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Pemetrexed alters folate phenotype and inflammatory profile in EA.hy 926 cells grown under low-folate conditions

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Abstract

Elevated homocysteine is a risk marker for several major human pathologies. Emerging evidence suggests that perturbations of folate/homocysteine metabolism can directly modify production of inflammatory mediators. Pemetrexed acts by inhibiting thymidylate synthetase (TYMS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT). EA.hy 926 cells grown under low (“Lo”) and high (“Hi”) folate conditions were treated with pemetrexed. The concentrations of several intracellular folate derivatives were measured using LC-MRM/MS. Lo cells had lower total folate concentrations and a different distribution of the intracellular folate derivatives than Hi cells. Treatment with pemetrexed caused a decrease in individual folate analytes. Microarray analysis showed that several genes were significantly up or down-regulated in pemetrexed treated Lo cells. Several of the significantly up-regulated transcripts were inflammatory. Changes in transcript levels of selected targets, including C3, IL-8, and DHFR, were confirmed by quantitative RT-PCR. C3 and IL-8 transcript levels were increased in pemetrexed-treated Lo cells relative to Lo controls; DHFR transcript levels were decreased. In Lo cells, IL-8 and C3 protein concentrations were increased following pemetrexed treatment. Pemetrexed drug treatment was shown in this study to have effects that lead to an increase in pro-inflammatory mediators in Lo cells. No such changes were observed in Hi cells, suggesting that pemetrexed could not modify the inflammatory profile in the context of cellular folate sufficiency.

Keywords

Pemetrexed; Folate; Inflammation; EA.hy 926 cells

1. Introduction

Elevated homocysteine, which is usually associated with low folate levels, is a risk marker for several major human pathologies, including cardiovascular disease, stroke (Refsum et al., 1998) neural tube defects in offspring (van der Put et al., 2001), and certain cancers

(Ueland et al., 2001). These conditions originate, at least in part, in the disruption of functions underpinned by folate; such disruption can be caused by low intake of B vitamins, including folate itself, and/or mildly dysfunctional genetic polymorphisms of key enzymes in the folate metabolic pathway.

The folate metabolic pathway is made up of two sub-pathways (Fig. 1): one facilitates the methylation of substrates such as DNA and proteins and the other supports the generation of thymidylate and purines.

The processes of purine and pyrimidine synthesis, methylation, and glutathione production are necessary for cell proliferation and the maintenance of a stable cellular phenotype, and as such are especially important for rapidly dividing cancer cells. Thus the folate metabolic pathway is an attractive target for the development of chemotherapeutic agents.

Methotrexate and 5-fluorouracil, folate anti-metabolites that have been used for decades to treat cancer, work by inhibiting one primary target in the folate metabolic pathway, dihydrofolate reductase (DHFR) and thymidylate synthetase (TYMS) respectively. A new generation of multi-targeted antifolates is designed to have more global effects; the exemplar for this class of drugs is pemetrexed, which acts by inhibiting TYMS, DHFR, and glycinamide ribonucleotide formyltransferase (GARFT) (Shih et al., 1997).

Inflammation is a feature of several of the pathologies that are associated with folate/homocysteine dysregulation, although the relationship between folate/homocysteine metabolism and the initiation/maintenance of inflammatory processes has not been fully defined. Emerging evidence suggests that perturbations of folate/homocysteine metabolism can directly modify production of inflammatory mediators. In human aortic endothelial cells (Poddar et al., 2001) and human monocytes (Zeng et al., 2003) treatment with homocysteine has been shown to increase secretion of monocyte chemo-attractant protein-1 (MCP-1) and interleukin 8 (IL-8), a neutrophil chemo-attractant (Zeng et al., 2003). A cell culture model using the EA.hy 926 cell line has been used to investigate the mechanisms whereby chronic “low-folate stress” increased synthesis of MCP-1, a chemokine that recruits monocytes into the subendothelial cell layer of blood vessels. EA.hy 926 cells grown in low folate culture medium expressed MCP-1 mRNA and protein at higher concentrations than cells grown in high folate medium (Brown et al., 2006), probably through the action of p38 stress kinases (Lu et al., 2009). These *in vitro* observations are supported by a study of young healthy adults in whom serum MCP-1 levels were positively associated with circulating homocysteine concentrations and inversely associated with serum and red blood cell folate concentrations (Hammons et al., 2009).

