Homocysteine, a Risk Factor for Premature Vascular Disease and Thrombosis, Induces Tissue Factor Activity in Endothelial Cells

Richard H. Fryer, Brent D. Wilson, David B. Gubler, Laurence A. Fitzgerald, George M. Rodgers

Elevated blood levels of homocysteine represent an independent risk factor for premature arterial vascular disease and thrombosis. We investigated whether homocysteine could induce tissue factor (TF) procoagulant activity in cultured human endothelial cells. Homocysteine increased cellular TF activity in a time- and concentration-dependent manner. Low concentrations of homocysteine (0.1 to 0.6 mmol/L), similar to those found in the blood of patients with homocystinuria, enhanced TF activity by 25% to 100%. Other sulfur-containing amino acids (cystine, homocystine, cysteine, and methionine) induced less TF activity than did homocysteine; however, β-mercaptoethanol and dithiothreitol were more effective than homocysteine in increasing TF activity. Preincubation of homocysteine with a sulfhydryl inhibitor such as JV-ethylmaleimide prevented homocysteine induction of TF activity. A quantitative polymerase chain reaction method indicated that homocysteine increased TF mRNA in endothelial cells. These results support the hypothesis that perturbation of vascular coagulant mechanisms may contribute to the thrombotic tendency seen in patients with homocystinuria. (Arterioscler Thromb. 1993;13:1327-1333.)

Key Words • homocysteine • tissue factor • thrombosis • endothelium

Endothelial cell (EC) injury is postulated to initiate atherosclerosis. Two conditions that are associated with atherosclerotic vascular disease are elevated serum levels of cholesterol and homocysteine. While previous studies have focused on the mechanisms by which hyperlipidemia initiates atherogenesis and its complications, recent attention has been devoted to homocysteine as a risk factor for vascular disease.

Homocystinuria is an autosomal recessive metabolic disorder that usually results from cystathionine-β-synthase deficiency. Homozygous patients will experience arterial and/or venous thromboembolic events at an early age, accelerated vascular disease, and other abnormalities, including mental retardation and osteoporosis. Investigation of the mechanism(s) by which homocysteine induces vascular injury has revealed that cultured ECs that are derived from an obligate heterozygote are deficient in cystathionine-β-synthase and are more susceptible to homocysteine-mediated injury than are normal cells and that sulfur-containing amino acids perturb ECs in vitro by a mechanism that involves hydrogen peroxide generation. Thus, these studies have suggested that EC dysfunction might be important in homocysteine-mediated vascular disease.

The importance of homocystinuria as a cause of vascular disease in a larger number of patients has been emphasized in a series of clinical studies performed over the past several years. These studies investigated patients with premature arterial disease (coronary or cerebrovascular thrombosis or peripheral arterial disease). Cumulatively, these studies reported that 14% to 30% of patients with premature arterial disease had heterozygous homocystinuria as an independent risk factor for thrombosis.

Our laboratory and others have studied the vascular mechanisms by which homocysteine might promote thrombosis. Our previous studies have noted that homocysteine increases arterial EC factor V activity by inducing an activator of factor V and that homocysteine downregulates EC activation of protein C. These effects of homocysteine would result in an enhanced vascular procoagulant activity and a thrombotic tendency. The purpose of this study was to investigate homocysteine-induced EC tissue factor (TF) expression. Our data indicate that homocysteine, in concentrations that are found in the blood of patients with homocystinuria, can initiate coagulation.

Methods

Tissue-culture plastic ware was obtained from Falcon Plastics (Oxford, Calif). Medium 199 (M199) and other tissue-culture reagents were obtained from the University of California, San Francisco Cell Culture Facility.
Fetal calf serum was supplied by Hyclone (Logan, Utah). D,L-Homocysteine, D,L-homocystine, D,L-methionine, D,L-cysteine, D,L-cystine, glutathione, N-ethylmaleimide (NEM), superoxide dismutase, β-mercaptoethanol, dithiobiotreitol, Trizma base, rabbit brain thromboplastin, catalase, and gel electrophoresis supplies were provided by Sigma Chemical Co (St Louis, Mo). Proplex-T concentrate was supplied by Baxter-Hyland (Glendale, Calif). The chromogenic substrate S-2222 was purchased from Kabi Vitrum (Franklin, Ohio). Plasmas deficient in factors VII, VIII, or X were obtained from George King Biomedical (Overland Park, Kan).

