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Received: 30 August 2010
Final form accepted: 01 December 2010
Published online: 15 December 2010

Volume 16 (2011) pp 69-78
DOI: 10.2478/s11658-010-0040-2
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Short communication

ENDOTHELIAL MICROPARTICLE FORMATION IN MODERATE CONCENTRATIONS OF HOMOCYSTEINE AND METHIONINE *in vitro*

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Abstract: Microparticles (MPs) are small membrane vesicles released by stimulated or apoptotic cells, including the endothelium. Hyperhomocysteinemia (HHcy) is a blood disorder characterized by an increase in the plasma concentrations of total homocysteine (Hcy). The plasma Hcy level is determined by environmental factors (dietary habits, i.e. the intake of folic acid, FA) and genetic factors (N^5,N^{10}-methylenetetrahydro-folate reductase, MTHFR, polymorphism 677C>T). To evaluate whether moderate Hcy concentrations induce endothelial MP formation, the role of FA supplementation and the influence of MTHFR polymorphism were analysed. Human umbilical vein endothelial cells (HUVEC) were treated *in vitro* with 50 μM of Hcy and methionine (Met). The MP number and apoptotic phenotype were analyzed using flow cytometry. Increasing doses of FA (5, 15 and 50 μM) were used to reduce the HHcy effect. The MTHFR 677C>T polymorphism was determined. HUVEC stimulated by Hcy produced significantly more MPs than HUVEC under the control conditions: 3,551 ± 620 vs 2,270 ± 657 kMP ($p = 0.02$). Supplementation with FA at concentrations of 5, 15 and 50 μM reduced the MP count in the cell culture supernatant to 345 ± 332, 873 ± 329, and 688 ± 453 kMP, respectively ($p = 0.03$). MTHFR 677C>T heterozygosity was associated with a significant increase in MP formation after stimulation with Hcy compared to

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Abbreviations used: BB – binding buffer; CAD – coronary artery disease; FA – folic acid; FBS – fetal bovine serum; FITC – fluorescein isothiocyanate; Hcy – homocysteine; HHcy – hyperhomocysteinemia; HUVEC – human umbilical vein endothelial cells; Met – methionine; MI – myocardial infarction; MP(s) – microparticle(s); MTHFR – N^5,N^{10}-methylenetetrahydrofolate reductase; PECAM-1 – platelet endothelial cell adhesion molecule-1; PBS – phosphate buffered salt solution; PE – phycoerythrin

the control conditions: 3,617 ± 152 vs 1,518 ± 343 kMP ($p = 0.02$). Furthermore, the MTHFR genotype altered MP formation after Met loading. On average, 24% of the entire MP population was apoptotic (annexin V-positive). Endothelial function impairment due to HHcy is related to MP shedding, which may involve platelets and other blood and vascular cells. MP shedding is a physiological response to moderate HHcy.

Key words: Flow cytometry, Homocysteine, Hyperhomocysteinemia, Microparticles, Human umbilical vein endothelial cells, Methylenetetrahydrofolate reductase

INTRODUCTION

Homocysteine (Hcy) is a sulphur amino acid synthesized during the metabolic conversion of methionine to cysteine. In the trans-sulphuration pathway, homocysteine is converted to cystathionine, but it can also be remethylated via the folate cycle. This pathway requires the methionine synthase enzyme and cobalamin (vitamin B12) as well as the N^5,N^{10}-methylenetetrahydrofolate reductase enzyme (MTHFR) and folic acid (FA), which enters the cycle as tetrahydrofolate [1].

Normal Hcy levels oscillate between 5 and 15 μM. Higher concentrations are considered to indicate a metabolic disorder called hyperhomocysteinemia (HHcy) [1]. While severe HHcy (over 100 μM) is a rare condition, 5 to 7% of the general population suffers from mild to moderate hyperhomocysteinemia (15 to 100 μM). Numerous epidemiological and clinical studies have indicated an association between elevated plasma Hcy concentrations and vascular diseases, including coronary artery disease (CAD), stroke, myocardial infarction (MI) and venous thromboembolism [2-4]. Plasma Hcy levels are determined by demographic, environmental and genetic factors. Normal homocysteine metabolism is dependent on an adequate supply of three dietary vitamins: FA, vitamin B12 and vitamin B6. Thus, a nutritional deficiency of these vitamins may cause moderate HHcy [3, 5]. Several genetically inherited enzyme mutations responsible for elevated homocysteine levels have been described, of which probably the most common one is the cytosine to thymine transition at nucleotide 677 (677C>T) of the MTHFR enzyme [5]. This polymorphism decreases MTHFR activity, which is associated with a decrease in the redundancy ratio of 5,10-MTHF to 5-MTHF and an increase in the level of 5,10-MTHF bioavailability for the oxidation of folate to formyl forms [6].