Results from ongoing studies in our laboratory indicate that methotrexate increases synthesis of a range of inflammatory mediators, including IL-8 and complement component 3 (C3), in EA.hy 926 cells in the context of low folate conditions (unpublished). The aim of the current study was to assess the impact of pemetrexed treatment of EA.hy 926 cells on folate phenotype and inflammatory protein expression in the context of low and high folate culture conditions.

2. Materials and methods

2.1. Culture

EA.hy 926 cells (Edgell et al., 1983) are a fusion product between human umbilical vascular endothelial cells and the epithelial cell line A549 derived from a human lung carcinoma. They have an endothelial-like morphology and produce a number of proteins characteristic of endothelial cells. They were adapted to growth under low folate conditions in Medium 199 (Gibco, Invitrogen, Carlsbad, CA), which contains only 10 ng/L (23 nM) of folic acid (FA), supplemented with 10% FCS, non-essential amino acids, gentamycin, penicillin G, and fungizone to generate “Lo” cells. Parallel cultures of EA.hy 926 cells were grown under standard folate concentrations for that cell line, in Medium 199 containing 4 mg/L (9 μ M) FA and supplemented as above to generate “Hi” cells (Brown et al., 2006). The pemetrexed-treatment experiments reported here were performed in parallel with experiments under similar conditions using methotrexate, with shared control data.

2.2. Cell viability assays

Lo and Hi cells, grown to confluence in 6-well plates, were maintained for 24 h in fresh medium prior to the addition of 0, 0.05, 0.1, 0.25, 0.5, 1.0, 2, 5, and 10 μ M pemetrexed (Alimta, gift from Eli Lilly and Co, Indianapolis, IN) in duplicate cultures. After a further 48 h the numbers of live cells remaining were determined with an electronic cell counter (Scepter, Millipore, Bedford, MA).

2.3. Alamar Blue assays

Fresh medium was added to confluent Lo and Hi cell cultures grown in 96-well plates, and treated 24 h later with 0, 0.05, 0.1, 0.25, 0.5, 1.0, 2, 5, and 10 μ M pemetrexed. After 48 h metabolic activity was measured by Alamar Blue assay according to the manufacturer's directions (Trek Diagnostic Systems, West Lake, OH).

2.4. Biochemical phenotyping

Confluent Lo and Hi cells were maintained for 24 h in fresh medium prior to treatment with 0.5 μ M pemetrexed for 48 h. Intracellular folate derivatives, i.e. 5-methyltetrahydrofolate (5-MTHF), tetrahydrofolate (THF), 5,10-methenyltetrahydrofolate (5,10-MTHF), and unmetabolized FA, were measured by stable isotope dilution liquid chromatography, multiple reaction monitoring, mass spectrometry (LC-MRM/MS) as described previously (Huang et al., 2008).

2.5. Affymetrix microarrays

RNA isolated from triplicate cultures of cells using RNeasy kits (Qiagen Inc., Valencia, CA) was reverse transcribed using the Affymetrix WT Expression kit (Ambion, Austin, TX). An Agilent Bioanalyzer and RNA6000 Nano LabChips (Agilent, Palo Alto, CA) were used to assess the purity and size distribution of the products, and quantitation was performed using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) at the University of Pennsylvania Microarray Core. The resulting cDNAs were hybridized to Affymetrix Human Gene 1.0 ST microarrays. Robust multi-array analysis (RMA) filtered results were imported

into Partek Genomics Suite version 6.5 (Partek Inc., St Louis, MO) for analysis. A fold change of >2 and false discovery rate of 5%, corresponding to a P -value of <0.05 , were chosen as the cutoffs. The microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO, <http://www.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE31986.

