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Homocystine Metabolism in Endothelial Cells of a Patient Homozygous for Cystathionine β-synthase (CS) Deficiency

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Summary

Homocystinuria due to cystathionine β-synthase (CS) deficiency is the most common inborn error of methionine metabolism. Patients with CS-deficiency have an extremely high risk of vascular disease. The underlying mechanism is still unsolved. Dysfunction of endothelial cells could be the trigger in the formation of atherosclerosis and thrombosis. Therefore, differences in cell function were studied between normal and CS-deficient human umbilical endothelial cells (HUVECs). Total homocysteine (tHcy) concentrations in culture media as a measure of homocysteine export increased in all cell lines, including the cell line with CS-deficiency, with constant amounts of approximately 2.5 μM every 24 h. von Willebrand factor (vWF), tissue plasminogen activator (tPA) and plasminogen activator inhibitor (PAI-1) in culture media were used as markers of endothelial function and increased also with progression of culture time. The effects of additions of folic acid, vitamin B6 and methionine to the culture medium were studied. The homocysteine export and the markers of endothelial function did not differ between the control and the CS-deficient HUVECs under various test conditions. These data show that CS-deficient endothelial cells have normal homocysteine export and normal endothelial cell function. In CS-deficient patients the very high blood levels of homocysteine, probably due to deficient CS function in liver and kidney, seems to be the hazardous factor to endothelial cells, thus promoting atherosclerosis and thrombosis in CS-deficient patients.

Introduction

Deficiency of cystathionine β-synthase (CS) resulting in very low or absent enzyme activity is an autosomal recessively inherited disorder of methionine metabolism and causes high levels of homocysteine in blood (hyperhomocysteinemia) and in urine (homocystinuria). Clinically, the most life-threatening complications of homocystinuria are severe atherosclerosis or thrombosis at young age. Homocystinuria is treated with vitamin B6 (pyridoxine) as first choice; approximately 50% of patients with homocystinuria respond to this vitamin treatment with a decrease of the homocysteine levels. Vitamin B6 non-responding patients could be treated with betaine, folic acid, vitamin B12, or even methionine restriction (1). The most common mutation in the CS-gene among Dutch homocystinuric patients is the T833C transition (2). Patients homozygous for this transition respond very well to vitamin B6 therapy (3).

The underlying mechanism causing vascular disease in hyperhomocysteinemia is still unsolved. In vitro high levels of homocysteine can generate H2O2 and free radicals are formed which in vivo theoretically could result in increased lipid peroxidation (4, 5), causing accumulation of oxidised low density lipoprotein inside the vessel wall. However, in homocystinuric patients no increased lipid peroxidation had been found (6, 7).

The first step in the development of atherosclerosis or thrombosis is dysfunction of endothelial cells. Elevated levels of von Willebrand factor (vWF), plasminogen activator inhibitor (PAI-1), and tissue plasminogen activator (tPA) are considered as markers of endothelial dysfunction (8-10). To study the hypothesis, that homocysteine itself can cause endothelial cell dysfunction, endothelial cells have been cultured with additions of various concentrations of homocysteine to the culture medium (11-14). However, these studies used very high concentrations of homocysteine (1 to 10 mM) in its reduced form (i.e. with free SH-groups), which is highly unphysiologic. In blood only 1% of total homocysteine is present in its reduced form. This means that applying 10 mM reduced homocysteine in vitro is about 50,000 times higher than the concentration normally present in blood of patients with mild hyperhomocysteinemia (15).

In vitro lymphoma cells, liver cells, and fibroblasts exported homocysteine into the extracellular medium (16-18). Enhanced homocysteine export is associated with increased homocysteine production or inhibition of metabolism (19). In our previous study with human umbilical vein endothelial cells (HUVECs) we showed a strong dependency of the homocysteine export on folic acid levels in the culture medium. No effects of additions of other vitamins involved in homocysteine metabolism, such as vitamin B6 and vitamin B12, were observed (20). Because increased homocysteine export reflects an imbalance in the homocysteine metabolism, we concluded that endothelial cells are vulnerable to subnormal folate levels. The underlying mechanism for this phenomenon is not known, but this imbalance in the homocysteine metabolism is likely influenced by activities of key enzymes in homocysteine remethylation (methylene tetrahydrofolate reductase: MTHFR and methionine synthase: MS) and transsulfuration (CS) (Fig. 1).