Endothelial dysfunction, i.e. impairment of the homeostatic properties of the vascular endothelium, leads to adverse cardiovascular outcomes. The hyperhomocysteinemic state causes endothelial dysfunction in humans, animals and in the experimental *in vitro* model [7-9]. Endothelial cell activation followed by apoptosis is also related to endothelial dysfunction. In this state, endothelial cells release qualitatively and quantitatively diverse small membrane vesicles called microparticles (MPs), which vary in diameter between 0.1 and 1.5 μm.

MPs are used as a marker of endothelial dysfunction and the procoagulation state [10, 11]. MPs may harbor a diversity of cell surface proteins, among them CD31 (PECAM-1, platelet endothelial cell adhesion molecule-1), which is a selective marker of matured and progenitor endothelial cells [12]. Additionally, phosphatidylserine, which is specifically bound to annexin V, is exposed on the endothelial MP surface [12], and annexin V-positive MPs are observed during apoptosis [10]. There is little evidence that homocysteine influences MP formation. In patients with chronic renal failure, both dialyzed and undialyzed, the elevation of apoptotic, annexin V-positive, endothelial MPs was observed [13]. However, the role of *p*-cresol and indoxyl sulfate rather than Hcy was documented [13, 14].
In our study, we tested the hypothesis that moderate concentrations of homocysteine induced the formation of endothelial MPs *in vitro* and investigated whether this activity is regulated by folate supplementation and the MTHFR polymorphism. We also tried to determine the phenotype of endothelium-released microparticles.

MATERIALS AND METHODS

Endothelial cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated by digestion using 0.25% trypsin in Ca^{2+} free Hanks balanced salt solution (No. 27250018, Gibco®). The cells were cultured in 25-cm^2 flasks with Human Endothelial Serum Free Medium (SFM, 11111044) supplemented with 10% Fetal Bovine Serum (FBS, No. 10100139, Gibco®) purchased from Invitrogen (Paisley, UK), 2 mM of L-glutamine (No. G8540, Sigma-Aldrich, Steinheim, Germany) and antibiotics (50 U/ml penicillin, 50 µ/ml streptomycin, 0.1 mg/ml neomycin, Polfa Tarchomin, Poland). HUVEC from passages 3-4 after overnight serum starvation were used in the study. All of the experiments were performed in duplicate, using nine different primary cell lines.

Microparticle formation and isolation

After reaching full confluence, the HUVEC were treated for 24 h with 50 µM DL-Hcy (No. H4628), 5, 15, 50 µM FA (No. F8758) or 50 µM DL-methionine (No. M2768) purchased from Sigma-Aldrich (Steinheim, Germany). The cell culture supernatant was collected and centrifuged for 15 min at 4,500 g. Then the supernatant was centrifuged for 1.5 h at 100,000 g at 4ºC using an ultracentrifuge (Avanti J-30I, Beckman Instruments Inc. Fullerton, USA). Next, the pellets were resuspended in 500 µl of Binding Buffer (BB, Pharmingen™, BD Bioscience, Bedford, USA) and analysed.

Flow cytometry analysis of microparticles

Microparticle acquisition was performed using a mix of fluorescent beads of various diameters to cover the MP size range from 0.5 to 0.9 µm (Megamix, Biocytex, France) by means of the FACS Calibur instrument (BD Immunocytometry Systems, San Jose, USA). MPs resuspended in BB (50 µl)

were incubated in the dark (30 min, 4ºC) with 5 µl of PE-conjugated monoclonal antibody against CD31 (PECAM-1) and 5 µl of FITC-conjugated annexin V (Annexin V-FITC Apoptosis Detection Kit I, Pharmingen™, BD Bioscience, Heilderberg, Germany) and then centrifuged (1.5 h, 100,000 g, 4ºC) [12]. Afterwards, the pellets were resuspended in 500 µl of PBS, and flow cytometry analysis was performed. The data was analyzed using CellQuest Pro software (BD Immunocytometry Systems, San Jose, USA). The MP number was calculated according to Brodsky *et al*. [11]. Then the number of MPs shed by the HUVEC was standardized to the number of cells cultured in each flask (kMP).

