Possible Roles for Folic Acid in the Regulation of Trophoblast Invasion and Placental Development in Normal Early Human Pregnancy[1]

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ABSTRACT

In addition to its role in the prevention of neural tube defects, folic acid has many other physiological functions, including cell proliferation, DNA replication, and antioxidant protection. The aim of this study was to determine the role that folic acid has in regulating placental trophoblast development. Placental explants from placentae at gestational age 7 wk (n = 3) were cultured in folic acid at concentrations of 10^{-6} M, 10^{-8} M, and 10^{-10} M. Extravillous trophoblast (EVT) invasion was assessed following 6-day culture, and explants were used for immunohistochemical evaluation of proliferation (MKI67) and apoptosis (active caspase 3). In addition, an array was performed on cell culture supernatants to examine a range of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs). Folic acid increased the invasion of EVT cells in this explant model by between 83% and 19% ($P = 0.005$), and this was associated with increased MKI67 positivity and decreased active caspase 3 positivity; this effect was concentration dependent and showed a biphasic response. In addition, culture in folic acid increased vascular density, as determined by anti-CD31 immunostaining ($P = 0.05$). The increase in EVT invasion correlated with increased placental explant secretion of MMP2 ($P = 0.01$), MMP3 ($P = 0.01$), and MMP9 ($P = 0.02$). This study demonstrates that folic acid is potentially important in a number of crucial early stages of placental development, including EVT invasion, angiogenesis, and secretion of MMPs, and highlights the need for further studies to address the benefit of longer-term folic acid supplementation throughout pregnancy to prevent pregnancy disorders associated with deficient placental development, including preeclampsia.

folic acid, placenta, pregnancy, trophoblast

INTRODUCTION

Folic acid, the synthetic form of folate, is involved in a number of important functions within the body, including synthesis of proteins required for DNA replication and as an essential substrate for a range of enzymatic reactions needed for amino acid synthesis and vitamin metabolism; as such, folic acid is essential for cell multiplication and differentiation processes [1, 2]. With regard to pregnancy, the importance of folic acid in the prevention of neural tube defects has long been known [3]. However, folic acid may also have important roles in other physiological pathways needed for successful pregnancy, including angiogenesis and vasculogenesis [4], methylation of the harmful, sulphur-containing homocysteine [5], antioxidant protection [6], and endothelial-dependent vascular relaxation [7]. These processes are essential for the establishment of fetoplacental circulation, enabling successful pregnancy outcome.

Placentation requires the invasion of fetally derived extravillous trophoblast (EVT) cells into maternal uterine spiral arteries, converting these arteries from highly resistant low-capacity vessels into a high-capacity system capable of transporting large volumes of blood to the intervillous space [8]. This process of EVT invasion requires the degradation and remodeling of the extracellular matrix, which is performed mainly by matrix metalloproteinases (MMPs) [9], whose function and substrate specificity is regulated by their specific inhibitors, tissue inhibitors of MMPs (TIMPs) [10]. Between 8 and 12 wk of pregnancy, trophoblast plugs that have accumulated in the spiral arteries are released, allowing the complete perfusion of the syncytiotrophoblast with maternal blood and the establishment of hemotrophic nutrition [11, 12]. The sudden perfusion of placental villi at the onset of maternal-placental circulation has been associated with the generation of oxidative stress, although the placenta is protected from undue harm by a number of antioxidant enzymes [13, 14]. Folic acid also has antioxidant activity and is directly able to scavenge free radicals and also increase the bioavailability of nitric oxide [15, 16].

In parallel to gaining access to the maternal circulation, the placenta must also develop its own circulation. Endothelial cells differentiate from mesenchymal cells, forming the first capillaries of the fetal placental vasculature. This coincides with maternal vascular remodeling and the onset of maternal blood flow into the placental intervillous space [17]. Development of the placental circulation requires both vasculogenesis, the development of new blood vessels from primitive precursor cells, and angiogenesis, which is the development of new vessels from a preexisting vasculature [18]. Placental angiogenesis is critical for development of a normal placental circulation and, consequently, normal development of the baby [19]. Folic acid is able to induce angiogenesis, in part via a nitric oxide-dependent mechanism [4].

