with pemetrexed. An important role of Dex is to reduce the possibility of severe skin rash caused by pemetrexed. Dex is also included for its antiemetic properties particularly when pemetrexed is combined with cisplatin or carboplatin. The pharmacological actions of Dex are mediated by the glucocorticoid receptor (GR), which principally acts as a transcription factor.[11] Glucocorticoids regulate cell proliferation and apoptosis as well as inflammation and immune response.[12] The nature of the physiological response as well as sensitivity to synthetic glucocorticoids such as Dex is tissue-dependent and variable. This variability occurs among different individuals as well as among different tissues in the same individual.[13]

A single GR gene generates two major splice variants, GRα and GRβ; each isoform has variants that result from multiple translation start sites within their mRNAs.[13] Although GRβ only diverges from GRα by substitution of the carboxyl-terminal 50 amino acids in GRα by a nonhomologous 15 amino acid sequence, GRβ is unable to bind GR ligands.[14] GRβ heterodimerizes with GRα to exert a dominant negative effect on the transcriptional activity of GRα.[14] Therefore, the degree of Dex sensitivity of a tissue may be related to the functional GR status, determined by the level of GR expression as well as the ratio of GRα to GRβ. Whereas GRα is expressed ubiquitously, GRβ expression is potentially also significant in the present study of lung cancer because the limited types of tissues expressing this isoform include epithelial cells of the terminal bronchioles.[14]

Given the profound tissue-specific effects of Dex, its ability to regulate many genes, and its reported ability to modulate cellular senescence in lung cancer cells,[15] it was of interest to examine the possibility that Dex may act on NSCLC cells to influence their responsiveness to pemetrexed. Immunohistochemical analysis of clinical NSCLC tumors shows considerable heterogeneity in total GR expression with relatively high total GR observed in approximately half of the tumors.[16] Further, approximately 33% of lung adenocarcinomas harbor p53 mutations,[17] and the p53 gene is a known target for activation by Dex.[18] It was therefore of interest to explore both the GR status and the p53 status (wild-type (wt) p53, p53 deletions, or p53 mutations) of NSCLC cells as possible determinants of responsiveness to Dex, particularly molecular and cellular effects of Dex that could influence the action of pemetrexed.

MATERIALS AND METHODS

Cell Culture and Reagents

The nonsquamous NSCLC cell lines A549, H1299, H358, H226, H460, H1650, ADLC-5M2, and H292 were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco; Life Technologies, Carlsbad, CA), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The origin, growth conditions, and responsiveness to selected agents of the NSCLC cell lines used in this study have been previously described.[19] All cell lines had been authenticated by single-nucleotide polymorphism (SNP) profiling within less than 12 months of in vitro propagation. The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay reagents were from MP Biomedicals (Solon, OH). Charcoal-stripped FBS was from Life Technologies (Carlsbad, CA). Dexamethasone was purchased from EMD Millipore (Billerica, MA). Dimethyl sulfoxide and crystal violet were purchased from Fisher Scientific (Pittsburgh, PA). Propidium iodide/RNase solution was purchased from BD Biosciences (San Jose, CA). Polymerase chain reaction (PCR) primers and TaqMan probes were either purchased from the Life Technologies inventory or custom synthesized by Integrated DNA Technologies (Coralville, IA). The GRα-expressing lentivirus was from GenTarget Inc. (San Diego, CA). p53shRNA expressing lentivirus was a kind gift from Dr. Yubin Ge at Karmanos Cancer Institute. Blasticidin and puromycin were from Life Technogies and Sigma-Aldrich (St. Louis, MO). Anti-DHFR antibody (sc-14778) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-TS antibody (# 9045) was purchased from Cell Signaling Technology (Danvers, MA).

Measurement of Gene Expression

To measure gene expression, mRNA was quantified by real-time reverse transcription (RT) PCR. Total RNA from cells was isolated using RNeasy minikit (Qiagen, Georgetown, MD) according to the manufacturer's protocol. RT was performed using 500 ng of total RNA and High-Capacity cDNA Archive kit (Life Technologies) according to the vendor's protocol. cDNA was measured by quantitative real-time PCR using the StepOnePlus Real-Time PCR System (Life Technologies) and TaqMan Fast Universal PCR Master Mix (Life Technologies). All the mRNA measurements were carried out using biological triplicate samples and C_T values were normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The mRNA levels are represented as RQ (relative quantification), which is calculated using the formula $RQ = 2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T$ sample $- \Delta C_T$ calibrator ($\Delta C_T = C_T$ of gene of interest $- C_T$ of GAPDH).

Western Blot Analysis

Cell lysates were generated using radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% nonyl phenoxypolyethoxylethanol (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris of pH 8.0) containing protease inhibitor cocktail (Pierce Biotechnology, Thermo Fisher Scientific, Rockford, IL) and incubated on ice for 1 hour. Total protein concentration was determined by Bradford assay