2.6. Quantitative real time PCR (qRT-PCR)

RNeasy kits (Qiagen Inc.) were used to prepare RNA from Lo and Hi cells. Reverse transcription was carried out with MMLV reverse transcriptase (Promega, Madison, WI) as described previously (Brown et al., 2006). qRT-PCR was carried out in 20 μ l reactions containing 1 μ l cDNA, 1 μ l Taqman Gene Expression Assay (Applied Biosystems, Foster City, CA) in Taqman Universal master mix (Applied Biosystems). The Applied Biosystems assay ID numbers were Hs01100879_m1 for C3, Hs00174103_m1 for IL-8 (CXCL8), and Hs00758822_s1 for DHFR. GAPDH and MCP-1 (CCL2) qRT-PCR assays were previously described (Brown et al., 2006).

2.7. ELISA

Confluent Lo and Hi cells were maintained for 24 h in fresh medium prior to 48 h of treatment with 0.5 μ M pemetrexed, and media were collected. Concentrations of inflammatory mediators of interest were measured in biological triplicates using MCP-1 (PeproTech, Inc, Rocky Hill, NJ), IL-8 (BD Biosciences, San Diego, CA) and C3 (Innovative Research, Novi, MI) ELISA kits, with adjustment for protein content of the cell fraction corresponding to each sample. The specificity of these assays has been established using Western blotting and/or multi-targeted ELISA analysis by the manufacturers. Each sample was assayed in duplicate.

2.8. Statistical methods

The two-tailed Student's t -test method was used to compare differences between means using SAS version 9.2 (SAS Institute, Cary, NC). Results with a P value <0.05 were considered statistically significant.

3. Results

3.1. Determination of pemetrexed concentration

In order to find a concentration of pemetrexed that was clinically relevant without causing excessive cell death or greatly decreasing metabolic activity, we tested a range of concentrations guided by a report that suggested a clinically achievable range of pemetrexed concentrations from 0 to 10 μ M (O'Kane et al., 2010). We selected a range of concentrations (0, 0.05, 0.1, 0.25, 0.5, 1.0, 2, 5, and 10 μ M) to explore using live cell counting and Alamar Blue experiments.

3.2. The effect of pemetrexed on EA.hy 926 cell survival

Direct counting of live cells using an electronic cell counter indicated that survival of pemetrexed-treated Lo cells decreased relative to control Lo cells following treatment with increasing concentrations of pemetrexed. At concentrations of pemetrexed ranging from

0.05 to 0.5 μ M, survival of pemetrexed-treated Lo cells was above 60% that of control Lo cells. Survival of pemetrexed-treated Hi cells was not significantly affected by any concentration of pemetrexed up to 10 μ M relative to untreated Hi cells (data not shown).

3.3. The effect of pemetrexed on EA.hy 926 cell metabolic activity

Confluent Lo and Hi cell cultures were maintained for 24 h in fresh medium and then treated with 0.5 μ M pemetrexed for 48 h. Alamar Blue assays were performed with all test concentrations of pemetrexed. Lo cells treated with concentrations of pemetrexed from 0.05 to 5 μ M retained Alamar Blue readouts above 90% of Lo controls. In Hi cells, results indicated that treatment with pemetrexed at any concentration did not affect cell metabolic activity (data not shown).

Based on the results from live cell counting and the Alamar Blue assays, a concentration of 0.5 μ M pemetrexed was selected for this study as it is the highest concentration at which metabolic activity is retained and live cell numbers of pemetrexed-treated Lo cells are above 60% of control cells.