In the present study the homocysteine export of HUVECs of a CS-deficient patient homozygous for the T833C transition is compared with the export of normal HUVECs. The influence of additions of folinic acid [5-formyl-THF (tetrahydrofolate)], vitamin B6 (pyridoxine HCl), and methionine is explored. Changes in endothelial cell function are studied by measurements of vWF, tPA, and PAI-1 in culture media.


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Materials and Methods

Cell Culture

Endothelial cells from the human umbilical vein (HUVECs) were obtained from umbilical cords of healthy fetuses from uncomplicated pregnancies and deliveries of healthy mothers (non-smoking and without medication). Within 24 h after cord collection endothelial cells were isolated by collagenase (Worthington, New Jersey, USA) treatment according to the procedure of Jaffe et al. (21). HUVECs were cultured under standard conditions of which more details were described in our previous article (20). All HUVEC experiments were performed in 12-well plates with cells of 3 days in confluence in the third or fourth passage.

After the experiments the cells were counted by using the methylene blue (3,7-bis(dimetylamino)phenothiazine-5-ium chloride) cell counting method according to Oliver et al. (22). This method is based on the ability of methylene blue to bind electrostatically at pH 8.5 to negatively charged groups inside the cell, mostly phosphate moieties of nucleic acids. The stained cells were eluted with 1:1 (v/v) ethanol:0.1 M HC1 and optical density was read at wavelength 620 nm with Titertek Multi scan 340 CC (ICN Flow, Irving, Scotland). Before it was used in routine, a linear function between the optical density and cell number was established.

All experiments with normal HUVECs were performed in duplo with at least three different cell lines. All experiments with CS deficient HUVECs were performed in triplo.

Homocysteine Analysis

Homocysteine concentrations were measured by an automated high performance liquid chromatography (HPLC) method with reverse phase separation and fluorescent detection (Gilson 232-401 sample processor, Spectra Physics 8800 solvent delivery system and Spectra Physics LC 304 fluorometer) (23). The detection limit was 0.5 M. The intra- and inter run variation coefficient was <5%.

cystathionine /β-synthase (CS) Assay

CS activities were measured following the procedure described by Fowler et al. (24). The determination was carried out in the presence or absence of Pyridoxal-5-Phosphate and the final product 14C-cystathionine was measured radiochemically. Protein concentrations were determined by the method of Lowry et al. (25).

DNA Analysis

Genomic DNA was isolated from HUVECs and polymerase chain reaction (PCR) was performed with 100 ng of genomic DNA. The PCR product was subjected to BstI and the restriction fragments were detected and visualized according the procedure described by Kluijtmans et al. (2).

ELISAs

a) von Willebrand factor (vWF): vWF antigen was determined by a sandwich ELISA modified from Jagerslev et al. (26). In brief: 96-well plates (Nunc, Roskilde, Denmark) were coated with rabbit anti-human vWF polyclonal antiserum (A082; Dako A/S, Denmark). The reference line was made from dilutions of unincubated standard culture medium to washing buffer (PBS, 0.05% Tween 20 [ICN Flow, Irving, Scotland], and 0.5 mg/ml BSA [Sigma Chemical Co, St Louis, USA]). Blanks were standard culture medium without serum. Test samples were diluted 1:80 by washing buffer. After incubating and washing rabbit anti-human vWF peroxidase conjugate (P226; Dako A/S, Denmark) was applied. After incubating and washing the substrate solution (20 μl of 0.05 M citric acid and 0.1 M Na2HPO4 pH 5.0, 40 mg ortho-diphenylalanine [OPD], and 20 μl 30% H2O2) was added. The reaction was stopped with 50 μl/well of 2 M H2SO4. The optical density was measured at wavelength 492 nm by Titertek Multiscan 340 CC. Measurements were performed in triplo or duplo.

b) Tissue plasminogen activator (tPA) and plasminogen activator inhibitor I (PAI-1): tPA and PAI-1 antigens were measured with a commercial kit (Biopool Imulyse tPA, PAI-1, Umeå, Sweden) according to the procedure of the manufacturer. The tPA assay detects single- and two-chain tPA in complex with antiplasmin, PAI-1, and PAI-2. The PAI-1 assay detects active and latent PAI-1, whereas tPA/PAI-1 and tPA/PAI-1 complexes are poorly detected.

