Homocysteine-respondent Genes in Vascular Endothelial Cells Identified by Differential Display Analysis

GRP78/BiP AND NOVEL GENES

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An elevated blood level of homocysteine is associated with arteriosclerosis and thrombosis. The mechanisms by which homocysteine may promote vascular diseases have not been elucidated yet. In the present study, we have applied a modified nonradioactive differential display analysis to evaluate changes in gene expression induced by homocysteine treatment of cultured human umbilical vein endothelial cells (HUVEC). We identified six up-regulated and one down-regulated genes. One up-regulated gene was GRP78/BiP, a stress protein, suggesting that misfolded proteins would accumulate in the endoplasmic reticulum because of redox potential changes caused by homocysteine. Another up-regulated gene encoded a bifunctional enzyme with activities of methylene tetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase, which is involved in a homocysteine metabolism. A third up-regulated gene encoded activating transcription factor 4, and a fourth was a gene whose function is not identified yet. The remaining three were novel genes. We isolated a full-length cDNA of one of the up-regulated genes from a HUVEC library. It encoded a novel protein with 394 amino acids, which was termed reducing agents and tunicamycin-responsive protein (RTP). Northern blot analysis revealed that RTP gene expression was induced in HUVEC after 4 h incubation with homocysteine. RTP mRNA was also observed in unstimulated cells and induced by not only homocysteine but also 2-mercaptoethanol and tunicamycin. The mRNA was ubiquitously expressed in human tissues. These observations indicate that homocysteine can alter the expressivity of multiple genes, including a stress protein and several novel genes. These responses may contribute to atherogenesis.

Injury of vascular endothelium is postulated to initiate atherogenesis. One of the conditions associated with arteriosclerosis and thrombosis is an elevated blood level of homocysteine (reviewed in Refs. 1–4), which is a sulphydryl group-containing amino acid.

Homocyst(e)inemia or homocyst(e)inuria is an autosomal recessive metabolic disorder that results from inherited deficiency of several enzymes. The best known is cystathionine-β-synthase deficiency. A much rarer form of homocysteinemia has been caused by 5-methyltetrahydrofolate-homocysteine methyltransferase deficiency. A more recent study indicated that mutations of 5,10-methylenetetrahydrofolate reductase also caused homocysteinemia. This type of mutant protein occurred at a quite high frequency of approximately 38% of unselected chromosomes in French Canadian people (5). Patients with severe homocysteinemia suffer from arterial and/or venous thromboembolic events at early age. Furthermore, several lines of statistical evidence suggest that mild homocysteinemia probably caused by the heterozygosity also represents a significant independent risk factor for premature vascular diseases (1–3). However, the mechanisms by which homocysteine may promote vascular diseases have not been defined yet.

Recently, the hemostatic consequences of homocysteinestimulated endothelial cell (EC)1 perturbation have been intensively investigated. Endothelial tissue factor (6) and factor V (7) activities were enhanced by incubating EC with homocysteine. Homocysteine treatment of EC reduced generation of activated protein C (8), thrombomodulin activity (9, 10), antithrombin III binding to anticoagulant heparan sulfate on EC (11), and cellular binding sites for tissue plasminogen activator (12). Furthermore, homocysteine increased the affinity of atherogenic apolipoprotein(a) for fibrin (13). In addition to these thrombogenic properties, inhibitory effects of homocysteine on protein transport system were reported. Thrombomodulin transport to the cell surface was prevented by homocysteine (9), and the same secretion problems were observed during the biosynthesis of multimeric glycoprotein, von Willebrand factor (14). It was also reported that vascular smooth muscle cell growth, not EC growth, was promoted by homocysteine (15), which might lead to homocysteine-induced arteriosclerosis.

All these observations suggest that homocysteine works against the cells in three ways. One possible mechanism would be that it serves as a trigger directly or indirectly to switch on the expression of certain genes. Another would be that it prevents protein exit from the endoplasmic reticulum (ER) to the cell surface or the extracellular space. The third one would be that it partially reduces certain proteins such as thrombomodulin and lipoprotein(a) to modify their functional activities. Consequences of all these changes cause vascular cell dysfunction that could be important in homocysteine-induced vascular diseases.