3.4. Modulation of folate phenotype in EA.hy 926 cells by nutrient deprivation and pemetrexed

Total folate was significantly lower in control Lo cells than in control Hi cells (98.1 and 2011.1 ng per mg protein, respectively, $P=0.002$) (Fig. 2). This low folate stress is accompanied by a difference in the distribution of key folate derivatives. 5-MTHF was the most abundant form of folate found in Hi cells, constituting almost 43% of the total, and THF was the least abundant, at approximately 23% of the total. In Lo cells, the proportion of 5-MTHF is dramatically lower, making up just over 17% of the total amount of folate; the percentage of THF is also lower, at 8.6%, the net result being that 5,10-MTHF was the most abundant folate derivative at 74.3% of the total.

Pemetrexed treatment caused a quantitatively large decrease in the concentration of total folate in Lo cells, from 98.1 to 56.9 ng per mg protein. This is reflected by a decrease in each measured analyte, i.e. 5-MTHF (from 16.8 to 4.3 ng per mg protein), THF (from 8.4 to 0.58 ng per mg protein), and 5,10-MTHF (from 72.9 to 51.9 ng per mg protein). These quantitatively large changes did not reach statistical significance for any of the variables in Lo cells. In Hi cells, pemetrexed treatment caused a statistically significant but proportionally more modest decrease in total folate (from 2099.1 to 1506.3 ng per mg protein, $P=0.02$). This was reflected in the individual analytes THF (from 506.3 to 340.7 ng per mg protein, $P=0.04$), 5,10-MTHF (from 690.9 to 489.8 ng per mg protein, $P=0.05$), and 5-MTHF (from 901.8 to 675.8 ng per mg protein, $P=0.08$).

Both Lo and Hi cells treated with pemetrexed demonstrated an increase in unmetabolized FA, increasing from 12.4 to 17.8 ng per mg protein in Lo cells ($P=0.17$) and from 32.9 to 43.3 ng per mg protein in Hi cells ($P=0.0007$). These observations suggest that the cells were able to take up FA in the presence of pemetrexed but that the drug was effective at inhibiting DHFR-mediated conversion of FA to DHF and THF, thereby limiting the amount of FA that enters into the pool of natural folates.

3.5. Pemetrexed-attributable changes in gene expression

In control Lo cells C3 mRNA was 15-fold higher and IL-8 mRNA was more than 6-fold higher than in control Hi cells ($P=0.03$ and 0.01 , respectively). MCP-1 mRNA concentration was not significantly different between Lo control and Hi control samples.

Affymetrix Human Gene 1.0 ST microarrays were used to assess changes in gene expression due to pemetrexed treatment in Lo and Hi cells. A number of gene transcripts were significantly up- or down-regulated after pemetrexed treatment in Lo cells. These include several down-regulated transcripts relevant to the cell cycle and various up-regulated inflammatory transcripts, including tumor necrosis factor alpha-induced protein 3, interleukin 1 receptor-like 1, IL7R, TNF receptor-associated factor 1, IL1A, killer cell lectin-like receptor subfamily C member 1, tumor necrosis factor receptor superfamily member 9, interferon, gamma-inducible protein 30, IL11, IL13RA2, IL32, and IL33. This was not seen to be the case in Hi cells (data not shown).

Genes of particular interest include C3, IL-8 and genes important in folate metabolism. In the current study, C3 transcripts were significantly up-regulated in pemetrexed-treated Lo cells relative to control Lo cells by 3.6-fold ($P=0.0008$, step-up $P=0.02$); C3 was ranked within the top ten up-regulated genes. IL-8 transcripts were up-regulated in pemetrexed-treated Lo cells by 2.2-fold ($P=0.04$, step-up $P=0.2$). DHFR transcripts were down-regulated in pemetrexed-treated Lo cells relative to control by 2.3-fold ($P=0.0001$, step-up $P=0.002$).