Maternal folic acid deficiency has been associated with miscarriage [20], placental abruption, and intrauterine growth restriction [21]. Low folate levels have also been found in plasma samples from women with preeclampsia [22]. Increasing folic acid intake and prolonging the period of folic acid supplementation from the currently recommended first 12 wk of pregnancy to throughout the length of gestation may serve to

help prevent preeclampsia by allowing optimal placental development. A recent study has shown the beneficial effect of extending folic acid supplementation into the second trimester of pregnancy, with a reduced incidence of preeclampsia [23]. Longer-term folic acid supplementation during pregnancy may also help ameliorate the secondary systemic symptoms of preeclampsia because of its beneficial effects on endothelial function and antioxidant protection.

We hypothesized that folic acid would increase both invasion by EVT cells and angiogenesis in placental explants from early pregnancy.

MATERIALS AND METHODS

After local ethics committee approval and with appropriate informed consent, placental tissue was obtained from women undergoing elective surgical termination of pregnancy at 7 wk gestational age calculated from ultrasound measurement of crown-rump length. Placental tissue was identified by its branching, fronded appearance.

Invasion Assays

The effect of folic acid on the ability of EVTs from placental explants (7 wk gestational age) to invade through Matrigel growth factor-reduced basement membrane (BD, supplied by VWR, Lutterworth, U.K.) was assessed as described previously [24]. Briefly, chorionic villous tips were minced to approximately 0.5 mm^3 and resuspended in culture medium (Dulbecco modified Eagle medium (DMEM):F12 [Biosource, Camarillo, CA] containing 10% fetal bovine serum, penicillin/streptomycin, and amphotericin B [all from Sigma Aldrich, Dorset, U.K.]). Dissected placental tissue was resuspended in 500 μl of culture media such that 15 μl constituted approximately 10 mg of tissue. Using a pipette, 15 μl of placental tissue explant suspension was drawn up and carefully spread onto the surface of the Matrigel-coated cell culture insert. To assess the effect of folic acid on EVT invasion, exogenous folic acid (10^{-6} M, 10^{-8} M, or 10^{-10} M) was added to the culture media; these concentrations were chosen because they represent physiological ranges [25]. Each experiment was performed in duplicate and performed on three separate placentae. Assays were incubated in standard tissue culture conditions of 37°C, 5% CO_2 (20% O_2) in an air incubator. At the end of the 6-day incubation period, the Matrigel and filters were removed, and the upper side of the membrane was cleaned using a cotton wool bud. For assessment of invaded cells, the filters were stained with hematoxylin and eosin and mounted on glass microscope slides with aqueous mountant. Each slide was blinded, and the total number of cells that had invaded to the underside of the filter was counted by P.J.W. at 100× magnification for the entire area of the filter. To account for the considerable variation between different placental samples, mean cell counts for each invasion assay with explants from a single placenta were normalized with respect to the control values for that experiment.

To determine whether folic acid altered trophoblast cell viability and numbers (apoptosis and proliferation), at the end of the invasion assay, explants were removed and fixed in 10% neutral-buffered formalin for 24 h and processed into paraffin wax. Serial 3-μm sections were immunolabeled using a standard avidin-biotin peroxidase method (Vector Laboratories, Peterborough, U.K.) with rabbit anti-active caspase 3 (pretreatment citrate buffer, pH 6.0, with 10-min microwave heating, 1:400 dilution for 45 min; R&D Systems, Abingdon, U.K.), mouse anti-MKI67 (pretreatment pressure cooking for 1 min in citrate buffer, pH 6.0, 1:200 dilution for 30 min; Novocastra Laboratories, Newcastle-upon-Tyne, U.K.), and anti-CD31 (pretreatment pressure cooking for 1 min in citrate buffer, pH 6.0, 1:20 dilution for 60 min; Novocastra Laboratories). CD31 labeling was used as a marker of angiogenesis [26]. In addition, conditioned medium was collected from each chamber and stored at −80°C until required for analysis.

