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Homocysteine Induces Trophoblast Cell Death with Apoptotic Features[1]

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ABSTRACT

Hyperhomocysteinemia has been suggested as a possible risk factor in women suffering from habitual abortions, placental abruption or infarcts, preeclampsia, and/or intrauterine growth retardation. However, little is known about the pathogenic mechanisms underlying the action of homocysteine. The present study investigated the in vitro ability of homocysteine to affect trophoblast gonadotropin secretion and to induce cell death. In primary human trophoblast cells, homocysteine treatment (20 μmol/L) resulted in cellular flattening and enlargement, extension of pseudopodia, and cellular vacuolization. Cellular detachment, apoptosis, and necrosis were favored. With in situ nick end labeling, we investigated DNA degradation, and we used M30 CytoDEATH to selectively stain the cytoplasm of apoptotic cells. Cytochrome *c* release from mitochondria to the cytosol and DNA cleavage in agarose gel have been investigated. Homocysteine, but not cysteine, induced trophoblast apoptosis and significantly reduced human chorionic gonadotropin secretion. These findings suggest that trophoblast cell death might represent a pathogenic mechanism by which homocysteine may cause pregnancy complications related to placental diseases.

apoptosis, human chorionic gonadotropin, placenta, trophoblast

INTRODUCTION

The hypothesis that hyperhomocysteinemia is a risk factor for vascular disease was proposed approximately 30 years ago by McCully [1], who observed advanced vascular lesions in children with inherited disorders of homocysteine (Hcy) metabolism. An association between severe hyperhomocysteinemia (fasting plasma Hcy concentration, >100 μmol/L) and thrombotic vascular disease was subsequently demonstrated in patients with hereditary deficiency of cystathionine β-synthase [2]. Recent interest in this field has been heightened by the growing recognition that moderate hyperhomocysteinemia (fasting plasma Hcy concentration, 25–100 μmol/L) also is a common finding and is associated with increased risk of stroke, cardiovascular disease, and venous thrombosis [3]. Mild hyperhomocysteinemia (fasting plasma Hcy concentration, 16–24 μmol/L) can be caused by deficiencies of vitamins B_6, B_{12}, or folate related to malnutrition or by genetically determined reduction in the enzyme activity.

Studies have confirmed that the homozygosity of C677T methylenetetrahydrofolate reductase mutation is associated with high levels of Hcy in healthy women [4], and it could be related to recurrent pregnancy losses or vascular problems, such as placental abruption, infarcts, and fetal growth retardation [5]. Several mechanisms were advanced to explain the toxicity of Hcy on the vascular endothelium. Some of them concerned hydrogen peroxide production: With the catalytic help of copper ion in serum, Hcy produces reactive oxygen species and, thus, injures cells by oxidative stress [6, 7]. However, cysteine, which is present at a much higher concentration than Hcy in vivo, is capable of generating reactive oxygen species but does not cause endothelial cell injury [8, 9]. This argues that a stimulus other than an oxidative burst might mediate Hcy-induced endothelial cell injury.

In human umbilical vein endothelial cells, Hcy has been found to reduce DNA synthesis and to increase the percentage both of apoptotic cells and of cells with a necrotic morphology [10]. The premature cell detachment, apoptosis, and necrosis of vascular endothelial cells under the influence of high Hcy concentrations might be the underlying causes of vascular disorders related to this amino acid. Several reports have clearly shown an association of elevated Hcy concentrations and obstetrical diseases that are connected with vascular disorders of pregnancy or of the uteroplacental unit [11–14]. Recently, Khong and Hague [15] reviewed the placental pathology in women diagnosed retrospectively to have hyperhomocysteinemia 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. Even if these findings suggest that elevated concentrations of Hcy could be a marker and, perhaps, a cause of a wide range of obstetrical disorders, the mechanisms of action by Hcy, as a causal factor of pregnancy complications, are still not understood.

The purpose of the present study was to investigate the possibility that Hcy could interfere with trophoblast functions. When trophoblast cells were exposed to Hcy (20 μmol/L), consecutive events were observed: the cytosolic release of cytochrome *c*, an increase in M30-positive trophoblast cells, an increased number of positive nuclei by TUNEL, and the internucleosomal DNA fragmentation. The cellular death and, consequently, the inhibition of trophoblast functions might represent one of the key mechanisms by which Hcy affects implantation and induces pregnancy complications.