The increased levels of IL-8 and C3 transcripts in pemetrexed-treated Lo cells relative to Lo control cells were confirmed by qRT-PCR (Fig. 3). In pemetrexed-treated Lo cells, C3 mRNA levels were up-regulated 4.3-fold relative to Lo controls ($P=0.0008$). IL-8 mRNA levels were up-regulated 2.1-fold in pemetrexed treated Lo cells relative to control Lo cells ($P=0.003$). MCP-1 mRNA was lower in pemetrexed-treated Lo cells ($P=0.003$). DHFR mRNA was down-regulated by almost 3-fold in pemetrexed-treated Lo cells ($P=0.001$). In Hi cells, pemetrexed drug treatment had no measurable effect on C3, IL-8, MCP-1 or DHFR mRNA levels.

3.6. Pemetrexed attributable changes in C3, IL-8 and MCP-1 protein concentrations

MCP-1 levels were significantly higher in control Lo cells than control Hi cells (19.7 vs. 11.4 ng per mg protein, $P=0.03$), which is consistent with previous published findings (Fig. 4). IL-8 protein concentration was significantly higher in control Lo cells than in control Hi cells (28.4 vs. 3.9 ng per mg protein, $P=0.0009$). C3 protein was not detected in either control or pemetrexed-treated Hi cells.

MCP-1 concentration in pemetrexed-treated Lo cells (20.4 ng per mg protein) was not significantly different from that of control Lo cells. Treatment with pemetrexed increased the concentration of IL-8 from 28.4 ng per mg protein in control to 42.9 ng per mg protein ($P=0.01$). In Lo control cells, the C3 level was 18.5 ng per mg protein and in pemetrexed-treated Lo cells it increased to 26.0 ng per mg protein ($P=0.04$).

Pemetrexed-treated Hi cells had an IL-8 concentration of 3.4 ng per mg protein, similar to the concentration in untreated cells. Likewise, the concentration of MCP-1 in pemetrexed-

treated Hi cells (10.2 ng per mg protein) was not significantly different from that of control Hi cells, suggesting that treatment with pemetrexed cannot readily modify IL-8 and MCP-1 protein levels in folate-replete Hi cells.

4. Discussion

Emerging evidence suggests that perturbations of folate/homocysteine metabolism can directly modify production of inflammatory mediators. EA.hy 926 cells grown in low folate culture medium expressed MCP-1 mRNA and protein at higher concentrations than cells grown in medium with high folate levels (Brown et al., 2006). Results from ongoing studies in our laboratory indicate that methotrexate increases synthesis of a range of inflammatory mediators, including IL-8 and C3, in EA.hy 926 cells (unpublished). This study was designed to examine the effects of pemetrexed treatment of EA.hy 926 cells on folate phenotype and inflammatory protein expression in the context of low and high folate growth conditions.

EA.hy 926 cells grown under low folate conditions had significantly lower levels of total folate than cells grown under normal folate conditions and a different distribution of the individual folate analytes. In Hi cells, 5-MTHF was the primary form of folate and THF was the least abundant, while in Lo cells 5-MTHF was the least abundant folate derivative and 5,10-MTHF was the most abundant.

Dysregulation of folate/homocysteine phenotype caused by treatment with antifolate drugs could compromise cellular functions and elicit pathogenic phenotypes. Pemetrexed, an antifolate drug used in the treatment of lung cancer, inhibits TYMS, DHFR and GARFT. Treatment with 0.5 μ M pemetrexed caused a reduction in total folate concentrations in both Lo and Hi cells, which is reflected in the individual analytes. In pemetrexed treated Lo cells, there were trends toward a decrease in the mean concentrations of all three folate analytes, although this did not reach statistical significance. Pemetrexed treatment caused a statistically significant decrease in total folate in Hi cells, with decreases in all of the folate analytes. Folic acid levels were significantly higher in both Lo and Hi cells treated with pemetrexed than in their respective controls, indicating that FA was taken up by the cells but remained unmetabolized due to inhibition of DHFR. This is most likely attributable to direct inhibition of the enzyme, though we have no data to confirm this. In the case of Lo cells, there may be an additional contribution via a decrease in DHFR transcript levels identified by Affymetrix microarrays and qRT-PCR.