Human umbilical vein endothelial (HUVE) cells were prepared as described. Human umbilical cords were digested with collagenase and cultured in 80% M199, 20% fetal calf serum, antibiotics (penicillin [final concentration, 100 U/mL], streptomycin [100 U/mL], and amphotericin [0.06 μg/mL]), and supplemental l-glutamine (2 mmol/L). HUVE cells were used during the first passage. TF assays were performed on cells plated in 24-well trays (17-mm diameter). Homocysteine-induced HUVE cell TF activity was assayed by incubating varying concentrations of homocysteine (dissolved in serum-containing or serum-free medium) with confluent cells at 37°C and then by using a chromogenic substrate assay. This method homocysteine or other agents were diluted in medium and sterile filtered. After incubation, the cells were washed and the chromogenic substrate assay was performed by adding phenol-free M199 (0.4 mL), S-2222 (0.1 mL; final concentration, 200 μg/mL), and Proplex-T (0.010 mL; final factor VII concentration, 1.0 U/mL) to each well. After a 30-minute incubation the absorbance of each well’s supernatant was determined. The samples consisted of dilutions of the stock thromboplastin was equivalent to 1000 μL of TF activity. This assay measured cell-surface expression of TF activity. The procoagulant activity induced by homocysteine was confirmed as TF by using homocysteine-treated cell lysates (10 mmol/L, 8 hours). After incubation the cell lysates were prepared by washing control and treated cells with tris(hydroxymethyl)aminomethane (Tris)-saline and then adding 0.1 mL Tris-saline to each dish. Cells were scraped with a rubber policeman into an Eppendorf tube and then incubated with the TF extract for 5 to 10 minutes before determination of the clotting time.

To determine whether sulfur-containing amino acids directly affected TF procoagulant activity in a cell-free system, we used an acetone powder of rabbit brain TF (Sigma) and a recalcification clotting method. In this assay 0.1 mL of normal human plasma, 0.1 mL of sample in Tris-saline buffer, and 0.1 mL of 25 mmol/L CaCl2 were incubated at 37°C, and an automated clotting time was determined. The samples consisted of dilutions of the commercial TF preparation with or without sulfur-containing amino acids. Concentrations of this commercial TF preparation (0.25 to 0.5 mg/mL) were found to result in clotting times of 50 to 60 seconds compared with buffer clotting times of >300 seconds. Varying concentrations of amino acids (0.3 to 3.3 mmol/L) were then incubated with the TF extract for 5 to 10 minutes before determination of the clotting time. The ability of homocysteine to induce TF mRNA production was assessed by using a quantitative polymerase chain reaction (PCR) technique. For these studies, control fibroblasts (which constitutively express TF activity) and untreated HUVE cells were compared with HUVE cells that were treated with homocysteine for 1 to 5 hours. The treated and control cells (104 to 105) were scraped from the flask, washed once in serum-free medium, and frozen at -70°C; total RNA was isolated at a later time by the guanidinium thiocyanate–phenol-chloroform-methanol method by using a kit from Stratagene (La Jolla, Calif). To produce a PCR primer set, single-strand cDNA was synthesized by using M-MuLV reverse transcriptase (200 U, BRL, Gaithersburg, Md) on 1 μg of total RNA, standard protocols, and reagents supplied with the enzyme. cDNA synthesis was primed by using random 9-mer oligonucleotides.

To detect the presence of TF, a PCR primer set was synthesized on the basis of the cDNA sequence of Morrissey et al to give an expected product of 310 bp. To eliminate a potential contribution of genomic DNA, the primers were located on exons 2 and 4 of the published TF gene: TF forward primer: 5'-CAC AAT TTT GGA GTG GCA ACC C-3' (cDNA No. 189-210); TF reverse primer: 5'-CAC TTT TGT TCC CAC CTG-3' (cDNA No. 498-481).