Protease Array

Concentrations of MMP1, MMP2, MMP3, MMP7, MMP8, MMP9, MMP10, MMP12, MMP13, TIMP1, TIMP2, and TIMP4 were assayed in conditioned media collected following 6-day explant culture, using the ExcelArray Human MMP/TIMP array kit per the manufacturer's instructions (Thermo Fisher Scientific, Loughborough, U.K.) and as described previously [27]. Multiplex standards were prepared per instructions in the ranges of 123 to 10 000 pg/ml (MMPs 1, 3, 7, 8, 9, 10, 12, and 13, and TIMP4) and 309 to 25 000 pg/ml (MMP2, TIMP1, and TIMP2). A total of 100 μl of cell culture supernatant was assayed. Samples and controls were assayed in triplicate. The microarray slide was imaged using an Axon GenePix 4200AL slide scanner (MDS Analytical Technologies, Wokingham, U.K.), and spot densitometry was performed using GenePix 2.0 software. The spot densitometry data generated was imported into Microsoft Excel.

Quantification of Immunohistochemistry

Quantification of MKI67, active caspase 3, and CD31 immunostaining was performed using the Positive Pixel Algorithm of Aperio ImageScope software (Oxford, U.K.). This software is able to discriminate between positively and negatively stained pixels, and combines the number of positive pixels stained with the intensity of staining of these same pixels to produce the value "positivity." Analysis of the entire placental explant was performed for all assays. For each folic acid concentration, slides were counted from the duplicate wells (i.e., a total of six slides were counted for each concentration for the three assays performed). Analysis was performed at 200× magnification by P.J.W., who was blinded to sample identity.

Statistical Analysis

All analyses were performed using SPSS for Windows version 16.0 (Chicago, IL). The Kolmogorov-Smirnov test was used to assess the distribution of data. All data were found to be normally distributed, and therefore between-group comparisons were made using one-way ANOVA; if significant, they were followed by the Bonferroni post hoc test. Concentration response effects for invasion assays were determined using Kendall τ B test. A value of $P = 0.05$ was considered significant for invasion assays and immunohistochemistry. Unless otherwise stated, all data are expressed as mean (SEM).

RESULTS

Folic Acid Increases EVT Invasion

Culture in folic acid produced a concentration-dependent increase in the mean invasion index of EVT (Fig. 1). When compared with control wells, incubation with folic acid at concentrations of 10^{-6} M increased invasion by 83% ($P < 0.0001$), at 10^{-8} M by 51% ($P < 0.0001$), and at 10^{-10} M by 19% ($P = 0.01$). Kendall τ B test showed this increased EVT invasion to be concentration dependent ($P = 0.005$; Fig. 1).

Trophoblast Cell Apoptosis and Proliferation Following Culture in Folic Acid

As shown in Figure 2a, placental explant culture in folic acid at 10^{-6} M resulted in increased active caspase 3 positivity compared with controls, with staining predominantly observed in villous cytotrophoblast and villous stromal cells ($P = 0.028$). In contrast, active caspase 3 positivity decreased when
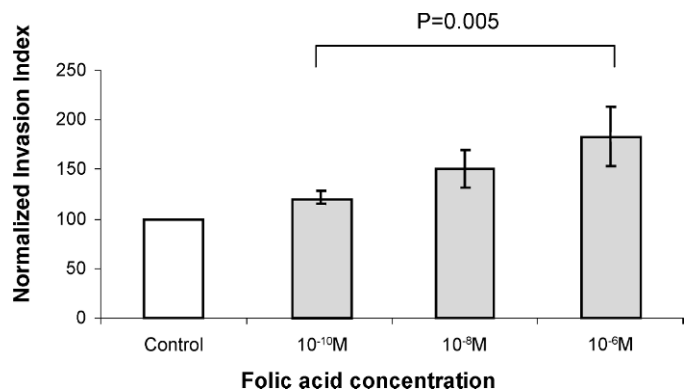
FIG. 1. Effect of culture in folic acid at 10^{-6} M, 10^{-8} M, and 10^{-10} M concentrations on the invasive capacity of placental villous explants. Data are expressed as mean normalized to DMEM:F12 control ± SEM.