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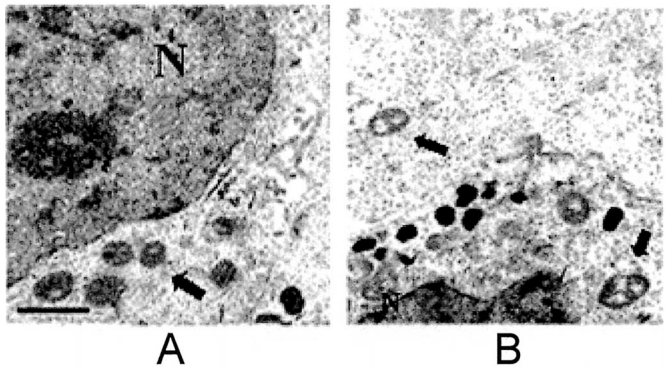

FIG. 1. Electron microscopic studies of Hcy-treated placental cells. **A**) Untreated trophoblast cell. The arrow points to mitochondria that show normal structural integrity and finely preserved cristae. N, nuclei. **B**) Hcy (20 μmol/L)-treated trophoblast cell. The arrows to mitochondria that are swollen and vacuolated. Bar = 1 μm. Magnification ×8000.

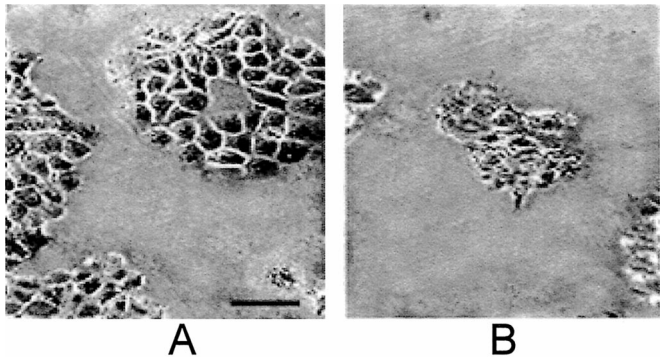

FIG. 2. Effects of Hcy on the morphology of trophoblast cells. **A**) Phase-contrast microscopy of untreated cells. **B**) Cells treated for 48 hours with DL-Hcy (20 μmol/L). Morphological changes are evident in treated cells. Bar = 50 μm. Magnification ×400.

MATERIALS AND METHODS

Primary Trophoblast Cells

Placentae were obtained from healthy women immediately after uncomplicated vaginal delivery at 36 wk of gestation. Informed consent for the use of human tissues in the present study was obtained from all patients.

The cytotrophoblast cells were isolated as detailed elsewhere [16]. Briefly, placental tissues were rinsed two to three times in cold Dulbecco modified Eagle medium (DMEM)-10% fetal calf serum (FCS; Gibco BRL, Grand Island, NY). After mincing, the tissues were submitted to repeated enzymatic digestions in Ringer-bicarbonate buffer containing 0.25% trypsin (Gibco BRL) and DNaseI (0.2 mg/ml; Sigma-Aldrich S.r.L., St. Louis, MO) at 37°C in a shaking water bath for 2 h. The supernatants were filtered through a 42-μm mesh filter and centrifuged (200 × *g* at room temperature for 7 min); then, the cell suspension was layered over a preformed Percoll (Pharmacia-Biotech, Uppsala, Sweden) gradient in Hanks balanced salt solution (HBSS; Gibco BRL). The gradient was made from 5% to 70% Percoll (v/v) by dilutions of 90% Percoll (nine parts Percoll and one part 10× HBSS) and then layered in a 50-ml conical polystyrene centrifuge tube. After centrifugation (200 × *g* at room temperature for 20 min), the middle layer was removed, washed, and then resuspended in DMEM. Cell viability, as evaluated by trypan blue exclusion, was greater than 95%. Cell purity was determined by immunostaining for markers of fibroblasts (2%, as determined using a polyclonal antivimentin antibody; Labsystem, Helsinki, Finland) and macrophages (3%, as determined using a polyclonal anti-α_1-chymotrypsin antibody; DAKO, Santa Barbara, CA).

The enriched (95%) cytotrophoblast cells, stained with polyclonal anticytokeratin antibody (Labsystem), were cultured at 37°C in DMEM-10% FCS at 37°C in 5% CO_2/95% air.