Statistics

Data are expressed in mean and standard deviation. The mean and standard deviation of experiments with the CS-deficient cell line was calculated from results from at least three different experiments. The mean and standard devia-
tion of the control cell lines was calculated from the means of at least three different control cell lines. The value obtained from a control cell line is the mean (without a standard deviation) of a duplo culture experiment of this cell line. Statistical analyses were performed with the t-test and p <0.05 was considered statistically significant.

Results

Characterisation of the HUVECs Deficient in Cystathionine β-synthase (CS)

In 6 different HUVECs from healthy controls and in the HUVECs from a patient with CS-deficiency (a sister of a CS-deficient patient homozygous for the T833C transition) the CS-activity was measured with and without addition of pyridoxal-5-phosphate to the enzyme assay mixture (Table 1). The mean enzyme activity of the six controls with and without pyridoxal-5-phosphate was 6.9 and 5.6 nmol cystathionine/h/mg protein respectively. The enzyme activity of the CS-deficient cell line with and without pyridoxal-5-phosphate was 0.88 and 0.18 nmol cystathionine/h/mg protein respectively (Table 1). The CS-activities of the control HUVECs were comparable with those observed in cultured normal fibroblasts and the CS-activity of the CS-deficient cell line was within the range of cultured fibroblasts of CS-deficient patients (2).

Genomic DNA was isolated from HUVECs of the CS-deficient patient and homozygosity for the T833C transition was demonstrated. This mutation is the most common cause of CS-deficiency in Dutch homocystinuria patients (2).

Homocysteine Export

1. Standard Conditions and the Effect of Folic Acid Supplementation

Under standard culture conditions HUVECs exported a fairly constant amount of homocysteine into the culture medium despite normal folate levels in the medium (20). The mean (± SD) homocysteine export in four different normal HUVECs under standard culture conditions after 24, 48, and 72 h of culture was 1.74 (± 0.68), 4.04 (± 1.23) and 6.40 (± 1.71) µM respectively. Addition of 3, 30, and 100 nM folinic acid (a stable precursor of MeTHF) to the standard culture medium reduced the homocysteine export in a dose-dependent manner (Table 2).

The mean homocysteine export of the CS-deficient HUVECs under standard culture conditions after 24, 48, and 72 h of culture was in the range as observed in normal HUVECs. Furthermore addition of folinic acid in various concentrations showed the same dose dependency as observed in normal HUVECs (Table 2).

When the homocysteine export was expressed in amount homocysteine per cell (pmol/cell) the CS deficient HUVECs showed also export in the range of normal HUVECs (Table 3).

Vitamin B12 supplementation to the standard culture medium (M199 did not contain Vitamin B12) did not result in differences in homocysteine export in similar experiments (data not shown).

2. Pyridoxine HCl Additions

Standard culture medium contained 200.5 nM pyridoxine according to the manufacturer. The homocysteine export did not show differences between standard culture medium and the media with additions of 0.3, 1, 10, 100, 1000 µM pyridoxine. No differences were found between control HUVECs and CS deficient HUVECs (Table 4).

When homocysteine export was expressed in homocysteine per amount of cells (pmol/cell) in normal and CS deficient HUVECs, also no differences were found under conditions as mentioned above (data not shown).

3. Methionine Loading

The homocysteine export was studied under various concentrations of methionine additions to the culture medium of normal and CS-deficient HUVECs.

Methionine deficient conditions of the culture medium were achieved by using M199 without methionine. In these experiments the only source of methionine to the culture medium was derived from the 20% serum which resulted in a methionine concentration of 5 µM in the culture medium. Under these conditions the homocysteine export was very low: in normal HUVECs the mean (± SD) of the homocysteine export after incubations of 24, 48, and 72 h was 0.6 (± 0.5), 1.2 (± 0.6), and 2.1 (± 0.9) µM respectively. In CS-deficient HUVECs similar results were obtained (Table 4).