1 The abbreviations used are: EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; ER, endoplasmic reticulum; PCR, polymerase chain reaction; 5′-RACE, rapid amplification of 5′-ends of cDNA; pfa, plaque-forming units; NMDMC, NAD-dependent methenyltetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase; RTP, reducing agents and tunicamycin-responsive protein; MOPS, 4-morpholinepropanesulfonic acid; bp, base pair(s).
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In the present study, to evaluate changes in gene expression in homocysteine-stimulated vascular EC, a differential display analysis was performed. As the result, we have identified seven genes under the influence of homocysteine.

**EXPERIMENTAL PROCEDURES**

*Cell Culture and Homocysteine Treatment—* HUVEC was purchased from Clonetics and used between passages 2 and 3. The cells were cultured in MCDB131 medium (Sigma) supplemented with 20 mM HEPES-NaOH (pH 7.4), 2% fetal bovine serum (Life Technologies, Inc.), 10 ng/ml recombinant human basic fibroblast growth factor (R&D Systems), and 10 units/ml porcine heparin (Novo Nordisk) on collagen type I-coated plastic dishes (Sumitomo) at 37°C in 5% CO2 at 95% humidity.

**First-strand cDNA synthesis.** A mixture (28.5 µl) containing 0.4 µg of total RNA, 15 pmol of oligo(dT) primer 5’T-TP-7’-3’ (where V represents A, C, or G), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 µM dithiothreitol, and 0.5 mM each dNTP was heated to 70°C for 10 min. The temperature was then reduced to 45°C, and 1.5 µl of SuperScript II reverse transcriptase (200 units/µl) was added. This 30-µl reaction mixture was incubated at 45°C for 1 h, boiled for 5 min, and chilled to 4°C. Then, 20 µl of H2O was added, and the cDNA solution thus obtained (50 µl) was used for the following reaction.

**(ii) Polymerase chain reaction (PCR).** The reaction was started by adding 1 µl of the cDNA solution and 2 µl (2 units) of TaKaRa Taq DNA polymerase (Takara Shuzo) to 7 µl of a solution such that the final composition of each reaction was 0.5 µM of oligo(dT) primer 5’T-TP-7’-3’ (where V represents A, C, or G), 100 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP, 10 mM dithiothreitol, and 1 µM each primer (Takara Shuzo). The thermal cycling parameters were 94°C (1 min) followed by 35 cycles of 94°C (1 min), 45°C (1 min), and 72°C (1 min), and the extension step at 72°C was for 7 min.

**DNA Preparation—ISOGEN (Wako).** This method described below instead of plasmid preparation. Well isolated plasmid DNA was subjected to electrophoresis in 1% agarose gel containing 0.66 µM formamide, 40 mM MOPS-NaOH (pH 7.2), 10 mM sodium acetate, 1 mM EDTA, and 0.01% SYBR Green II (Molecular Probes). DNA was transferred onto GeneScreen nylon membrane (DuPont NEN) by standard capillary blotting techniques. Specific probes of the insert DNA in plasmid vector pCR II were generated by PCR as follows. The reaction mixture (50 µl) contained 10 µM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 50 µM dATP, 50 µM dCTP, 50 µM dGTP, 10 µM dTTP, 40 µM fluorescein-12-dUTP (DuPont NEN), 1 µM each primers, 2.5 units of TaKaRa Taq DNA polymerase, and 5 µl of the PCR product above described. The primers used were forward primer 5’-ATACCTGCGAGATTTTCGCACTTTCCGG-3’ (which flank the insert site of the vector). The thermal cycling parameters were 94°C (1 min) followed by 35 cycles of 94°C (1 min), 45°C (1 min), and 72°C (1 min), and the extension step at 72°C was for 7 min.