FIG. 2.   Effect of culture in folic acid at concentrations of 10^{-6} M, 10^{-8} M, and 10^{-10} M on (**a**) caspase 3 expression and (**b**) MKI67 expression of placental villous explants. Data are shown as mean positivity ± SEM. Original magnification ×200.
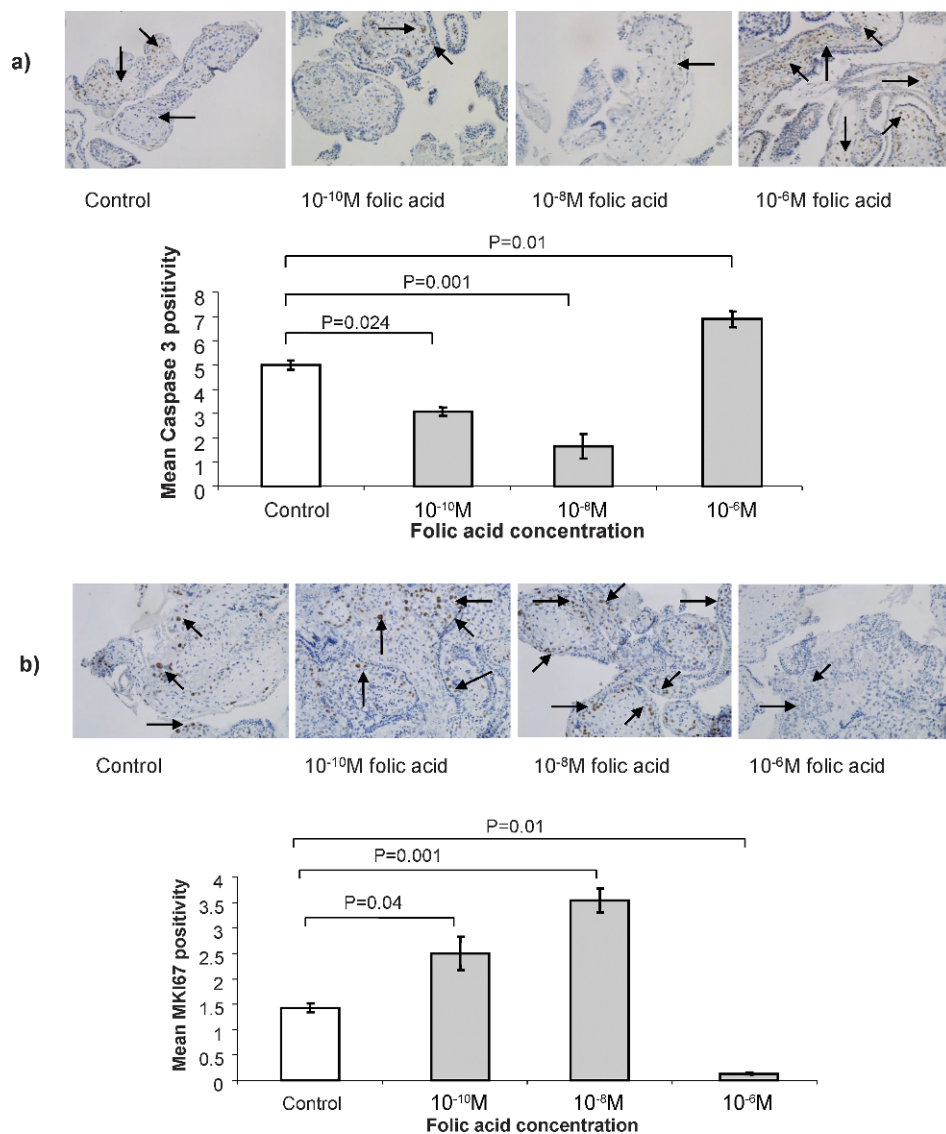
compared with controls cultured in folic acid at concentrations of 10^{-8} M ($P = 0.001$) and at 10^{-10} M ($P = 0.024$).

MKI67-positive staining was mainly found in the villous cytotrophoblast of placental explants. When compared with controls, MKI67 positivity was reduced in placental explants following culture in 10^{-6} M folic acid ($P = 0.012$). However, culture in folic acid at 10^{-8} M produced an increase in MKI67 positivity compared with controls ($P = 0.001$), and this increased MKI67 positivity was also found following culture with folic acid at 10^{-10} M ($P = 0.039$ vs. control; Fig. 2b).

Alteration in CD31 Staining Following Culture with Folic Acid

Vascular density, as determined by CD31 immunostaining, increased following culture with folic acid (Fig. 3a). When compared with controls, CD31 positivity in placental explants was higher following culture with folic acid at concentrations of 10^{-6} M ($P = 0.001$), 10^{-8} M ($P = 0.002$), and 10^{-10} M ($P = 0.02$). Furthermore, the increased CD31 expression following culture with folic acid was found to be a concentration-dependent response ($P = 0.05$; Fig. 3b).