After additions of methionine in the culture medium (0, 5, 10, 50, 100, 200 µM) the homocysteine export increased. This increase was
dose dependent with maximal homocysteine export of approximately 2 μM per 24 h at a methionine addition of 50 μM or more. Under all conditions studied, no differences could be seen between normal and CS deficient HUVECs (Table 4). Again no difference was found if the homocysteine export was expressed per cell (data not shown).

4. von Willebrand Factor (vWF)

In the culture medium of normal and CS-deficient HUVECs, vWF was measured in culture medium after various additions of folinic acid (0, 3, 30, and 100 nM) at various culture times (24, 48, and 72 h).

Table 3 At culture times of 24, 48, and 72 h homocysteine export was measured in culture medium of normal versus CS-deficient HUVECs was measured after additions to medium of folinic acid of 0, 3, 30, and 100 nM.

<table>
<thead>
<tr>
<th>folinic acid (nmol/l)</th>
<th>normal</th>
<th>CS-deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean (±SD)</td>
<td>mean (±SD)</td>
</tr>
<tr>
<td>0</td>
<td>6.9 (± 0.2)</td>
<td>5.4 (± 0.2)</td>
</tr>
<tr>
<td>3</td>
<td>4.0 (± 0.4)</td>
<td>2.9 (± 0.3)</td>
</tr>
<tr>
<td>20</td>
<td>2.0 (± 0.3)</td>
<td>1.5 (± 0.2)</td>
</tr>
<tr>
<td>100</td>
<td>0.1 (± 0.2)</td>
<td>1.6 (± 0.5)</td>
</tr>
</tbody>
</table>

Under standard culture conditions the mean (± SD) concentration of vWF increased slightly. The results of vWF measurements in culture media of CS-deficient HUVECs were similar to those of normal HUVECs (Table 5).

Additions of 3, 30 and 100 nM folinic acid to the culture medium of normal HUVECs did not result in significant changes of vWF concentrations compared with the results obtained by using standard culture medium alone. vWF levels in various culture media of CS-deficient HUVECs were similar to normal HUVECs (Table 5).

Similar results were obtained when vWF concentration in culture medium was expressed per cell (data not shown).

Table 4 At culture times of 24, 48, and 72 h homocysteine export was measured in culture medium of normal versus CS-deficient HUVECs was measured after additions to medium of pyridoxine HCl of 0, 0.3, 1, 10, 100, and 1000 μM and in methionine free M199 with additions of methionine of 0, 5, 10, 50, 100, 200 μM.

<table>
<thead>
<tr>
<th>Pyridoxine HCl (μmol/l)</th>
<th>24 hours tHcy (μmol/l) mean (±SD)</th>
<th>48 hours tHcy (μmol/l) mean (±SD)</th>
<th>72 hours tHcy (μmol/l) mean (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.4 (± 0.9)</td>
<td>4.5 (± 0.7)</td>
<td>5.4 (± 0.9)</td>
</tr>
<tr>
<td>0.3</td>
<td>2.8 (± 0.4)</td>
<td>4.3 (± 0.8)</td>
<td>6.4 (± 0.5)</td>
</tr>
<tr>
<td>1</td>
<td>2.7 (± 0.4)</td>
<td>4.6 (± 0.9)</td>
<td>5.7 (± 1.0)</td>
</tr>
<tr>
<td>10</td>
<td>2.6 (± 0.3)</td>
<td>4.0 (± 0.9)</td>
<td>5.8 (± 1.0)</td>
</tr>
<tr>
<td>100</td>
<td>2.5 (± 0.6)</td>
<td>4.0 (± 1.0)</td>
<td>5.6 (± 0.6)</td>
</tr>
<tr>
<td>1000</td>
<td>2.1 (± 0.5)</td>
<td>4.3 (± 0.9)</td>
<td>6.2 (± 0.8)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Methionine (μmol/l)</th>
<th>24 hours tHcy (μmol/l) mean (±SD)</th>
<th>48 hours tHcy (μmol/l) mean (±SD)</th>
<th>72 hours tHcy (μmol/l) mean (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.6 (± 0.3)</td>
<td>1.2 (± 0.6)</td>
<td>2.1 (± 0.9)</td>
</tr>
<tr>
<td>5</td>
<td>1.3 (± 0.7)</td>
<td>2.3 (± 0.7)</td>
<td>3.4 (± 0.9)</td>
</tr>
<tr>
<td>10</td>
<td>2.1 (± 0.6)</td>
<td>4.1 (± 0.7)</td>
<td>5 (± 1.3)</td>
</tr>
<tr>
<td>50</td>
<td>2.6 (± 0.3)</td>
<td>5.3 (± 1.3)</td>
<td>6.7 (± 1.0)</td>
</tr>
<tr>
<td>100</td>
<td>2.4 (± 0.1)</td>
<td>5.1 (± 0.8)</td>
<td>6.3 (± 0.8)</td>
</tr>
<tr>
<td>200</td>
<td>2.5 (± 0.1)</td>
<td>5.1 (± 0.6)</td>
<td>6.6 (± 0.8)</td>
</tr>
</tbody>
</table>
Table 5: At culture times of 24, 48, and 72 h vWF, tPA, and PAI-1 concentrations in culture medium of normal and CS-deficient HUVECs were measured after additions of 0, 3, 30, and 100 nM of folinic acid to medium.

