European Journal of Pharmacology 732 (2014) 60–67

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Immunopharmacology and inflammation

Methotrexate modulates folate phenotype and inflammatory profile in EA.hy 926 cells ☆

Carolyn M. Summers, Andrea L. Hammons, Jasbir Arora, Suhong Zhang, Jeanine Jochems, Ian A. Blair, Alexander S. Whitehead *

Centers for Cancer Pharmacology, Pharmacogenetics, and Excellence in Environmental Toxicology, Department of Pharmacology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, United States

ARTICLE INFO

Article history:
Received 4 December 2013
Received in revised form
21 February 2014
Accepted 4 March 2014
Available online 18 March 2014

Keywords:
Inflammation
Folate
Methotrexate
Immune modulation
Gene expression

ABSTRACT

EA.hy 926 cells grown under low folate conditions adopt a "pro-atherosclerotic" morphology and biochemical phenotype. Pharmacologically relevant doses of the antifolate drug methotrexate (MTX) were applied to EA.hy 926 cells maintained in normal (Hi) and low (Lo) folate culture media. Under both folate conditions, MTX caused inhibition of cell proliferation without significantly compromising metabolic activity. MTX treated Hi cells were depleted of folate derivatives, which were present in altered proportions relative to untreated cells. Transcript profiling using microarrays indicated that MTX treatment modified the transciptome in similar ways for both Hi and Lo cells. Many inflammation-related genes, most prominently those encoding C3 and IL-8, were up-regulated, whereas many genes involved in cell division were down-regulated. The results for C3 and IL-8 were confirmed by quantitative RT-PCR and ELISA. MTX appears to modify the inflammatory potential of EA.hy 926 cells such that its therapeutic properties may, at least under some conditions, be accompanied by the induction of a subset of gene products that promote and/or maintain comorbid pathologies.

1. Introduction

Hyperhomocysteinemia, in which circulating concentrations of the intermediate amino acid homocysteine (Hcy) are elevated, has been associated with a wide range of human pathologies including atherothrombotic diseases (Refsum et al., 1998), Alzheimer's disease (Mattson and Shea, 2003), some cancers (Weinstein et al., 2001), and the birth defect spina bifida (Mitchell et al., 2004). It is generally underpinned by low folate status (Jacques et al., 1996; Harmon et al., 1996) and the relative concentrations of intracellular folate derivatives may be altered (Mitchell et al., 2009). The folate/Hcy metabolic pathway is the means whereby one carbon units are channeled into important biological processes including methylation, glutathione production, and nucleic acid synthesis (Lucock, 2006) (Fig. 1). The key folate derivative, 5-methyltetrahydrofolate (5-MTHF), provides the methyl group for the remethylation of Hcy to methionine. The latter is subsequently converted to S-adenosylmethionine (SAM), the methyl donor for many methyltransferase reactions on substrates such as DNA, proteins, and lipids. The loss of the methyl group from 5-MTHF generates tetrahydrofolate (THF) which is in turn converted to 5,10-methyleneTHF. This derivative can be reduced by 5,10-methylenetetrahydrofolate reductase (MTHFR) to regenerate 5-MTHF or used to initiate a series of reactions to generate thymidylate and purines. Historically, elevated Hcy was considered to be the pathogenic component in the conditions with which hyperhomocysteinemia has been associated because of its direct toxic effects on redox thiol status and ER stress response (Koch et al., 1998). However, alternative causative mechanisms implicating low folate concentrations and their negative impact on processes such as nucleic acid synthesis and methylation have been suggested (Lucock, 2000).

