Effect of folic acid on homocysteine-induced trophoblast apoptosis

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In trophoblast cells exposed to homocysteine (Hcy) we observed cellular apoptosis and the inhibition of trophoblast functions. Because folate and Hcy, linked in the same metabolic pathway, are inversely related, we investigated the role of folic acid in reversing the Hcy effect in human placenta. In primary trophoblast cells we examined the cytosolic release of cytochrome c, both M30 and terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) and DNA laddering. Hcy (20 μmol/l) treatment resulted in cytochrome c release from mitochondria to the cytosol, and an increased number of M30-positive trophoblast cells and TUNEL positive nuclei. Furthermore, DNA cleavage in agarose gel and the determination of histone-associated DNA fragments have been investigated. Homocysteine induced DNA fragmentation and significantly reduced hCG secretion. The addition of folic acid (20 nmol/l) resulted in inhibition of the effects of Hcy on human trophoblast. These results suggest a protective role of folic acid in the prevention of trophoblast apoptosis linked to Hcy.

Key words: apoptosis/folic acid/homocysteine/placenta/trophoblast

Introduction

Spontaneous abortion is the most common adverse pregnancy outcome affecting between 12 and 15% of clinically recognized pregnancies (Wicox et al., 1988). Although as many as half of clinical spontaneous abortions may be caused by chromosomal abnormalities, maternal factors, including poor folate status, may contribute to their occurrence (Nelen, Bolm et al., 2000). Currently, it is believed that folate deficiency affects DNA stability (Duthie et al., 2002; Wang and Fenech, 2003) and it is well recognized that maternal nutrition, specifically multivitamin supplementation during the periconceptional period, is a significant modulator of risk for some congenital malformations (Kakutani et al., 1996; Alonso-Aperete et al., 1999). Although the exact mechanism underlying this effect is yet to be elucidated, one the hypothesis is that folic acid supplementation is able to reduce the plasma total homocysteine (Hcy) concentrations (Selhub et al., 1993). The metabolism of folate and Hcy are interrelated, since folate is a necessary cofactor for the enzyme that mediates conversion of Hcy to methionine (Fiskerstrand et al., 1997). Several reports have clearly shown an association of elevated Hcy concentrations and obstetric diseases that are connected with vascular disorders of pregnancy or of the utero-placental unit (Li et al., 1992; Powers et al., 1998; Buemi et al., 2001). Nelen et al. (2000) studied women with repeated miscarriages and found a direct relationship between high levels of Hcy and defective chorionic villous vascularization: early miscarriages might be explained by the damage that excess Hcy may cause on chorionic and decidual vessels leading to defective implantation of the embryo. Khong and Hague (1999) reviewed the placental pathology in women diagnosed retrospectively to have hyper-Hcy, following a recent history of intrauterine fetal growth restriction or of thromboembolic disease. Most of the placental findings indicated abnormal placentation with absence of trophoblast-induced physiological vascular change in the spiral arteries of the placental bed.

Recently, we provided (Di Simone et al., 2003) the first demonstration that human placenta is a target for Hcy. When trophoblast cells were exposed to Hcy, a cascade of events was observed: the cytosolic release of cytochrome c, an increase in M30 positive cells, and of positive nuclei by terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) and the internucleosomal DNA fragmentation. We then suggested that trophoblast death might represent one mechanism by which Hcy causes pregnancy complications related to placental diseases. Several studies in vivo have shown the utility of folic acid supplementation in subjects with hyper-Hcy (Vermeulen et al., 2000). In vitro data demonstrated that folate deficiency, at the level of cytotrophoblast cells, induces apoptosis (Steengers-Theunissen et al., 2000) and the addition of folic acid to the culture medium caused a significant reduction in the effects of Hcy on the proliferation/necrosis balance of cells in culture (Buemi et al., 2001). Because placental functions play a central role in pregnancy, we studied the effect of folic acid on Hcy-induced trophoblast apoptosis. We examined its effect on both M30 and TUNEL, on cytosolic release of cytochrome c, DNA fragmentation and hCG secretion. Our finding demonstrated the utility of folic acid in the prevention of trophoblast damage linked to Hcy.