Cell Detachment Assay

In preliminary experiments, trophoblast cells were plated in 24-well plates (Falcon, Becton-Dickinson, Plymouth, U.K.) at 2 × 10^5 cells per well and cultured with medium containing 10, 20, and 40 μmol/L of DL-Hcy or L-cysteine. Following 24 h of incubation, cell counting of adherent and nonadherent cells was performed with a hemocytometer (Ortho Diagnostics, Raritan, NJ). Adherent cells were collected by trypsinization with a solution of 0.05% trypsin-0.02% EDTA (Gibco BRL). Duplicate hemocytometer counts of floating and attached cell number were determined, and cell detachment, which constitutes an index of cytotoxicity, was quantified [17].

Transmission Electron Microscopy

Cytotrophoblast cells were grown in six-well culture plates for 12 h. The cells, on the wells, were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, ph 7.4, for 2 h at room temperature. The samples were rinsed in 3% sucrose in cacodylate buffer. After fixation in 1% osmium tetroxide (OsO_4, Sigma-Aldrich, St. Louis, MO) for 1 h at 4°C, the samples were rinsed in distilled water, dehydrated in an alcohol series, and embedded in epoxy embedding medium (Epon 812, Sigma-Aldrich). Ultrathin sections were stained with uranyl acetate and lead citrate and were examined with a Philips 400 transmission electron microscope (Eindhoven, The Netherlands).

Apoptosis Detection

The percentage of apoptotic cells was determined by M30 Cyto-DEATH antibody [18]. This is a monoclonal mouse immunoglobulin (Ig) G2b antibody (clone M30; Roche, Mannheim, Germany) that binds to a caspase-cleaved, formalin-resistant epitope of cytokeratin 18 cytoskeletal protein. The immunoreactivity of the M30 antibody is confined to the cytoplasm of apoptotic cells. Trophoblast cells, treated with Hcy (20 μmol/L) for 24 h, were fixed in 10% neutral-buffered formalin for 15 min, treated with 0.3% hydrogen peroxide in methanol for 10 min to block the endogenous peroxidase activity, washed in PBS, and then incubated with M30 antibody at room temperature for 1 h. In negative controls, preimmune mouse serum instead of primary antibody was used. Immunoreactions were revealed by the avidin-biotin complex technique using diaminobenzidine (DAB) as substrate. We counted the number of M30-positive cells in all fields found at 400× final magnification. For each slide, three randomly selected microscopic fields were observed, and at least 100 cells/field were evaluated.

To investigate the DNA degradation, cells were prepared with shandon cytospin (Cheshiere, London, UK), fixed with acetone, and incubated for 5 min with the hybridization buffer (Boehringer-Mannheim, Mannheim, Germany). Then, 2.5 U of terminal deoxynucleotidyl transferase (TdT) and 100 pmol of biotin-dUTP in hybridization buffer were added and incubated for 1 h at 37°C. Thereafter, the cells were incubated with the streptavidin-biotin-peroxidase complex for 30 min at room temperature. The sites of peroxidase binding were detected with DAB. Negative controls in the absence of TdT were also performed to verify the aspecific reading caused by peroxidase. The percentage of TUNEL-positive cells was counted at 400× magnification. In the absence of TdT, no unspecific staining was observed. For each slide, three randomly selected microscopic fields were observed, and at least 100 cells/field were evaluated. Apoptosis was also recognized by scoring the morphological features of nuclear pyknosis, chromatin condensation and/or fragmentation, and cellular fragmentation into apoptotic bodies [19].

Preparation of Tissue Lysates and Western Blot Analysis

Cell cultures were performed for 6, 12, 24 and 48 h. At each time, cytochrome *c* release from the intermembrane space of mitochondria into

TABLE 1. Effect of Hcy on trophoblast cell detachment.

	Hcy (μmol/L)			
	0	10	20	40
Adherent cell count (n)[a]	2.1 ± 0.13	1.9 ± 0.1	1.6 ± 0.08[b]	1.2 ± 0[c]
Detachment	0.3%	2.3%	11.5%	30.0%

[a] The values are expressed as × 10^5. Each value represents the mean ± SD of three experiments.

[b,c] Significance versus controls (untreated cells; Hcy, 0 μmol/L) are shown ([b]$P < 0.034$ and [c]$P < 0.008$).