Many of the above pathologic conditions have inflammatory aspects and involve damage to, or dysfunction of, the vasculature and its constituent cell types, in particular endothelial cells. Inappropriate or sustained activation of immunologically active endothelial cell products might contribute to ongoing pathology at the local and possibly also systemic level. In recent studies EA.hy 926 cells, which are derived from the fusion of primary endothelial

Abbreviations: 5-MTHF, 5-methyltetrahydrofolate; 5,10-MTHF, 5,10-methenyltetrahydrofolate; CVD, cardiovascular disease; FA, folic acid; Hcy, homocysteine; MTX, methotrexate; THF, tetrahydrofolate

☆This publication was made possible by support from National Institutes of Health Grant nos. AR47663-06 and ES013508-08; and Pennsylvania Department of Health Grant no. 4100038714. Its content does not necessarily represent the official views of the funding agencies.

* Correspondence to: Alexander S. Whitehead, Department of Pharmacology, Perelman School of Medicine, University of Pennsylvania, 1311 BRB II/III, 421 Curie Boulevard, PA 19104-6160, Philadelphia, Pennsylvania, United States. Tel.: +1 215 898 2332; fax: +1 215 573 9889.
E-mail address: aswhiteh@exchange.upenn.edu (A.S. Whitehead).

http://dx.doi.org/10.1016/j.ejphar.2014.03.004
0014-2999/

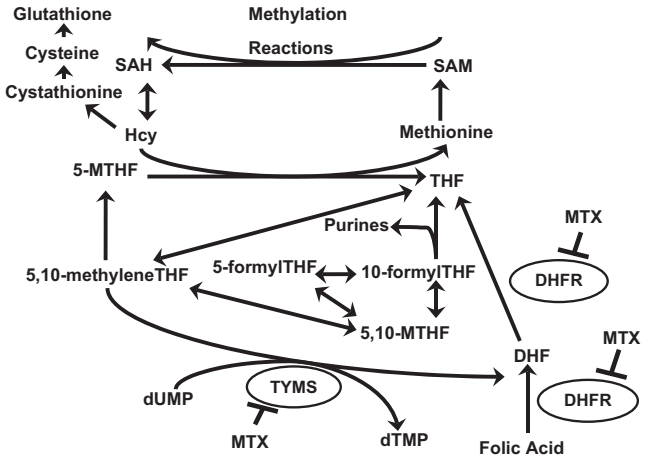

Fig. 1. Folate/homocysteine pathway. 5-MTHF, 5-methyltetrahydrofolate; 5,10-MTHF, 5,10-methenyltetrahydrofolate; DHF, dihydrofolate; DHFR, dihydrofolate reductase; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; Hcy, homocysteine; MTX, methotrexate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate; TYMS, thymidylate synthase.

cells and an epithelial tumor cell line but have an endothelial-like morphology and synthetic profile, were grown under low folate conditions and adopted a "pro-atherosclerotic" phenotype compared to cells grown under high folate conditions, without displaying any significant differences in intracellular or extracellular Hcy levels (Brown et al., 2006). This phenotype was characterized by elongated cell morphology with prominent networks of stress fibers and impaired barrier function. A significantly increased synthesis and export of monocyte chemoattractant protein 1 (MCP-1) was observed in the cells grown under low folate conditions (Brown et al., 2006). MCP-1, a potent chemokine that is synthesized by vascular smooth muscle cells in response to oxidized lipid, facilitates the transmigration of monocytes from the circulation across the endothelium and is a key contributor to the early stages of atheroma formation (Schwartz et al., 1991). In cultured human monocytes Hcy has been shown to induce the secretion of IL-8, a neutrophil chemoattractant, as well as MCP-1 (Zeng et al., 2003). The above in vitro observations have been corroborated in vivo in a study of young healthy adults in whom serum MCP-1 levels were inversely associated with serum and red blood cell folate concentrations, and positively associated with circulating Hcy concentrations (Hammons et al., 2009). Taken together, these findings have reinforced speculation that "folate stress," which is indicative of poor nutritional status, might augment aspects of baseline inflammatory preparedness to facilitate more vigorous initial responses to infectious challenges in individuals weakened by malnutrition (Lu et al., 2009). Such an adaptive response may have evolved if the consequent survival advantage offsets the negative effects of enhanced sub-clinical inflammatory processes.