<table>
<thead>
<tr>
<th>vWF(%) in medium with folinic acid of (nmol/l):</th>
<th>24 hours mean (±SD)</th>
<th>48 hours mean (±SD)</th>
<th>72 hours mean (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal</td>
<td>CS-deficient</td>
<td>normal</td>
<td>CS-deficient</td>
</tr>
<tr>
<td>0</td>
<td>108 (± 8) 117 (± 5)</td>
<td>122 (± 12) 129 (± 4)</td>
<td>137 (± 15) 143 (± 5)</td>
</tr>
<tr>
<td>3</td>
<td>103 (± 5) 113 (± 5)</td>
<td>115 (± 9) 126 (± 7)</td>
<td>135 (± 16) 145 (± 8)</td>
</tr>
<tr>
<td>30</td>
<td>100 (± 7) 111 (± 3)</td>
<td>116 (± 6) 119 (± 4)</td>
<td>130 (± 12) 144 (± 9)</td>
</tr>
<tr>
<td>100</td>
<td>107 (± 9) 113 (± 7)</td>
<td>116 (± 11) 121 (± 8)</td>
<td>138 (± 15) 142 (± 10)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>tPA (ng/ml) in medium with folinic acid of (nmol/l):</th>
<th>0</th>
<th>3</th>
<th>30</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>2.5</td>
<td>2.8</td>
<td>3.7</td>
<td>2.7</td>
</tr>
<tr>
<td>(± 1.3)</td>
<td>(± 0.3)</td>
<td>(± 1.1)</td>
<td>(± 0.6)</td>
<td>(± 1.2)</td>
</tr>
<tr>
<td>7.3</td>
<td>10.7</td>
<td>8.3</td>
<td>8.2</td>
<td>7.8</td>
</tr>
<tr>
<td>(± 2.6)</td>
<td>(± 1.0)</td>
<td>(± 1.4)</td>
<td>(± 1.7)</td>
<td>(± 0.9)</td>
</tr>
<tr>
<td>8.9</td>
<td>19.0</td>
<td>8.7</td>
<td>8.3</td>
<td>8.6</td>
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<tr>
<td>(± 3.7)</td>
<td>(± 3.0)</td>
<td>(± 4.7)</td>
<td>(± 3.5)</td>
<td>(± 4.7)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PAI-1 (ng/ml) in medium with folinic acid of (nmol/l):</th>
<th>0</th>
<th>3</th>
<th>30</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>452</td>
<td>376</td>
<td>424</td>
<td>414</td>
<td>584</td>
</tr>
<tr>
<td>(± 178)</td>
<td>(± 77)</td>
<td>(± 192)</td>
<td>(± 160)</td>
<td>(± 338)</td>
</tr>
<tr>
<td>938</td>
<td>791</td>
<td>811</td>
<td>861</td>
<td>784</td>
</tr>
<tr>
<td>(± 429)</td>
<td>(± 194)</td>
<td>(± 272)</td>
<td>(± 283)</td>
<td>(± 373)</td>
</tr>
<tr>
<td>1082</td>
<td>1092</td>
<td>962</td>
<td>898</td>
<td>904</td>
</tr>
<tr>
<td>(± 269)</td>
<td>(± 494)</td>
<td>(± 269)</td>
<td>(± 269)</td>
<td>(± 373)</td>
</tr>
</tbody>
</table>

(*) Significant difference between normal and CS-deficient HUVECs in incubation medium of 72 hrs. under standard culture conditions. (p<0.01).