Variation in MMP/TIMP Secretion Following Culture with Folic Acid

Standard curves generated for all analytes had R^2 values of 0.97 or above. Coefficients of variation for triplicate repeats were all less than 10%. Detectable levels of MMP2, MMP3, MMP7, MMP8, MMP9, MMP10, MMP12, MMP13, TIMP1, TIMP2, and TIMP4 were found in placental explant culture supernatants after incubation of 6 days in folic acid at 10^{-6} M, 10^{-8} M, and 10^{-10} M concentrations (Fig. 4).

The concentration of MMP2 secreted by placental explants increased following treatment with folic acid at 10^{-6} M, 10^{-8} M, and 10^{-10} M in a concentration-responsive manner ($P = 0.01$). The greatest effect on MMP secretion following culture with folic acid was seen for MMP3, which showed a concentration-responsive increase ($P = 0.01$) in secretion by placental explants following culture in folic acid at concentrations of 10^{-6} M, 10^{-8} M, and 10^{-10} M. MMP9 secretion was also increased in a concentration-dependent manner after culture in folic acid at concentrations of 10^{-6} M, 10^{-8} M, and 10^{-10} M ($P = 0.02$).

Levels of MMP1 fell below the range of detection of the ExcelArray kit. Concentrations did not vary between controls
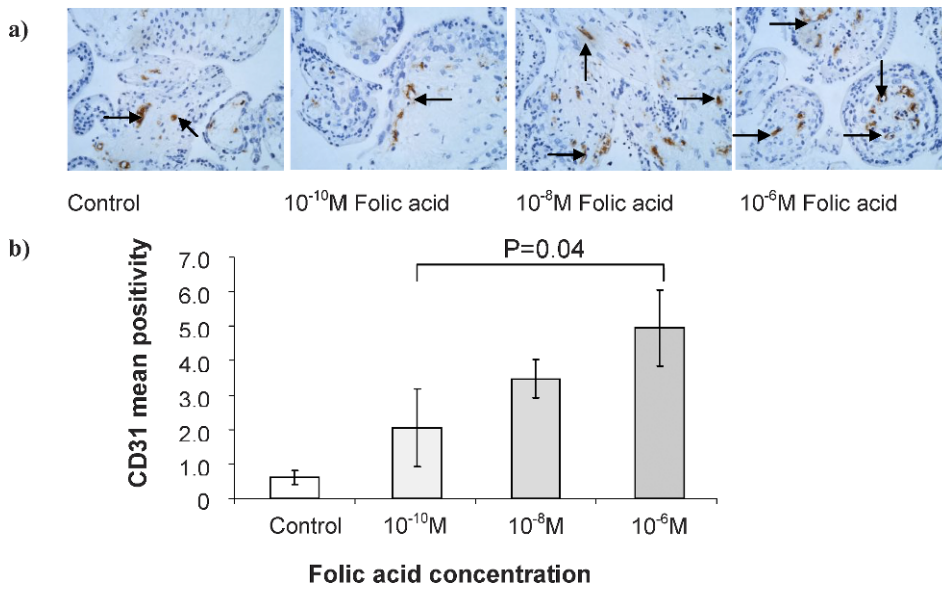
a)

Control 10^{-10}M Folic acid 10^{-8}M Folic acid 10^{-6}M Folic acid

b)

FIG. 3.   Effect of culture in 10^{-6} M, 10^{-8} M, and 10^{-10} M concentrations of folic acid on vascular density of placental villous explants, as determined by CD31 immunostaining. In photomicrographs (**a**) CD31-positive cells appear brown and are clearly visible around placental vessels (indicated by arrows). Original magnification ×200. In graphs (**b**) data are shown as mean positivity ± SEM.

and those treated with folic acid for MMP7, MMP8, MMP10, MMP12, MMP13, TIMP1, TIMP2, and TIMP4.

DISCUSSION

This study demonstrates for the first time that folic acid, at low concentrations, is able to increase EVT invasion and placental secretion of MMP2, MMP3, and MMP9. Additionally, folic acid displays a biphasic effect on placental explant proliferation and apoptosis in early pregnancy.