To normalize the PCR amplification in different cDNA preparations, a second set of PCR oligonucleotide primers against a control protein, glyceraldehyde-3-phosphate dehydrogenase (GPDH), was synthesized as described by Hua and Maciag: GPDH forward primer: 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3'; GPDH reverse primer: 5'-TCT AGA CGG CAG GTG AGG TCC ACC-3'. The GPDH primers produced an expected product of 650 bp and are also on separate exons. A Hybaid thermal cycler (model HB, TR-1) was used for 30 cycles with each cycle consisting of 90 seconds at 95°C, 55°C, and 73°C in the "plate mode" to regulate temperature. PCR products were separated by electrophoresis on 1.5% agarose Tris-glycine gels and stained with ethidium bromide. To quantitate the amount of specific TF and GPDH products, 0.5 μL of each TF primer oligonucleotides, 1 μL of a 25 mmol/L solution of each dNTP (Pharmacia), and a reaction buffer containing 1.5 mmol/L MgCl2, 10 mmol/L Tris-HCl, 50 mmol/L KCl, and 0.1 mg/mL gelatin, pH 8.3, from Boehringer Mannheim. To label the TF and GPDH PCR products for quantitation, 1 μCi of α[32P]-dCTP (Amersham, Arlington Heights, II) was added per 100 μL of reaction volume to give a final concentration of 1.16 pmol/L. Equal aliquots from the PCR products were fractionated on 1.3% agarose/Tris-borate-EDTA gels and stained with ethidium bromide. To quantitate the amount of specific TF and GPDH products, the [32P]-labeled GPDH (650 bp) and TF (310 bp) bands were excised and counted in a Beckman scintillation counter. We quantitated the amount of TF product as the ratio of TF disintegrations per minute per GPDH disintegrations per minute. This ratio from HUVE cells and monocyte samples was then compared
with that from fibroblasts to determine the fold increase in mRNA levels.

Results

Time-course studies of TF induction were initially done by incubating a large concentration of homocysteine (10 mmol/L) with HUVE cells (Fig 1). This concentration of homocysteine was selected because 10 mmol/L homocysteine induced maximal EC factor V activity and maximal suppression of protein C activation in our previous studies. In contrast to the time-course results reported for other inducers of TF activity such as cytokines and endotoxin, in which maximal TF activity occurred by 4 to 6 hours and with lesser TF activity after longer incubation times, homocysteine induced a steady increase in HUVE cell TF activity through 6 to 8 hours (Fig 1). Our previous experiments indicated that concentrations of homocysteine up to 10 mmol/L did not cause EC cytotoxicity (as measured with a Cr-release assay) when incubated with cells for up to 16 hours. Procoagulant activity induced by homocysteine was shown to be dependent on both factors VII and X but not factor VIII (recalcification clotting-time assay and a commercial TF assay). Each value represents TF activity of homocysteine-treated cells, expressed as a percentage of control cells at that interval. The mean value of triplicate experiments is shown.

Fig 2 demonstrates the concentration-response relations for homocysteine-induced HUVE cell TF expression. As depicted in the left panel of Fig 2, homocysteine increased HUVE cell TF activity in a concentration-dependent manner, with a sevenfold increase in TF activity seen with 10 mmol/L homocysteine. Higher concentrations of homocysteine induced HUVE cell cytotoxicity, with cell retraction and detachment.

To determine whether the concentrations of homocysteine found in the blood of affected homocystinuric patients could induce HUVE cell TF activity, substantially lower homocysteine concentrations (0.1 to 0.6 mmol/L) were incubated with cells for 10 hours (Fig 2, right). This longer incubation period was chosen on the basis of results of time-course experiments with a 0.5 mmol/L homocysteine concentration (data not shown).

Homocysteine at a concentration of 0.1 mmol/L increased TF activity by 25% over control values, while 0.2 to 0.3 mmol/L homocysteine enhanced HUVE cell TF activity by 44%, and 0.6 mmol/L homocysteine increased TF activity >100% over control values. We observed that similar results were observed when homocysteine was incubated with cells in serum-free medium or serum-containing medium. Experiments were subsequently conducted with homocysteine dissolved in serum-containing medium.