5. Tissue Plasminogen Activator (tPA)

In the culture medium of normal and CS-deficient HUVECs tPA-antigen concentrations were measured under the same conditions as mentioned above in paragraph 4. With progression of culture time (24, 48 and 72 h) the mean level of tPA in the culture medium increased in normal HUVECs (Table 5).

The results of tPA measurements in culture media of CS-deficient HUVECs were similar to the results of normal HUVECs. Only the tPA concentration in culture medium of CS-deficient HUVECs without folinic acid addition after 72 h was significantly elevated compared with tPA concentration in culture medium of normal HUVECs under the same conditions (Table 5). Additions of the various concentrations of folinic acid to the culture medium at various incubation times (see paragraph 4) showed no significant differences in tPA concentrations between normal and CS-deficient HUVECs.

Expression of tPA concentration per cell showed similar results (data not shown).

6. Plasminogen Activator Inhibitor (PAI-1)

Under the same conditions as mentioned above in paragraph 4, PAI-1 antigen concentrations were measured in the culture medium of normal and CS deficient HUVECs. With progression of culture time (24, 48 and 72 h) the mean level of PAI-1 in the culture medium in-
creased in normal HUVECs (Table 5). The results of PAI-1 measurements in culture media of CS-deficient HUVECs were similar to the results of normal HUVECs (Table 5).

Additions of the various concentrations of folic acid to the culture medium did not result in any difference in PAI-1 concentrations within or between the different cell lines (Table 5).

Expression of PAI-1 concentrations per cell showed similar results (data not shown).

Discussion

HUVECs lacking virtually all CS-activity due to homozygosity for the T833C transition, showed the same homocysteine export as normal HUVECs. Furthermore no differences between these CS-deficient and normal cell lines were observed in response to additions to the culture medium of:

1. Folinic acid: in both the normal and the CS-deficient cell lines the homocysteine export was reduced in a dose dependent manner.
2. Pyridoxine: additions of pyridoxine to the culture medium did not affect homocysteine export.
3. Methionine: additions of methionine ("methionine loading") increased the homocysteine export in a dose dependent manner until a maximal level was reached at approximately 50 μmol methionine/L.

Again, there was no difference in response to this methionine loading between the CS-deficient and the normal HUVECs.

Even under these various extreme conditions the homocysteine export did not differ between the control and the CS-deficient HUVECs, which suggests that CS does not contribute to the homocysteine metabolism in endothelial cells. Therefore the high blood homocysteine levels in CS-deficient patients seem to be due to deficient CS function in liver and kidney. In these organs CS is expressed at a high level (27).

Also no differences between control and CS-deficient HUVECs were present in concentrations of vWF, tPA, and PAI-1 (markers of endothelial function). These concentrations increased with almost constant amount in the culture medium every 24 h, which is a normal property of cultured endothelial cells (28-30). Folinic acid addition to the medium, which reduced the homocysteine export, did not affect these endothelial cell function parameters. In this experiment again the CS-deficient endothelial cells were indistinguishable from normal HUVECs lacking virtually all CS-activity due to homozygosity for the T833C transition.

The possible contribution of endothelial stress factors such as mechanical stress, oxidant stress or other stress factors to our results could not be studied. The present investigation showed that CS-deficient endothelial cells have normal homocysteine export and normal endothelial cell function. Therefore, in CS-deficient patients the very high blood levels of homocysteine, probably due to deficient CS function in liver and kidney, seem to be the hazardous factor to endothelial cells and thus promoting atherosclerosis and thrombosis in CS-deficient patients.

Acknowledgments

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