Hcy analysis

To control the real Hcy concentration under our experimental conditions, High Performance Liquid Chromatography (HPLC) with ultraviolet detection was used (HP 110 Series System, Hewlett-Packard, Waldbronn, Germany) [15]. The level of Hcy was calculated using calibration standards.

MTHFR (677C>T) polymorphism analysis

To establish the relationship between the MTHFR (677C>T) polymorphism and MP formation, DNA was extracted from every cell of the primary culture using a NucleoSpin Blood Mini Kit (740 951.250 Macherey-Nagel, Düren, Germany). The genotyping of the MTHFR polymorphism was performed by real-time quantitative polymerase chain reaction (ABI PRISM® 7900HT Fast Real-Time PCR System, Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems): the wild allele (C)-complementary probe sequence conjugated with VIC fluorochrome and the mutant allele (T)-complementary probe sequence conjugated with FAM:
5'GAAAGCTGCGTGATGATGAAATCG**G**CTCCCGCAGACACCTTCTCCT TCAA3'
5'GAAAGCTGCGTGATGATGAAATCG**A**CTCCCGCAGACACCTTCTCCTT CAA3'.

Statistical analysis

The results are presented as the means ± SD. The data was analyzed by the Kruskal-Wallis test and the Mann-Whitney U test, using the Statistica 8.0 PL package (StatSoft, Inc. 2008). P-values lower than 0.05 were considered significant.

RESULTS

Hcy turnover in HUVEC culture

The analysis of Hcy turnover in the cell culture supernatant showed that the concentrations of Hcy changed significantly after treatment with FA (Fig. 1). The mean concentration of Hcy in the presence of 50 µM of FA significantly decreased, from 48.14 ± 7.84 to 29.62 ± 7.93 µM ($p = 0.03$) after a 24-h incubation. Similarly, treatment with 5- and 15-µM doses of FA resulted in

a reduction in the Hcy concentration: 54.43 ± 6.89 vs 34.84 ± 4.79 μM (p = 0.01); and 45.95 ± 7.22 vs 33.92 ± 8.71 μM (p = 0.03), respectively. Treatment with Met alone caused increases in the Hcy levels in the cell culture supernatant: 0.79 ± 0.12 vs 33.42 ± 3.72 μM (p = 0.001).

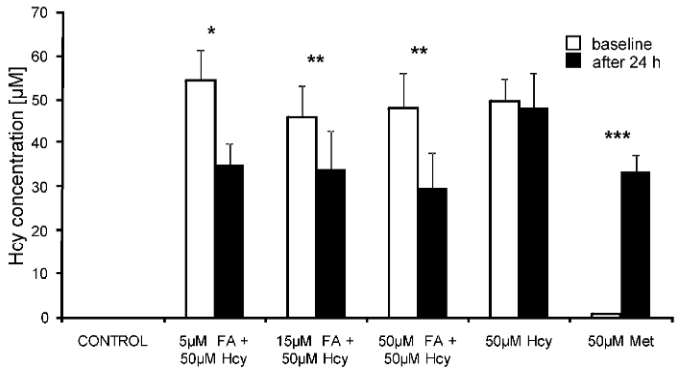

Fig. 1. An analysis of the homocysteine concentrations in the cell culture supernatant after HUVEC incubation with homocysteine (Hcy), or Hcy and folic acid (FA) or with methionine (Met) alone. *p = 0.01, **p = 0.03, ***p = 0.001. The data is presented as the means ± SD.

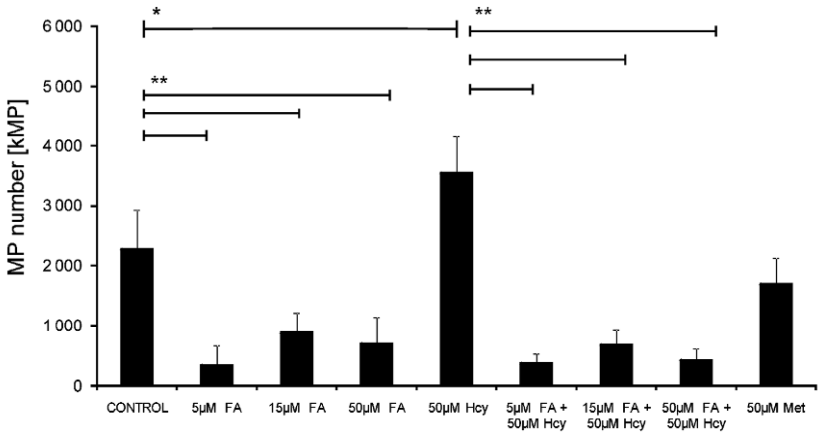

Fig. 2. The formation of microparticles (MPs) in HUVEC after stimulation with Hcy and Met with the presence of FA in the cell culture; *p = 0.02, **p = 0.03. The data is presented as the means ± SD. The number of MPs is standardized to one million cells per culture flask.