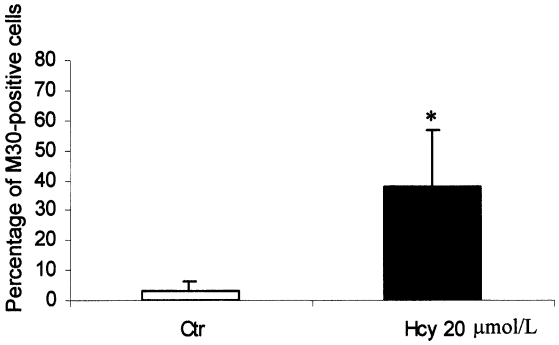

FIG. 3. Effect of Hcy on M30 staining of trophoblast cells. Cytokeratin 18 neoepitope formation was assessed by quantifying the number of M30-positive cells after 24 h of culture in the presence of DL-Hcy (20 μmol/L). The values represent the mean ± SD from four independent experiments. *$P < 0.029$ vs. untreated cells.

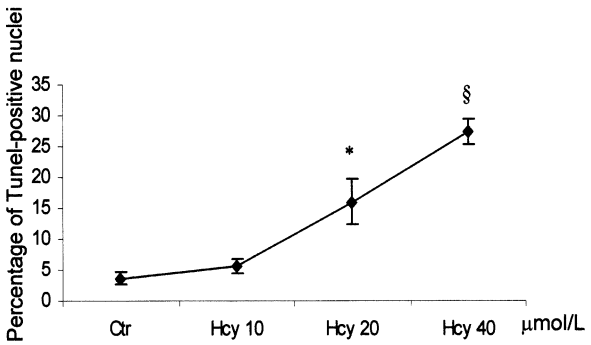

FIG. 5. Dose-dependent increase in TUNEL staining of trophoblast cells treated with Hcy. Trophoblast cells were incubated for 24 h in medium containing DL-Hcy (10, 20, or 40 μmol/L). The values represent the mean ± SD from three independent experiments. *$P < 0.014$ and §$P < 0.003$ vs. untreated cells.

the cytosol was investigated by Western blot analysis [20]. Briefly, nonadherent and adherent cells were collected, washed with PBS, and suspended in ice-cold buffer (5 mM Tris-HCl [pH 7.4], 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EGTA, and 0.2% Triton X-100) supplemented with protease inhibitors (1 mM dithiothreitol, 0.2 mM PMSF, 5 μg/ml of leupeptin, 5 μg/ml of aprotinin, 0.7 μg/ml of Pepstatin A (Sigma-Aldrich), and 50 mM NaF). The nuclei were centrifuged at 500 × *g* for 5 min at 4°C. The supernatant was further centrifuged at 10^5 × *g* for 1 h at 4°C in a Beckman TLA 100.4 rotor (Beckman Instruments Inc., Fullerton, CA), and the resulting supernatants were used as the cytosolic extracts. The pellet, containing mitochondria, was used as positive control in each experiment. The protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Eighty micrograms of each protein sample were separated on a 15% SDS-polyacrylamide gel, and after electroblotting onto polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA), the membranes were incubated with 6% nonfat dry milk in 1 M Trizma base, 1.5 M NaCl, and 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 Biotechnology, Santa Cruz, CA) directed against cytochrome *c*, washed twice with TBST, incubated with alkaline phosphatase-conjugate goat anti-rabbit antibody (Bio-Rad Laboratories) for 2 h at room temperature, and then washed twice more in TBST. The immunoblot was revealed using BCIP/NBT Phosphatase Substrate System (Kinkegaard & Perry Laboratories, Gaithersburg, MD). Images of the blots were acquired with a Cohu charged-coupled-device camera (Cohu, Inc., San Diego, CA), and quantification of the bands was performed by Phoretix 1D (Phoretix International, Newcastle, U.K.). The rising level of the release of cytochrome *c* into the cytosol from adherent plus nonadherent 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.).

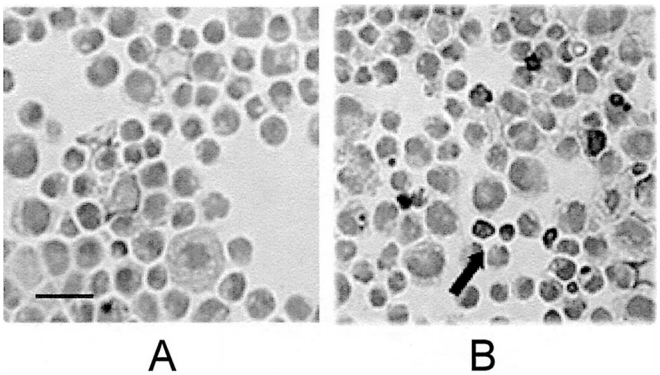

FIG. 4. TUNEL staining induced by DL-Hcy added to cytotrophoblast cells at the concentration of 20 μmol/L. **A**) Controls (untreated cells). **B**) Hcy (20 μmol/L) treatment. Note the increased number of TUNEL-positive cells in the presence of Hcy (15%) with respect to untreated cells (3% in **A**). Chromatin condensation is indicated by the arrow. Bar = 50 μm. Magnification ×400.