The central role of folate in nucleotide synthesis has been exploited pharmacologically via the development of potent antifolate drugs for the treatment of neoplastic and auto-immune conditions. One of the most widely used antifolate drugs is methotrexate (MTX), which inhibits the key enzymes dihydrofolate reductase (DHFR), thymidylate synthase (TYMS), glycinamide ribonucleotide transformylase (GART), and aminoimidazolecarboxamide ribonucleotide transformylase (AICART) (Kremer, 2004) (Fig. 1). High dose MTX is a component of diverse therapeutic regimens for several cancers including acute lymphoblastic leukemia (Jonsson and Kamen, 1991), while lower doses are used to treat inflammatory diseases such as rheumatoid arthritis (RA) (Williams et al., 1985). In the latter condition, MTX tends to be well tolerated with relatively minor side effects and there is clear therapeutic benefit in reducing the inflammatory aspects of the disease that contribute to joint damage (Coury and Weinblatt, 2010). However, RA patients have significant cardiovascular comorbidity (Nurmohamed, 2009) and there is controversy as to whether MTX exacerbates or ameliorates this serious source of mortality. An early study on the use of MTX in the treatment of rheumatoid arthritis patients with existing CVD indicated that mortality was increased (Landewe et al., 2000). Conversely, several more recent studies have suggested that MTX use is associated with a decrease in the incidence of CVD events and mortality (Choi et al., 2002; van Halm et al., 2006; Naranjo et al., 2008), although it remains unclear whether such a decrease would reflect a full or only partial amelioration of inflammation-attributable CVD.

The possibility that low folate status, due to nutritional variables or the use of antifolate drugs, contributes to human disease by inducing a subset of potentially pathogenic inflammation-associated molecules, including MCP-1, is of considerable public health interest. The characterization of changes to the inflammatory profile that might be induced by drugs such as MTX would serve as the foundation for future studies to define the precise relationship between dysregulation of folate metabolism and inflammation. This study was designed to investigate the effect of pharmacologically relevant doses of MTX on the absolute and relative concentrations of key folate derivatives and gene expression in the Ea.hy 926 cell line. The potential implications of observations concerning the up-regulation of key inflammatory proteins are discussed.

2. Materials and methods

2.1. Cell culture

EA.hy 926 cells (Edgell et al., 1983) are a fusion product between human umbilical vascular endothelial cells (HUVECs) and the epithelial cell line A549 derived from a human lung carcinoma. EA.hy 926 cells were adapted to growth under low folate conditions (Lo cells) in Medium 199 (Gibco, Invitrogen, Carlsbad, CA), which contains 23 nM of folic acid, supplemented with 10% FCS, non-essential amino acids, gentamycin, penicillin G, and fungizone. Parallel cultures of EA.hy 926 cells were grown under standard folate concentrations for that cell line (Hi cells), in Medium 199 with folic acid increased to 9 μM and supplemented as above (Brown et al., 2006).

2.2. BrdU cell proliferation assays

Hi and Lo cells were seeded into 96-well plates in their respective media at a density that would yield 50% confluence after overnight incubation. Triplicate cultures were then maintained in fresh media containing 0, 0.1, 0.25, or 0.5 μM MTX (Sigma-Aldrich, St. Louis, MO) for 24 and 48 h, after which media were removed and adherent cells were fixed and stained using the Cell Proliferation ELISA, BrdU Colorimetric kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Colorimetric analyses were performed with an ELISA plate reader (Dynex Technologies, Chantilly, VA).

2.3. Cell viability assays

Hi and Lo cells, grown to confluence in 6-well plates, were maintained for 24 h in fresh medium prior to the addition of 0, 0.1, 0.25, or 0.5 μM MTX. After a further 48 h the numbers of live cells remaining were determined in duplicate with an electronic cell counter (Scepter, Millipore, Bedford, MA). The numbers of live and dead cells in each treatment group were also determined by