Folic acid may have a key role in regulation of EVT invasion, which is a crucial part of placental development. Invasion of EVT into the maternal decidua and inner third of the myometrium is crucial for the remodeling/transformation/conversion of the maternal spiral arteries, allowing the establishment of uteroplacental circulation [28]. Inadequate EVT invasion is associated with defective placental development, with a reduction in both the number of remodeled spiral arteries in decidua and myometrium and in the depth of remodeling, which is deficient particularly in myometrium; this is suggested to be the primary defect that occurs in preeclampsia and intrauterine growth restriction [29]. Supplementation with folic acid during the very early stages of pregnancy may therefore be beneficial by promoting EVT

invasion, which could be of benefit in preventing pregnancy disorders associated with inadequate EVT invasion. Indeed, a recent observational study identified that continuation of folic acid supplementation into the second trimester of pregnancy was associated with reduced incidence of preeclampsia [23] and that exposure to folic acid antagonists increases the risk of placental abruption, intrauterine growth restriction, and fetal death [21]. Furthermore, treatment with the antifolate drug methotrexate in ectopic pregnancy has been shown to reduce placental growth and EVT invasion due to reduced trophoblast proliferation, although caution must be used when comparing ectopic pregnancy and normal placental development [30].

CD31 labeling confirmed that culture with folic acid increased angiogenesis in placental explants. Placental angiogenesis is crucial for the appropriate development of the fetoplacental circulation, thereby maximizing blood flow throughout the placenta and, correspondingly, the rate of nutrient and gas exchange between the mother and her baby. The observed doubling of vascular density following culture with folic acid also provides a possible explanation for the reported reduction in vascular density in preeclampsia and intrauterine growth restriction when supply of folic acid is limited [21]. Placental explants have previously been used for the assessment of angiogenesis [31]; however, it is acknowl-
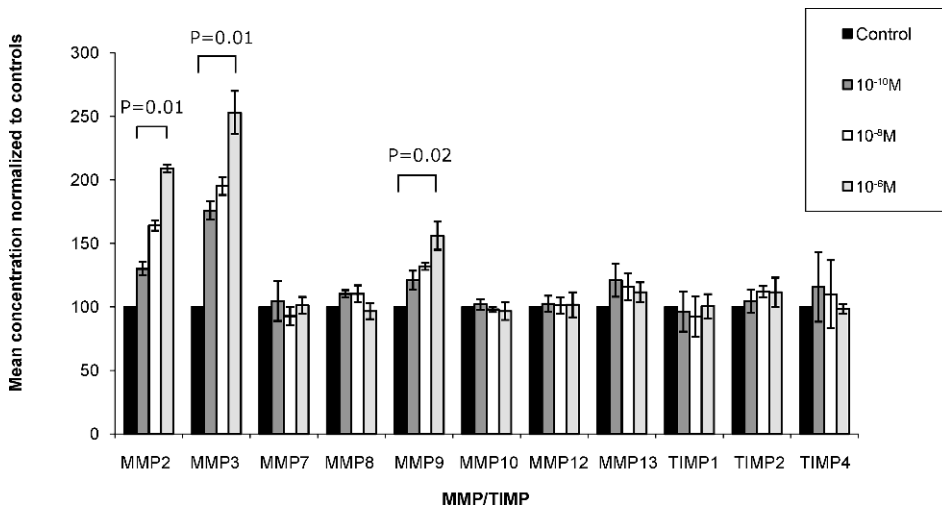

FIG. 4.   Effect of culture in folic acid at concentrations of 10^{-6} M, 10^{-8} M, and 10^{-10} M on secretion of MMPs and TIMPs from placental explants following 6-day culture. Data are shown as mean positivity ± SEM.

edged that further studies are needed to further delineate the involvement of folic acid in placental angiogenesis.

The increase in EVT invasion associated with folic acid supplementation may be explained by the increased production of MMP2, MMP3, and MMP9. The importance of MMP activity in trophoblast invasion has been described previously [32, 33], and their expression has been confirmed in both early and late pregnancy placental tissue [34, 35]. Increased production of the gelatinases (MMP2 and MMP9) may be associated with increased EVT invasion due to increased degradation of type IV collagen in the basement membrane, allowing increased numbers of EVT cells to invade through the maternal decidua. MMP3, also known as stromelysin-1, may also be involved in the increased invasion of EVT via degradation of a variety of extracellular matrix components, including proteoglycans, fibronectin, laminin, and type IV collagen, and has been shown to be present throughout pregnancy [36]. Although the assay used does not detect MMP activity, we suggest that the increased production of MMP2, MMP3, and MMP9 would also be associated with increased enzymatic activity of these metalloproteinases, because folic acid was not found to alter the production of the TIMPs, which downregulate the activity of MMPs. The use of zymography to determine the activity of MMP2, MMP3, and MMP9 would allow a more complete assessment of the effect of folic acid on MMP activity.