The effect of Hcy and Met on MP formation in HUVEC

The flow cytometry analysis showed that HUVEC formed MPs *in vitro* under the control conditions (Fig. 2). Stimulation of HUVEC with Hcy significantly induced MP formation when compared to the control conditions: 3,551 ± 620 vs 2,270 ± 657 kMP (p = 0.02). Moreover, supplementation with FA at concentrations of 5, 15 and 50 μM significantly reduced the MP count in the cell

culture supernatant: 345 ± 332; 873 ± 329; and 688 ± 453 kMP (p = 0.03), respectively. The protective effect of FA supplementation was also noticeable in presence of the moderate concentration of Hcy (50 μM): 376 ± 155 vs 670 ± 258 vs 420 ± 193 kMP; p = 0.03. Interestingly, Met treatment did not increase MP formation in the HUVEC, compared to the result obtained under the control conditions (1,665 ± 439 vs 2,270 ± 657 kMP). The protective effect of FA on Hcy-stimulated MP formation was not dose-dependent, and no correlation between the FA concentrations and the MP number was observed: r = -0.4, p = 0.44.

The MTHFR (677C>T) polymorphism and Hcy-induced MP formation in HUVEC

The effect of the MTHFR polymorphism on MP formation was analyzed (Fig. 3). Three of six cell lines analyzed were heterozygous for the 677C>T polymorphism and three were homozygous (677CC). The MTHFR genotype of the HUVEC altered MP formation after Met loading (50 μM): the heterozygotic (677C>T) HUVEC produced twice as many microparticles per million cells as the homozygotic (677CC) ones: 2,341 ± 1,027 vs 1,015 ± 103kMP (p = 0.001). Surprisingly, the MTHFR 677CC polymorphism did not alter the MP release under control conditions nor in the presence of Hcy (50 μM): p = 0.25 and p = 0.66, respectively (Fig. 3). A significant increase in MP formation after stimulation with Hcy compared to the results obtained under the control conditions was observed both in heterozygotic (677CT) and homozygotic (677CC) HUVEC: respectively, 1,518 ± 343 vs 2,617 ± 152 kMP (p = 0.02); and 2,982 ± 906 vs 4,399 ± 1,127kMP (p = 0.03).

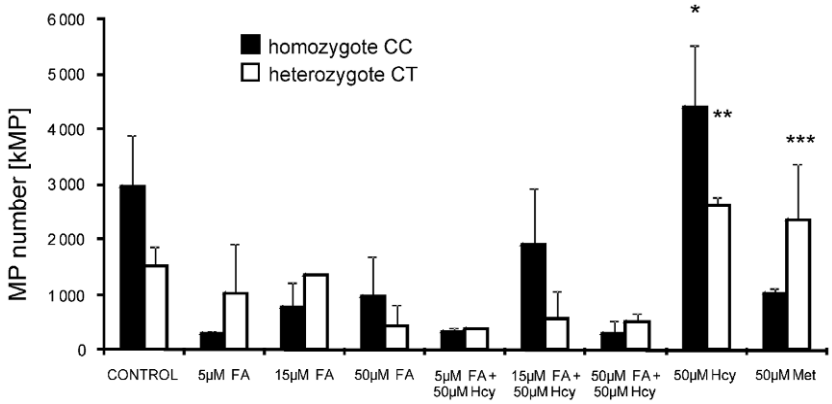

Fig. 3. The role of the MTHFR (677C>T) polymorphism in MP formation in HUVEC after stimulation with Hcy and Met with the presence of FA in the cell culture. *p = 0.03 and **p = 0.02 vs control conditions; ***p = 0.001 vs homozygote 677CC. The data is presented as the means ± SD. The number of MPs is standardized to one million cells per culture flask.

The role of Hcy in apoptotic MP formation

Annexin V-positive MPs are a marker of apoptosis [10]. On average, 24% of the entire MP population was conjugated with annexin V. No difference in the level of MP formation was observed between stimulation with Hcy and Met ($p = 0.15$). Surprisingly, folate had no significant effect on the MP phenotype. Neither increasing concentrations of FA ($p = 0.71$) nor the presence of Hcy ($p = 0.45$) influenced the proportion of annexin V-positive MPs in the overall population of endothelial cell-derived MPs.