DNA Fragmentation Analysis

Cytotrophoblast cells (2×10^5 cells/ml) were cultured in complete medium and treated for 48 h with DL-Hcy (10, 20, and 40 μmol/L) or L-cysteine (20 μmol/L). At the end of the incubation period, the cells were washed twice in PBS. Cell pellets were resuspended and incubated in lysis buffer (50 mM Tris-HCl, 100 mM EDTA, and 0.5% SDS) supplemented with proteinase K (0.7 mg/ml; Sigma-Aldrich S.r.L.) and incubated for 1 h at 55°C [21]. The DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) followed by absolute ethanol and addition of 70% ethanol. The DNA was dissolved in 10 mM Tris (pH 7.5) and 1 mM EDTA (pH 8) after evaporation of ethanol. The DNA was loaded into wells of a 1.5% agarose gel and electrophoresed at 75 mV using 100 mM Tris, 100 mM boric acid, and 0.2 mM EDTA as running buffer. The DNA was visualized by ethidium bromide staining.

Hormone Secretion

Primary trophoblast cells were cultured in complete medium in the presence of DL-Hcy (10, 20, and 40 μmol/L) or L-cysteine (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 radio-

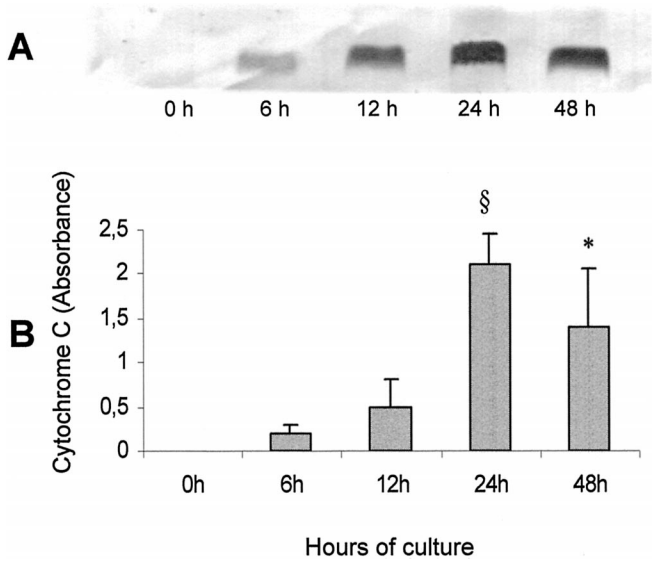

FIG. 6. Release of cytochrome *c* into the cytosol. The rising level of cytochrome *c* into the cytosol from adherent plus nonadherent cells was estimated in comparison with the constant level of β-actin present in the cytosolic extracts. The cytosolic proteins were separated on SDS-polyacrylamide gel electrophoresis for Western blot analysis using specific antibody to cytochrome *c* as described in *Materials and Methods*. Significant increase of the DL-Hcy (20 μmol/L)-induced cytochrome *c* release is shown. §$P < 0.008$ and *$P < 0.014$ vs. untreated cells.

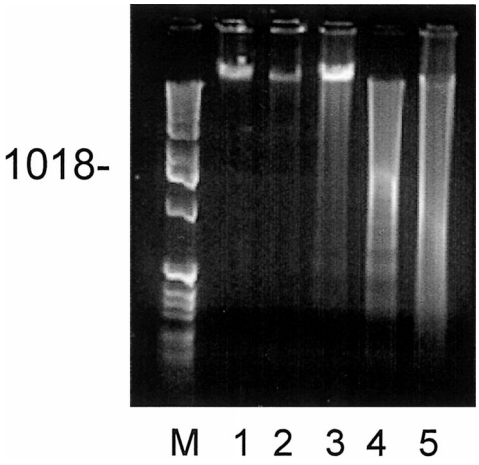

FIG. 7. Gel electrophoresis of fragmented DNA. Trophoblast cells were cultured in the presence of DL-Hcy for 48 h. Chromosomal DNA was separated on a 2% agarose gel. Lane 1: untreated cells, controls; lane 2: Cysteine (20 μmol/L); lanes 3–5: Hcy (10, 20, and 40 μmol/L, respectively). M, DNA size markers (bp).

immunoassay kit (generously provided by Radim, Rome, Italy). The intra- and interassay coefficents of variation were less than 12% and 8%, respectively.