The effects of other related sulfur-containing amino acids were investigated, and these results are shown in Fig 3. Homocystine and cystine were minimally soluble in the medium, and neither of these oxidized amino acids induced significant TF activity over control cell values when tested at a 1 mmol/L concentration (data not shown). Cysteine and methionine, two amino acids related to homocysteine, had less activity than homocysteine. However, β-mercaptoethanol had greater activity than homocysteine in inducing HUVE cell TF activity (Fig 3). When a number of free-thiol reagents were compared for their ability to induce HUVE cell TF activity at 2.5 and 5 mmol/L concentrations, the following order of potency was observed: β-mercaptoethanol was the most effective, followed by dithiothreitol, homocysteine, and glutathione in decreasing order (data not shown).

Other investigators have reported that the suppressive effect of homocysteine on EC protein C activation results from the free sulfhydryl group of the amino acid. To determine whether TF induction was dependent on homocysteine’s sulfhydryl group, titration experiments were conducted with the sulfhydryl inhibitor NEM. This inhibitor was incubated with homocysteine in molar ratios (NEM to homocysteine) of 2.5:10, 5:10, and 10:10 for 15 minutes and then diluted in HUVE cell medium for the TF assay. There was a concentration-dependent decrease in homocysteine-induced TF activity by NEM (Table 1).

The ability of sulfur-containing amino acids to directly affect TF procoagulant activity was assessed by a recalcification clotting-time assay and a commercial TF
FIG 2. Concentration-response relations for homocysteine-induced human umbilical vein endothelial (HUVE) cell tissue factor (TF) expression. Left panel, Concentration-response curve showing the relation between high concentrations of homocysteine and HUVE cell TF expression. Homocysteine was dissolved in serum-containing medium (0.6 to 10 mmol/L) and incubated with HUVE cells for 8 hours. TF activity was measured with a chromogenic substrate assay\(^\text{17}\) and expressed as TF activity (milliunits per microgram of cell protein) on a semilogarithmic scale. Each value represents the mean of triplicate experiments. Right panel, Low concentrations of homocysteine (0.1 to 0.6 mmol/L) were dissolved in serum-containing medium and incubated with cells for 10 hours. TF activity was measured with a chromogenic substrate assay\(^\text{17}\) and expressed as TF activity (milliunits per microgram of cell protein). Each value represents the mean of triplicate experiments.

preparation in a cell-free system. Coincubation of homocysteine, dithiothreitol, or β-mercaptoethanol with this TF preparation enhanced the ability of TF to shorten the clotting time of normal plasma by up to 25%. When these amino acids were incubated with buffer alone before determination of the clotting time, no shortening of the clotting time occurred. These results suggest that certain sulfur amino acids can directly increase TF activity; perhaps this effect is related to their ability to reduce disulfide bonds and is independent of their ability to induce TF mRNA.

Starkebaum and Harlan\(^\text{7}\) reported that homocysteine-induced EC injury occurred as a result of copper-catalyzed hydrogen peroxide generation from homocysteine and that catalase prevented EC injury. We investigated whether catalase and superoxide dismutase (which scavenge hydrogen peroxide and superoxide anion, respectively) could inhibit the effects of homocysteine in inducing EC TF activity. However, catalase alone in concentrations of 1 to 2 U/mL induced TF activity; higher catalase concentrations induced a dose-dependent increase in TF activity (data not shown). Coincubation of catalase with homocysteine resulted in an additive procoagulant effect. When superoxide dismutase (200 U/mL) was coincubated with homocysteine, TF expression was not inhibited.

We also tested the effects of hydrogen peroxide directly in the EC TF model. Concentrations of H\(_2\)O\(_2\) of 60 μmol/L incubated with ECs for 4 hours increased TF activity.

**TABLE 1. Inhibition of Homocysteine-Induced Human Umbilical Vein Endothelial Cell Tissue Factor Activity by NEM**

<table>
<thead>
<tr>
<th>Sample</th>
<th>TF activity (％ of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Homocysteine (10 mmol/L)</td>
<td>406</td>
</tr>
<tr>
<td>NEM/homocysteine (molar ratio)</td>
<td></td>
</tr>
<tr>
<td>2.5:10</td>
<td>315</td>
</tr>
<tr>
<td>5:10</td>
<td>249</td>
</tr>
<tr>
<td>10:10</td>
<td>106</td>
</tr>
</tbody>
</table>