DISCUSSION

The formation of endothelial MPs is associated with a pathological state which may lead to tissue factor expression and procoagulant activity [11, 16]. Many studies suggest that endothelial cell-derived MPs have a paracrine role and contribute to the development of endothelial dysfunction in most cardiovascular diseases: CAD, MI, hypertension and congestive heart failure. Moreover, diabetes, end-stage renal failure and pulmonary or venous embolism are strong factors bringing about MP shedding [13, 14, 17].

Our study demonstrated that in moderate concentrations, Hcy stimulates endothelial cells (HUVEC) to MP formation, and that FA prevents HUVEC from MP shedding. We may assume that the MTHFR polymorphism is related to MP formation in HHcy and methionine-loading conditions, and that endothelial cell-derived MPs have a diverse phenotype, not only apoptotic.

A possible relationship was previously demonstrated between hyperhomocysteinemia after an oral methionine load and endothelial function in healthy adults and cardiovascular patients [18]. Increased Hcy levels, which are related to ischemic conditions, share similar mechanisms that lead to MP formation as a result of cardiac ischemia, myocardial infarction (MI) and stroke [9, 17, 19]. Moreover, endothelial MP numbers correlate inversely with the flow-mediated vasodilatation response and endothelial-related vasorelaxation [12, 20]. Our findings concur with those showing that circulating MPs promote the development of endothelial dysfunction. The different influences of free radicals on the inter- and intracellular mechanisms of bradykinin-dependent vasodilatation compared to vasodilatation in response to other endothelium-dependent stimuli may be regulated by means of MP release [21].

Usui *et al.* [22] demonstrated that in healthy volunteers, methionine loading acutely attenuated endothelial functions. Pretreatment with FA did not prevent the rise in homocysteine levels, but prevented the impaired vascular response. Our study confirms that FA is an active protector against endothelial cell dysfunction. We demonstrated that FA prevents HUVEC from MP formation under the control conditions, and that this effect was maintained after stimulation with Hcy. Interestingly, folate activity was not dose-dependent in our model, and the concentrations used were efficient. We supposed that the MTHFR polymorphism is involved in MP formation in endothelial cells; the

heterozygotic HUVEC responded more significantly to methionine loading and produced twice as many MPs than homozygotic ones. Our study is in accordance with some previous reports on the role of familial hyperhomocysteinemia in atherosclerosis and endothelial dysfunction [11, 18]. Oral administration of methionine at a dose of 100 mg/kg is usually used to induce moderate hyperhomocysteinemia (~25 μM) in healthy human subjects [7]. In our study, we observed that *in vitro* administration of Met (50 μM) resulted in moderate concentrations of Hcy (33.42 ± 3.72 μM) in the culture supernatant, and these conditions induced a significant increase in the number of MPs. However, we did not observe a similar relationship in the presence of Hcy alone (50 μM). Our observation is in line with Den Heijer's *et al.* study, which showed that the post-load homocysteine levels had stronger genetic determination than the fasting homocysteine levels [23]. In addition, current data suggests that MP number may be genetically regulated [12].

In our study, most MPs were not annexin V-positive (about 75%), which may suggest that the apoptotic process does not dominate in the HHcy response [10]. Unlike other uremic toxins, Hcy did not influence an increase in apoptotic MP numbers [13]. We might speculate that non-apoptotic MPs have proangiogenic activity or transfer coagulation factors (i.e. tissue factors) [14, 16, 17].

We did not analyze the detailed phenotype of HUVEC-derived MPs and this remains a subject for further study. The mechanism of MP formation after Hcy treatment is not clear, but we can postulate that endothelial cells can manage oxidative stress, and MP shedding is a physiological response to moderate hyperhomocysteinemia. We may conclude that the endothelial function impairment due to HHcy is related to MP shedding, which may involve platelets and other cells like monocytes or vascular cells.

The limitations of our study are that we used CD31 as the sole marker for endothelial MPs, and that we did not include other markers of endothelial activation, e.g. tissue factor, E-cadheris or cell adhesion molecules. Secondly, we used a low number of different cell lines and replicates in our experiments, but we may assume that *in vitro* conditions confirm our results. Thirdly, we did not take into consideration the 677TT genotype, which may have been helpful in confirming the role of Hcy metabolism in MP release. However, in certain cell types, an unfavourable phenotype might be driven directly by heterozygosity.

Acknowledgements. This study was supported by a grant from the Jagiellonian University Medical College in Kraków (K/ZDS/001009, to Ewa Stępień).

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