These experiments were performed using different trophoblast tissues obtained from three or four women, with comparable results.

Statistical Analyses

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

RESULTS

Microscopic Characterization of Cellular Changes Occurring after Treatment with Hcy and Influence on Cell Growth

For electron microscopic examination, cytotrophoblast cells were treated for 12 h, and adherent cells were fixed as above. The transmission electron microscopic studies revealed extensive mitochondrial vacuolation and swelling with complete loss of cristae (Fig. 1B). By contrast, the control cells (Fig. 1A) showed normal structural integrity and finely preserved cristae.

After 24 to 48 h of culture, cell growth characteristics were strongly modified by Hcy. Figure 2 shows the effect of Hcy treatment on the morphology of human trophoblast cells. Untreated cells showed a polygonal shape with the tendency to form nuclear nests (Fig. 2A). Exposure of trophoblast for 48 h to DL-Hcy (20 μmol/L) (Fig. 2B) resulted in cellular flattening and enlargement, extension of pseudopodia, and cellular vacuolization with intracellular granules. Cellular detachment was favored with a concentration-dependent decrease of adherent cells (Table 1). No differences between untreated (controls) or cysteine-treated cells were found (data not shown).

Characterization of Hcy-Induced Trophoblast Damage

The antibody M30 CytoDEATH recognizes a specific caspase cleavage site and, thus, is a reliable tool for identifying early apoptosis [22]. We found an increase in M30-positive trophoblast cells after Hcy treatment (Fig. 3). The morphological evidence for apoptosis in trophoblast cells is shown in Figure 4. The cells were incubated for 24 h in the presence (Fig. 4B) or in the absence (Fig. 4A) of Hcy (20 μmol/L). Treatment with Hcy induced DNA degradation, as evidenced by increased number of TUNEL-positive nuclei in Figure 4B with respect to Fig. 4A. This effect was greater at 40 μmol/L of DL-Hcy and similar to untreated cells at 10 μmol/L of DL-Hcy (Fig. 5). No differences between untreated (controls) or cysteine-treated cells were found (data not shown).

As shown in Figure 6, analysis of cytochrome *c* release from mitochondria to the cytosol showed only a weak release after 6 to 12 h of culture. Western blot analysis in samples taken 24 and 48 h after addition of DL-Hcy revealed an enhanced release of cytochrome *c*.

The induction of cell death was also estimated by agarose gel electrophoresis. Treatment of trophoblast cells with DL-Hcy (20 μmol/L) produced DNA cleavage (Fig. 7), in both floating and adherent cells, with the typical internucleosomal fragmentation in 180 to 200 base pairs (bp), characteristic of cell death by apoptosis. Exposure of trophoblast cells to 40 μmol/L of Hcy for up 48 h induced an extensive DNA degradation. In untreated cells and in presence of cysteine (20 μmol/L), no internucleosomal DNA fragmentation was observed.

Effect of Hcy on Trophoblast hCG Secretion

We evaluated the levels of hCG secreted into the culture medium by trophoblast cells in the presence of DL-Hcy or L-cysteine. After 48 h of culture, DL-Hcy significantly inhibited hCG secretion (Table 2); this inhibition was greater at 40 to 80 μmol/L than at 20 μmol/L, with hCG secretion reduced to 80% and 50% of the control levels (untreated cells), respectively. Cysteine did not display any significant effect at the tested concentration (40 μmol/L).

These experiments were performed using different trophoblast tissues obtained from four women, with comparable results.

DISCUSSION

Many pieces of evidence indicate that elevated Hcy concentrations can cause obstetrical diseases that are connected with vascular disorders of pregnancy [23].