**Hybridization and chemiluminescence detection procedures using anti-fluorescein-horseradish peroxidase conjugate (DuPont NEN) were performed according to the manufacturer’s instruction.**

**5’-RACE—** To obtain the upstream nucleotide sequence of the novel gene transcript containing a sequence of fragment GC4, the 5’-RACE assay was carried out using Marathon cDNA Amplification Kit (Clontech) and TaKaRa LA PCR Kit (version 2) (Takara Shuzo) as follows. In the first step, first-strand cDNA was synthesized with 1 µg of poly(A) RNA prepared from homocysteine-treated HUVEC, using cDNA synthesis primer 5’T-TCTTCTAGATTCGCGCGCGCTG7’-N-3’ and Moloney murine leukemia virus reverse transcriptase, and then double-strand cDNA was synthesized. In the second step, a cDNA adapter provided by the Clontech Kit was ligated to both ends of the double-strand cDNA, and the product was subjected to the following PCR. The 20-µl mixture was containing 0.2 µM AP1 primer (complementary to the 5’-end of GC4), 0.2 µM TdT CCTCTCCCTCC3’-complementary (to a part of the vector GC4), 200 µM each dNTP, 2.5 mM MgCl2, 1 X LA PCR buffer II, 1 unit of TaKaRa LA Taq, and 0.22 µg of TaqStart antibody (Clontech). The reaction mixture was subjected to a PCR step at 94°C for 1 min, followed by 16 cycles of 94°C (15 s), 68°C (30 s) and 72°C (30 s) for amplification. The additional final extension step was performed at 72°C for 7 min. (iii) Native polyacrylamide gel electrophoresis. Each PCR product (10 µl) was mixed with 2.5 µl of dye solution (50% glycerol, 0.1% bromphenol blue, 5 mM Tris-HCl, 1 M each dNTP, 0.5 µM 21M13 primers). The gel was prepared from a solution containing 0.9 M NaCl, 30 mM Tris-HCl and 1 M Tris-HCl gel (126 x 137 x 1 mm) in TGE buffer (25 mM Tris-HCl, 192 mM glycine, 1 M EDTA), and the gel was run at 400 V for 1 h. The gels were stained with SYBR Green I (Molecular Probes), excited at 254 nm by a transiluminator, and photographed with Polaroid 667 film through a yellow filter (Wratten gelatin filter No. 15, Kodak).

**Sequencing of the Interesting Bands—** Interesting bands (up- or down-regulated by homocysteine treatment) on gels were cut out with razors on UV-transilluminator, and each fragment was eluted by boiling in 50 µl of water for 10 min. Then, to reamplify the DNA fragments, 5 µl of the eluate was mixed with 45 µl of the reaction mixture containing 0.44 µM oligo(dT) primer Tp171, 0.44 µM decanucleotide primer, 11.1 mM Tris-HCl (pH 8.3), 55.6 mM KCl, 1.67 mM MgCl2, 0.22 mM each dNTP, and 2 units of TaKaRa Taq DNA polymerase. The thermal cycling parameters were as follows: 94°C (2 min), 40°C (1 min), and 72°C (1 min) followed by 30 cycles of 94°C (15 s), 60°C (30 s), and 72°C (30 s). The additional extension step was carried out at 72°C for 7 min. The amplified DNA fragments were cloned into the plasmid vector pCR II using T.A Cloning Kit (Invitrogen). For DNA sequence analysis, template DNA was prepared by a colony PCR method described below instead of plasmid preparation. Well isolated plasmid DNA was subjected to electrophoresis (with inserted clones were being 5 µl of water containing 0.1% (v/v) Tween 20, and 5 µl of the lysate was subjected to PCR using forward primer 5’-GTTTTTCCAGTCTACGAGC-3’ and reverse primer 5’-GAATTGTTCAGCGGATA-3’ which flank the multi-cloning site of the vector. Sequence reaction was performed by catalyst model 800 (Perkin Elmer) using a Dye Primer Cycle Sequencing FS Ready Reaction Kit with M13Rev primer or a 21M13 primer (Perkin Elmer) according to the manufacturer’s instruction. The products were analyzed with a DNA sequencer model 373A (Perkin Elmer). Comparison of DNA homology with the GenBank and the EMBL data bases was performed using BLAST (17) and FASTA (18). The PCR-amplified insert DNA sequences described here were also used for preparation of probes on Northern blot analysis.