Materials and methods

Trophoblast cell cultures

Four placentas were obtained from healthy women immediately after uncomplicated vaginal delivery at 36 weeks of gestation. This study was approved...
and adherent cells were collected, washed with PBS and suspended in ice-cold buffer (5 mmol/l Tris-HCl, pH 7.4, 5 mmol/l KCl, 1.5 mmol/l MgCl$_2$, 1 mmol/l EGTA and 0.2% Triton X-100), supplemented with protease inhibitors (1 mmol/l dithiothreitol, 0.2 mmol/l phenylmethylsulphonyl fluoride, 5 µg/ml leupeptin, 5 mg/ml aprotonin, 0.7 mg/ml pepstatin A and 50 mmol/l NaF). The whole cells were then centrifuged at 500 g for 5 min at 4°C. The supernatant was further centrifuged at 10,000 g for 1 h at 4°C in a Beckman TLA 100.4 rotor, and the resulting supernatants were used as the cytosolic extracts. The pellet was used as positive control in each experiment. The protein concentration was determined using the Bio-Rad Protein Assay (BioRad Laboratoires, USA). Eighty micrograms of each protein sample were separated on a 15% SDS–polyacrylamide gel, and after electroblotting onto polyvinylidene fluoride (PVDF) membrane (Millipore, USA), the membranes were incubated with 6% non-fat dry milk in 1 mol/l Trizma base, 1.5 mol/l NaCl, 0.05% Tween 20 (TBST, pH 7.4). The PVDF membrane was successively incubated overnight at 4°C with a rabbit polyclonal IgG antibody (clone H-104; Santa Cruz Biotecnology, USA) directed against cytochrome c, washed twice with TBST, incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody (Bio-Rad Laboratories) for 2 h at room temperature and washed twice more in TBST. The immunoblot was revealed using BCIP/NBT Phosphatase Substrate System (Kinkegaard & Perry Laboratoires, USA). Images of the blots were acquired with a Cuh charged-coupled-device camera, and quantification of the bands was performed by Phoretxt 1D (Phoretx International, UK). The rising level of the release of cytochrome c into the cytosol from adherent plus non-adherent cells was estimated versus the constant level of a 42 kDa protein present in the cytosolic extract (β-actin; mouse monoclonal, clone AC-15; Sigma–Aldrich S.r.L.).

**Measurement of fragmented DNA by enzyme-linked immunosorbent assay (ELISA)**

Cytotrophoblast cells were detached by EDTA-trypsin (Gibco BRL) treatment and collected by centrifugation at 250 g for 10 min at 4°C, then suspended in a fresh culture medium to make 1 x 10$^5$ cells/ml. A volume of 100 µl of the cell suspension was transferred to each well of a microculture plate and incubated with the test samples [follic acid (0, 2, 20, 200 mmol/l) with or without Di-Hcy (20 mmol/l)]. At 24 and 48 h, the plates were centrifuged, at 250 g for 10 min at 4°C the supernatant carefully removed and 200 µl of lysis buffer were added to each well; the amounts of fragmented DNA were measured with a Cell Death Detection ELISA plus kit (Roche Laboratoires, Italy).

**Preparation of the tissue lysates and western blot analysis**

To examine the effect of follic acid on Hcy-induced cytochrome c release, cell cultures were performed for 24 h and cytochrome c release from the intermembrane space of mitochondria into the cytosol was investigated by western blot analysis as previously described (Li et al., 2002). Non-adherent
The experiments were done three times on different placentas, in duplicate within each experiment.

**DNA fragmentation analysis**

Cytotrophoblast cells (2 x 10^5 cells/ml) were cultured in complete medium and treated for 48 h with folic acid (20, 200 nmol/l) with or without Dl-Hcy (20 nmol/l) at 37 °C. After 48 h of culture, the media were removed and stored at −20 °C for hCG determination. The assay was performed with a commercial radioimmunoassay kit (generously provided by Radim, Rome, Italy). The intra- and inter-assay coefficients of variation were <12% and <8% respectively.