The hypothesis that Hcy might induce vascular disease was originally advanced by McCully [1], based on the ob-

TABLE 2. Effect of Hcy on basal hCG production.[a]

Control	Cysteine (μmol/L) 40	Hcy (μmol/L) 10	20	40	80
157.1 ± 18.7[b]	176.4 ± 25.9	168.1 ± 17.7	79.1 ± 23.8[c]	28.2 ± 13[c]	24.9 ± 11.9[d]

[a] Values are the mean ± SD of four experiments.
[b] hCG (mIU/ml).
[c] $P < 0.004$ versus control or cysteine-treated cells.
[d] $P < 0.000$ versus control or cysteine-treated cells.

servation that thromboembolism and atherosclerosis were features of homocystinuria. In vitro, Hcy has been shown to directly damage endothelial cells and to increase proliferation of smooth muscle cells [24, 25]. In addition, Hcy might affect the blood-clotting mechanisms [26].

These data could suggest a responsibility of maternal hyperhomocysteinemia for the patterns of placental atherosclerosis and thrombosis found by histology [27]. Although data emphasized the role of thrombotic phenomena in placental tissues, studies in humans suggested that thrombotic events were not able to account for all the obstetrical complications in women with hyperhomocysteinemia [15, 23]. The molecular mechanisms of Hcy that cause cellular injury have been investigated. It was suggested that Hcy could elicit oxidative stress in cells [28, 29]. The resulting oxidative damage is evidenced by enhanced lipid peroxidation [30], elevated 8-hydroxy-2-deoxyguanosine levels in DNA [31], and impaired antioxidant enzymatic function [9].

The concentrations of Hcy (10–20 μmol/L) used in the present study are comparable to pathophysiological levels observed in subjects with mild hyperhomocysteinemia (16–24 μmol/L) [3, 23]. Thus, in mild hyperhomocysteinemia, human trophoblast cells in vivo are presumed to be exposed to apoptosis-inducing levels of Hcy (20 μmol/L).

Recent studies reported several Hcy-induced cellular responses. Wang et al. [32] showed that Hcy inhibited endothelial proliferation, presumably after its conversion to S-adenosylhomocysteine and subsequent hypomethylation of P21. In the human histocytic cell line U937 [33], Hcy induced cell death, characterized by a loss of cell adhesion, by cytosolic release of cytochrome *c* and internucleosomal DNA fragmentation. To our knowledge, this study is the first demonstration that trophoblast cells are a target for Hcy.

Apoptosis is a complex phenomenon that can involve different metabolic pathways depending on the inducer of cell death being considered. It occurs under normal physiological conditions and is an active process requiring energy [34]. Necrosis is a passive, catabolic, and pathological process that represents a cell's response to extreme accidental or toxic insult. Both types of cell death can be encountered following the same initial insult, and the balance between death by apoptosis or by necrosis appears to depend on the intensity of injury [35]. In our primary trophoblast cells, the DNA smear observed with 40 μmol/L of Hcy may be indicative of necrosis, whereas at 20 μmol/L, the predominant type of cell death seems to be apoptosis, as indicated by the M30 staining and by the internucleosomal DNA fragmentation.

We observed a cascade of events leading to trophoblast cell death: mitochondrial anomalies, such as swelling and vacuolation evident at 12 h of culture, suggested a loss of transmembrane mitochondrial potential accompanied by a translocation into the cytosol of some mitochondrial proteins, such as cytochrome *c* (24 h of culture). A translocation of cytochrome *c* into the cytosol might play an important role in the caspase activation cascade, such as caspase 3, which participates to the degradation of nuclear proteins, leading subsequently to the internucleosomal cleavage of DNA with the typical DNA fragmentation in multiples of 180 to 200 bp [36]. Thus, in our trophoblast cell cultures, the release of cytochrome *c* might be an event upstream of the internucleosomal DNA fragmentation observed at 48 h of culture. To investigate Hcy-induced apoptosis of placental cells, we used two different staining techniques. The TUNEL procedure has been postulated to stain apoptotic cells, but in necrotic cells, the DNA also is degraded by release of lysosomal enzymes. For this reason, we applied TUNEL as an additional procedure to show chromatin condensation and M30 staining as a specific marker for trophoblast apoptosis [18, 22]. The Hcy (20 μmol/L)-induced placental apoptosis consequently decreased hCG levels to approximately 50–80% of control levels.

In conclusion, the present study provides in vitro evidence of Hcy-induced trophoblast cell death through release of cytochrome *c*. A cell detachment apoptosis of human trophoblast cells under the influence of Hcy, with the inhibition of trophoblast gonadotropin secretion, might represent key mechanisms by which Hcy causes pregnancy complications related to placental diseases.

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