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(nos. 5, 17, and 21) were plated at a density of ~1,000 pfu/dish and resubjected to PCR-based screening method, respectively. Furthermore, positive plate lysates (nos. 5-6, 17-5, and 21-22) were plated at a density of ~20 pfu/dish and again subjected to the same method. Positive plate lysates (nos. 5-6-80, 17-5-7, and 21-22-16) were then plated at a density of ~20 pfu/dish, and several dozen well-isolated plaques were excised with the yellow tips, eluted in 100 μl of SM buffer, and subjected to the PCR. Finally, three positive phages (nos. 5-6-50-167, 17-5-7-10, and 21-22-189) were cloned. The plasmid DNAs (termed pGC4-a, pGC4-b, and pGC4-c, respectively) were purified using Qiagen Plasmid Midi Kit. The insert DNAs were sequenced in both directions using Dye Primer Cycle Sequencing FS Ready Reaction Kit with M13Rev and (+)M13primers, and Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin Elmer) with 10 synthesized specific primers.

RESULTS

Identification of Homocysteine-respondent Genes in HUVEC by Nonradioactive Differential Display Analysis—To identify differentially expressed genes as a consequence of homocysteine treatment on HUVEC, we have compared mRNA expression patterns of nontreated confluent HUVEC and cells incubated for 4 h with 1 or 10 mM homocysteine by a nonradioactive differential display analysis. The medium used in this study contained 2% fetal bovine serum, and 1 or 10 mM concentration of homocysteine did not produce cell detachment under these conditions. The differential display analysis was first performed by Liang and Pardee (16) using 35S-labeled dATP and urea-containing sequencing gels. In the present study, a more rapid and convenient method has been developed using a combination of a highly sensitive DNA stain, SYBR Green I, and non-denaturing gels. A total of 28 arbitrary decanucleotide primers was used for screening against all 9 cDNA pools prepared by T1,TTV primers (i.e. 756 PCR in total were performed among three-cell culture conditions). Fig. 1 shows representative band patterns of PCR products with three different primer combinations. Indicated in Fig. 1 are PCR products (CA27, CC17, and CC22) whose band intensities were reproducibly increased by homocysteine treatment. In total, we observed 13 PCR fragments that were differentially displayed between HUVECs cultured in the absence and presence of 10 mM homocysteine. However, sequencing analysis as described below revealed that one of them belonged to the Alu family, and two had sequences of L1 elements. Because these three were difficult to be characterized by Northern blot analysis, 5'-RACE, or library screening, they were excluded from further analyses. Finally, we identified 10 cDNA fragments designated CC17, CC22, CA27, AC27, CC1, CC15, CA13, GC4, GC5, and GC13 as shown in Table I.

Northern Blot Analysis with Differentially Displayed cDNA Fragments—To confirm the gene expression patterns observed in the differential display analysis, 10 cDNA fragments were recovered from gels and reamplified by PCR using the corresponding pair of primers. The reamplified fragments were then cloned into plasmid pCR II and used as probes for RNA blots prepared with total RNAs from control and 10 mM homocysteine-treated HUVECs. As a result of Northern blot analyses, 9 of the 10 fragments generated hybridization patterns that reproduced the increase in homocysteine-treated HUVEC (data not shown; the estimated sizes of the transcripts are shown in Table I). The remaining one (GC13) did not hybridize to any transcripts, probably due to the sensitivity of the Northern blot analysis.