Confluent human umbilical vein endothelial cells were incubated with either serum-free medium (control), 10 mmol/L homocysteine, or varying molar ratios of N-ethylmaleimide (NEM) to homocysteine for 8 hours. For example, the 1:4 NEM/homocysteine incubation consisted of a final concentration of 2.5 mmol/L NEM and 10 mmol/L homocysteine. NEM alone was grossly cytotoxic to the cells and induced a 10-fold increase in tissue factor (TF) activity. Each value represents the mean of triplicate determinations.
FIG 4. Agarose gel of the polymerase chain reaction using specific glyceraldehyde-3-phosphate dehydrogenase (GPDH) and tissue factor (TF) primers and a template cDNA input. Note consistent amplification of GPDH, a 650-bp product, and a variable TF primer-specific product of 310 bp. Primers were used in separate reaction tubes. The standard (Std.; lane 1) is the 1-kb ladder (BRL) with size fragments indicated. Lanes 2 and 8, human umbilical vein endothelial (HUVE) cells, control; lanes 3 and 9, HUVE cells, 2 hours, 10 mmol/L homocysteine; lanes 4 and 10, HUVE cells, 3 hours, 10 mmol/L homocysteine; lanes 5 and 11, HUVE cells, 5 hours, 10 mmol/L homocysteine; lanes 6 and 12, fibroblasts; lanes 7 and 13, polymerase chain reaction control, no cDNA added.

activity fivefold over control values; 100 μmol/L H2O2 increased TF activity eightfold. Higher concentrations of H2O2 (>100 μmol/L) caused gross EC injury and detachment.

To determine whether homocysteine treatment of HUVE cells resulted in de novo synthesis of TF by stimulating transcription, the effects of homocysteine on increasing TF mRNA were investigated with PCR. As a control to normalize the variables present in the cDNA synthesis and amplification processes, mRNA for GPDH was also quantitated, since GPDH is expressed constitutively and expression is probably not affected by homocysteine. In the quantitative studies, TF expression was calculated as a ratio of TF mRNA to GPDH mRNA.

Total RNA was isolated from control and homocysteine-treated cells, and single-strand cDNA was synthesized with M-MuLV reverse transcriptase. Preliminary experiments were performed to determine the optimal input of cDNA and primer concentrations. Fig 4 shows the results of these studies. Similar to other EC/monocyte agonists, homocysteine induced a transitory response in ECs such that the amount of TF mRNA peaked at 3 hours and was almost undetectable at 2 or 5 hours after induction. Quantitation of PCR products using 32P-dCTP showed that relative to fibroblasts, there was nearly a fourfold increase in TF mRNA synthesis after 3 hours of homocysteine treatment (Table 2).

Discussion

The recent appreciation of the potential importance of abnormal homocysteine metabolism in the pathogenesis of premature arterial disease, including myocardial infarction, thrombotic stroke, and peripheral arterial occlusion,8-11 has led to increased investigation into the mechanism(s) by which homocysteine induces a thrombotic state. A large number of studies have reported on the mechanism of homocysteine-induced EC injury; the free sulfhydryl group has been noted to be important in mediating hydrogen peroxide generation,12 which leads to cytotoxicity.6 However, a recent report studied the possibility of in vivo lipid peroxidation in homocysteinemic patients and concluded that lipid hydroperoxidation was not an important mechanism mediating vascular disease in this disorder.28 In terms of thrombotic mechanisms, homocysteine (the disulfide dimer of homocysteine that is also found in increased concentrations in the blood of homoycystinuric patients) activates factor XII,29 and homocysteine activates EC factor V12 and suppresses protein C activation.13 The present report indicates that homocysteine may also initiate coagulation by inducing TF activity in ECs. We observed that concentrations of homocysteine as low as 0.1 to 0.3 mmol/L, similar to those found in the blood of affected patients, could increase EC TF activity. The low homocysteine concentrations required a longer incubation time to induce maximal TF activity (10 hours) than did higher concentrations (8 hours).

Homocysteine also enhances atherogenesis by other mechanisms. Homocysteine has been reported to increase monocyte adhesion to human aortic ECs30 and to increase the binding of an atherogenic lipoprotein [lipoprotein(a)] to fibrin.31 Thus, homocysteine may modulate aspects of atherogenesis, vascular disease, and thrombosis.