**Statistical analyses**

Statistical differences were determined using two-way analysis of variance for multiple comparisons.

**Figure 2.** TUNEL staining of trophoblast cells. (A) Magnification ×400. Scale bar = 50 μm. (B) Note the reduced number of TUNEL positive cells, after 24 h of culture, in the presence of folic acid (FA; 20 nmol/l) with respect to homocysteine (Hcy; 20 μmol/l) alone. These values represent the mean SD from four independent experiments. *P < 0.003.

**Figure 3.** Release of cytochrome c into the cytosol. Cytosolic extracts were prepared and assessed by western blot analysis for cytochrome c. The level of cytochrome c (A) in the cytosol was estimated in comparison with the constant level of β-actin (B) present in the cytosolic extracts. Significant increase of the homocysteine (Hcy) (20 μmol/l)-induced cytochrome c release is shown [□P < 0.004 versus Hcy alone] (HBSS; Gibco BRL). The gradient was made from 5-70% Percoll (v/v) by dilutions of 90% Percoll (9 parts per cell, HBSS 10%, 1 part) and layered in a 50 μl conical polystyrene centrifuge tube. These values represent the mean SD from four independent experiments (C).

**Figure 4.** Effect of folic acid (FA) on quantitative in vitro determination of histone-associated DNA fragments. Trophoblast cells were incubated for 24 h (white bars) and 48 h (black bars) in the presence of homocysteine (Hcy) (20 μmol/l) and/or FA (2, 20, 200 nmol/l). Results are mean ± SD of three independent experiments. *P < 0.001 Hcy versus controls (Ctr); §P < 0.02 Hcy plus folic acid (20 nmol/l) versus Hcy; #P < 0.01 Hcy plus folic acid (200 nmol/l) versus Hcy.

**Hormone secretion**

To evaluate the effect of folic acid on Hcy-induced hormone secretion, primary trophoblast cells were treated with folic acid (2, 20 or 200 nmol/l) with or without Dl-Hcy (20 μmol/l). After 48 h of culture, the media were removed and stored at −20 °C for hCG determination. The assay was performed with a commercial radioimmunoassay kit (generously provided by Radim, Rome, Italy). The intra- and inter-assay coefficients of variation were <12% and <8% respectively.

**Figure 5.** Gel electrophoresis of fragmented DNA. Primary trophoblast cells were exposed to homocysteine (20 μmol/l) alone (lane 4) or with folic acid (FA; 2, 20 nmol/l; lanes 5, 6). Untreated cells (lane 1), or cells treated with FA (2, 20 nmol/l) alone (lanes 2, 3) are also shown. The DNA was extracted and electrophoresed on a 2% agarose gel. M = DNA size markers in base pairs.
Results

**Folic acid and Hcy-induced trophoblast damage**

After Hcy (20 μmol/l) treatment we found an increase in M30-positive trophoblast cells (Figure 1) and an increased number of TUNEL positive nuclei (Figure 2). We found that in Hcy-treated cells the number of M30 positive cells (35±16%) tended to be higher, even if not significant, than number of TUNEL positive cells (14±2%).

When folic acid (20 nmol/l) was added before Hcy (2 h), the number of M30 and TUNEL positive cells was significantly reduced (P < 0.03, Figure 1; P < 0.003, Figure 2), whereas no significant difference was observed when folic acid was added after Hcy (data not shown).

As shown in Figure 3, analysis of cytochrome c release from mitochondria to the cytosol showed an increase at 24 h of culture in Hcy-treated cells. When folic acid (20 nmol/l) was administered before Hcy, the cytosolic release of cytochrome c was significantly reduced (P < 0.004). No differences between untreated cells (controls) and cells treated with folic acid (20 nmol/l) alone have been found.

**In vitro effect of folic acid on Hcy-induced DNA fragmentation**

The time-course of apoptosis was monitored by the appearance of fragmented DNA derived from trophoblast cells. Incubation with DHcy (20 μmol/l) produced significant amounts of fragmented DNA in a time-dependent manner (Figure 4). When folic acid (2, 20, 200 nmol/l) was administered to cells before DHcy, a reduced DNA fragmentation was evident.