The biphasic concentration-dependent response of varying concentrations of folic acid on EVT proliferation and apoptosis (Fig. 2) was unexpected; micromolar concentrations were associated with increased apoptosis and reduced proliferation, whereas culture in nanomolar concentrations caused decreased apoptosis and increased proliferation. This biphasic response was mirrored between apoptosis and proliferation, suggesting involvement of a common pathway in these responses. It has previously been reported that reduced folic acid availability is associated with increased placental apoptosis [37]. However, that study used pooled cytotrophoblast samples from term placentae, 7 mo later in gestation than the tissues reported here. The response to folic acid may well be different in term tissues and following the process of parturition and labor. Furthermore, it is known that folic acid stores are depleted during pregnancy [38], which may alter placental responses. There is increasing interest in the use of micronutrient and vitamin supplementation during pregnancy to maximize health benefits for both mother and baby [39–41]. Despite public health campaigns recommending the use of preconceptual folic acid supplementation, the dietary intake of folic acid is still inadequate in around 13 million people within the United Kingdom [42]. However, the biphasic nature of the response to folic acid emphasizes the importance of careful evaluation of concentration before implementation of supplementation, an issue that was highlighted in a recent Cochrane review of micronutrient supplementation in pregnancy [43]. Concentrations higher than the current daily recommendation of 400 μg may be harmful with regard to placental development. A recent report showed median serum folate levels to be 23.5 nmol/L during the first trimester of pregnancy among women who took 400-μg folic acid supplements [44]. In the present study, this median serum folate concentration falls within the 10^{-8} M treatment group, which increased cell proliferation and decreased apoptosis of the EVT cells. Extrapolation from these preliminary data might suggest possible deleterious effects of very high-dose supplementation, but this is in any case contraindicated because of the risk of masking the effects of any vitamin B_{12} deficiency. In line with this are the contrasting reports regarding folic acid supplementation and

cancer treatment. A recent report has highlighted the need to limit folic acid supplementation to no more than 400 μg, rather than the 1000 μg more frequently prescribed for cancer patients to limit tumor cell proliferation [45]. In contrast, Hervouet et al. [46] have suggested that folate supplementation can act as a promising adjuvant for antiglioma therapies because of its role in DNA methylation, as well as increasing apoptosis and decreasing proliferation.

Possible limitations of the current study are the use of placental samples from one gestational time point (7 wk). Nevertheless, the use of placentae from the same time point allows assessment of the effects of folic acid with fewer confounding factors, because combining results from placental samples from a wider range of early pregnancy samples may lead to misleading interpretation of results, because the invasive capacity of EVTs during the first 20 wk of pregnancy has been shown to be different [47]. A further possible limitation may be the use of placental explants as the source of EVT, rather than isolating a pure population of EVTs using a Percoll gradient. Purified EVTs were not used in the present study because of the relatively low yield of cells obtained using this method, which would have reduced the number of folic acid concentrations that could have been assessed in each experiment. However, previous studies have confirmed the phenotype of the invaded cells from the placental explants to be consistent with an EVT population [48]. Future studies using placental samples from sequential gestational age points within the first trimester would be useful in determining whether the effect of folic acid on EVT function observed in the current study applies to a wider range of gestational ages, or whether it specific to 7 wk of gestation.

In summary, the current study highlights a further possible benefit of periconceptual and maintained folic acid supplementation, stimulating increased EVT invasion via increased secretion of MMP2, MMP3, and MMP9, as well as altered placental/trophoblast proliferation and apoptosis, and a doubling of vascular density. All of these aspects require further investigation to allow the potential for longer-term supplementation of folic acid throughout pregnancy as a means of preventing and/or alleviating symptoms of preeclampsia. Furthermore, the biphasic response of placental cells to folic acid emphasizes the need for carefully monitored functional studies to be performed prior to the implementation of supplementation trials.

ACKNOWLEDGMENTS

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