The majority of sulfhydryl amino acids present in the blood of these patients are in the form of mixed disulfides. However, Araki and Sako, using a fluorescent agent specific for binding free thiols,32 have identified the presence of homocysteine in patient plasma (0.3 μmol/L).

Our data on the mechanism for homocysteine-induced TF activity suggest that the activity of homocysteine is dependent on the free sulfhydryl group and is mediated in part by TF gene transcription. The inability of methionine (with its methylated sulfur group) and the marked ability of β-mercaptoethanol and other potent reducing agents to stimulate TF activity is consistent with an important role for the free thiol. However, cysteine also has a free thiol group and induces much less TF activity than does homocysteine. Thus, there may be additional requirements important for the induction of procoagulant activity.

Generation of hydrogen peroxide may also be important in the mechanism of homocysteine-induced TF activity. We were unable to demonstrate inhibition of

### Table 2. Induction of Tissue Factor mRNA by Homocysteine in Human Umbilical Vein Endothelial Cells by Quantitation of Specific Polymerase Chain Reaction Products

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Time of induction (h)</th>
<th>TF/GPDH*</th>
<th>Fold increase over fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVE</td>
<td>0</td>
<td>0.0742</td>
<td>0.0357</td>
</tr>
<tr>
<td>HUVE</td>
<td>2</td>
<td>0.0357</td>
<td>0.48</td>
</tr>
<tr>
<td>HUVE</td>
<td>3</td>
<td>0.2870</td>
<td>3.80</td>
</tr>
<tr>
<td>HUVE</td>
<td>5</td>
<td>0.0185</td>
<td>0.25</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>0</td>
<td>0.0752</td>
<td>...</td>
</tr>
</tbody>
</table>

*Disintegrations per minute of the primer-specific 310-bp tissue factor (TF) band divided by the specific glyceraldehyde-3-phosphate dehydrogenase (GPDH) 650-bp band.
†There were no measurable disintegrations per minute associated with the TF-specific polymerase chain reaction product compared with control polymerase chain reaction in which no templates were used.

HUVE, human umbilical vein endothelial.
he homocysteine effect with catalase because the enzyme itself induces TF activity. However, since the interaction of homocysteine with ECs is known to result in hydrogen peroxide generation,7 and we have demonstrated TF induction by hydrogen peroxide, we speculate that hydrogen peroxide may mediate TF expression in this model. A recent study has demonstrated in vivo oxidative changes in ECs after neutrophil activation.39 A fluorescent probe specific for hydroperoxides has detected oxidants released by activated neutrophils; these oxidants were associated with ECs. Thus, within the microenvironment of the perturbed vascular endothelium, hydrogen peroxide may accumulate because of metal catalysis of homocysteine,7 resulting in perturbation of EC coagulant functions.

Our finding of peak homocysteine-induced TF mRNA levels after 3 hours with a return to basal, nondetectable levels is similar to that reported for TF mRNA induction in other models.34 One question raised by the transient expression of TF in vitro is the relation to the persistent hypercoagulable state seen in patients with homocystinuria. EC desquamation and circulating ECs have been reported in an animal model of the disease and are associated with an increase in the [3H]thymidine-labeling index of vascular endothelium.35 We speculate that newly regenerated ECs would subsequently respond to homocysteine perturbation by expression of TF procoagulant activity. Increased turnover of the vascular endothelium and recurrent expression of TF activity might account for the persistent thrombotic tendency seen in homocystinuria.

Mild reducing agents such as homocysteine have been previously shown to inhibit EC protein C activation by reducing critical disulfide bonds in the thrombomodulin molecule.14 One possible explanation for the effect of homocysteine in increasing TF activity is that homocysteine may reduce disulfide bonds in TF, resulting in an enhanced procoagulant activity. It is conceivable that the free thiol group of homocysteine may modify TF activity once it has been expressed on the cell surface, and we observed enhanced TF activity in vitro after treating a commercial TF preparation with reducing agents. Other posttranscriptional events that may account for our findings include homocysteine-induced membrane alterations that may modulate catalytic activity of the TF-factor VIIa complex.36 Studies with homocysteine-treated relipidated TF will be necessary to exclude a role for this reducing activity of homocysteine.

Acknowledgments

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