Incubation with folic acid alone did not induce DNA fragmentation. The values for the negative controls were similar to those of the untreated cells.

Apoptosis was verified by electrophoretic observations (Figure 5).

**Effect of DHcy and folic acid on hCG secretion**

After 48 h of culture, exposure to folic acid alone (20–40 nmol/l) resulted in a significant increase (P < 0.03) in hCG levels (Table I). Administration of Hcy (20 μmol/l) to trophoblast cells inhibited hCG secretion (P < 0.02) with a reduction to 43% compared to controls (untreated cells). We then evaluated the potential role of folic acid on Hcy-induced hCG secretion. As shown in Table I, folic acid (20 nmol/l) restores hCG secretion, but only at 40 nmol/l was the hCG secretion significantly increased (P < 0.05) with respect to Hcy-treated cells.

**Discussion**

To our knowledge, this is the first in vitro evidence of the effect of folic acid on Hcy-induced trophoblast apoptosis. These findings agree with our previous observations (Di Simone et al., 2003): the presence of Hcy (20 μmol/l) is able to induce a translocation of cytochrome c into the cytosol, with an internucleosomal cleavage of DNA and the typical DNA fragmentation in multiples of 180–200 bp. Folic acid demonstrated a significant inhibition of the effects of Hcy on human trophoblast and reduced both M30 and TUNEL positive cells. When we compared the results of M30 and TUNEL, we found that Hcy-treated cells stained more positive with the M30 (35±16%) than with the TUNEL (14±2%) method, even if the difference was not significant. An exact overlap of M30 and TUNEL positive staining cannot be expected since the neoepitope of CK18 (Kadyrov et al., 2001; Austgulen et al., 2002), recognized by M30 CytoDEATH antibody, is unmasked early in the apoptotic cascade, whereas DNA degradation, detected with the TUNEL method, occurs late in the process of cellular death.

The effect of folate has been investigated extensively, even if not in trophoblast tissue. Recently, Ho et al. (2003) findings demonstrated that folate deprivation induced increases in reactive oxygen species, in cytosolic calcium and neuronal apoptosis.

Doshi et al. (2001) reported that exposure of endothelial cells to Hcy stimulated intracellular generation of superoxide and that folate can reduce levels of intracellular superoxide, suggesting a direct action of folic acid as a scavenger for superoxide. However, an indirect effect is also possible, by reduction of intracellular Hcy (Doshi et al., 2001) or by improvement of the cellular antioxidant defence system (Weiss et al., 2001).

The presence of folic acid in the medium might favour the catalysis of Hcy, via remethylation of Hcy in methionine, as demonstrated by Buemi et al. (2001) in vascular smooth muscle cells, or induce the expression of antioxidant enzymes (Outinen et al., 1999).

This study allowed cells to be exposed to Hcy levels observed in subjects with mild hyper-Hcy (16–24 μmol/l). The dose of folic acid used (20 nmol/l), is comparable to the nanomolar range of human plasma folate levels and it can also be achieved with dietary supplementation (Selhub et al., 1993; Brouwer et al., 1998). In fact the concentration of total folate in the serum of well-nourished human adults ranges from 14 to 34 nmol/l and most of the folate present in serum is free or loosely bound (Green and Ford, 1984), making it available for placental uptake (Antony et al., 1981).

However, even if the concentrations of Hcy and folic acid are similar to the plasma levels, we should consider the present model to be an in vitro system in which the short-term effect of these drugs was tested. This differs from the in vivo conditions, where the trophoblast tissue is exposed to a longer period of drug exposure and several pathogenic mechanisms may be present simultaneously.

In conclusion, the present study provides in vitro evidence of a protective role of folic acid in Hcy-induced placental disease. The intracellular mechanisms by which folate is able to protect trophoblast from Hcy-induced apoptosis will be investigated in ongoing studies.
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