The effect of pemetrexed on EA.hy 926 cells was examined using an Affymetrix microarray platform. The changes in transcript profile observed in pemetrexed-treated Lo cells could be attributable to alterations in the methylation status of select classes of genes, including some inflammatory genes. However, further studies will be needed to determine if this is the case. An ongoing body of work in our laboratory suggests that several genes are differentially regulated after treatment with different antifolate drugs. These studies suggest that inflammatory proteins, particularly C3 and IL-8, increase in EA.hy cells with antifolate drug treatment. Both IL-8 and C3 transcripts were up-regulated in pemetrexed-treated Lo cells according to the gene expression data, and warranted further investigation. qRT-PCR

confirmed that IL-8 and C3 mRNA were up-regulated and ELISA assays showed that IL-8 and C3 protein secretion were increased in pemetrexed treated Lo cells. In Hi cells, there was no such increase in the expression or secretion of IL-8 or C3 following pemetrexed treatment. Untreated Lo cells had higher levels of both of these proteins compared to untreated Hi cells.

The implication of these data is that cells under folate stress have different inflammatory potential than folate-replete cells. The altered potential is more pronounced when such cells are further stressed with antifolate drug treatment. Results are, at least in part, consistent with modification of the NF κ B pathway.

Antifolate drug treatment, which may be expected to have anti-inflammatory properties, was shown in this study to have effects that lead to increases in some pro-inflammatory mediators. The *in vitro* observations suggest that pemetrexed treatment in patients may have consequences for endothelial function such that there is modification of inflammation-related function. It is also possible that such modifications may occur in other tissues and may have a bearing on treatment efficacy as well as comorbidities. Results with folate replete cells suggest that depression in total folate caused by pemetrexed is insufficient to bring about a change in the relative proportions of folate analytes. This in turn suggests that the folate metabolic pathway is left intact, thereby precluding folate-attributable modification of inflammatory potential. Thus, folate replete cells, in contrast to folate stressed cells, are resistant to off target consequences of pemetrexed treatment, which might explain, at least in part, why patients who receive folate and B₁₂ prior to treatment with pemetrexed have fewer off-target (i.e. toxic) consequences of pemetrexed therapy (Vogelzang et al., 2003).

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Fig. 2. LC-MRM/MS measurement of folate derivatives. Lo and Hi cells were exposed to 0.5 μ M pemetrexed for 48 h. Results represent the mean $\pm$ S.D. of biological triplicates. (A) Hi cells, (B) Lo cells. **P* values <0.05 compared with control. PMX, pemetrexed.

Fig. 3.

Assessment of DHFR, C3, MCP-1 and IL-8 transcripts in Lo and Hi cells treated with pemetrexed by qRT-PCR. Lo and Hi cells were exposed to 0.5 μ M pemetrexed for 48 h. Expression of *GAPDH* was used for normalization. Results represent the mean $\pm$ S.D. of biological triplicates. (A) DHFR, (B) C3, (C) MCP-1, (D) IL-8. **P* values <0.05 compared with respective control. #*P* values <0.05 for Hi control compared to Lo control. PMX, pemetrexed.

Fig. 4.

Assessment of C3, MCP-1 and IL-8 protein in media of Lo and Hi cells treated with pemetrexed by ELISA. Lo and Hi cells were exposed to 0.5 μ M pemetrexed for 48 h. Secreted proteins were measured in media. Results represent the mean $\pm$ S.D. of biological triplicates. (A) C3, (B) IL-8, (C) MCP-1. **P* values <0.05 compared with respective control. #*P* values <0.05 for Hi control compared to Lo control. PMX, pemetrexed.