Nucleotide Sequencing and Homology Searching—All of 10 cloned cDNA fragments (~500 bp) were completely sequenced, and the results revealed that all fragments were flanked by the sequences corresponding to the particular oligo(dT) primer and the decanucleotide primer. Their nucleotide sequences except for a 30-base corresponding to the both-end primers were then

![Fig. 1. Representative band patterns on differential display analysis showing up-regulated PCR fragments in homocysteine-treated HUVEC. Total RNA was extracted from HUVEC incubated with the indicated concentrations of homocysteine for 4 h and subjected to differential display analysis. A nonradioactive, 6% polyacrylamide gel poststained with SYBR Green I is shown for three different primer combinations (A-C). Primer combinations used are as follows: A, T1,CA and 5'-GCTTGGAGG-3'; B, T1,CC and 5'-GATCTGACAC-3'; and C, T1,CC and 5'-GATCGCATTG-3'. Arrows in A-C (CA27, CC17, and CC22, respectively) indicate signals demonstrating altered expression. DNA size standards derived of X174 DNA/Hincl digest (Toyobo) are shown on the left.

### TABLE I

| cDNA fragments corresponding to mRNAs, where abundance is altered by homocysteine treatment on HUVEC |
| Fragment name | Primer used in cDNA synthesis | Primers used in PCR | Size of PCR product(\(\text{bp}\)) | Effect of homocysteine | mRNA size(\(\text{kb}\)) | Corresponding human genes(\(\text{a}\)) |
|----------------|-------------------------------|---------------------|---------------------------------|-------------------------|-----------------|--------------------------|
| CC17           | T1,CC                         | GATCTGACAC, T2,G    | 448                             | Up                      | 2.9             | 78-kDa glucose-regulated protein (GBP78), immunoglobulin heavy chain binding protein (IgH) |
| CC22           | T1,CC                         | GATCGCATGG, T5,G    | 373                             | Up                      | 2.9             | (A part of CC17)         |
| CA27           | T1,CA                         | GCTTGGAGG, T5,G     | 493                             | Up                      | 2.6             | NAD-dependent methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase (NMDMC) |
| AC27           | T1,AC                         | GCTTGGAGG, T2,A     | 482                             | Up                      | 2.6             | tax-responsive enhancer binding protein (TAXRE67), activating transcription factor 4 (ATF-4) |
| CC1            | T1,AC                         | TACAACGGAG, T2,G    | 297                             | Up                      | 1.6             | (A part of CC17)         |
| CC15           | T1,CC                         | GATCGCATGAC, T6,G   | 163                             | Up                      | 1.6             | (A part of CC17)         |
| CA13           | T1,CA                         | GTTTCTGCAG, T1,G    | 163                             | Up                      | 2.2             | HUMORF12*                |
| GC4            | T1,GC                         | TTTTGGTCTCC, T5,G   | 343                             | Up                      | 3.3             | No significant similarity |
| GC5            | T1,GC                         | GGAACCATAC, T5,G    | 231                             | Up                      | 3.3             | No significant similarity |
| GC13           | T1,GC                         | GTTTCTGCAG, T4,G    | 240                             | Down                   | ND*             | No significant similarity |

* Determined by nucleotide sequence of the insert in the vector multi-cloning site. This size contains the primer sequences at both ends.

* Estimated by Northern blot analysis.

* Searched with the GenBank and the EMBL databases using BLAST and FASTA.

* One of the randomly sampled human cDNA clones reported by Nomura et al. (24).

* ND, not detectable.
analyzed by searching for homologies against the GenBank and the EMBL data bases. The results are summarized in Table I. The sequence of CC22 was a part of the 3'-9-end of CC17, which was identical to bases 4737–5154 of human 78-kDa glucose-regulated protein (GRP78) gene (GenBank accession no. M19645) (19) or bases 1823–2240 of human immunoglobulin heavy chain binding protein (BiP) mRNA (GenBank accession no. X87949). Unexpectedly, the DNA sequences of CC17 and CC22 were corresponding to a part of the open reading frame and not the 3'-end. It was considered that the oligo(dT) primer on cDNA synthesis (T11CC) or on PCR (T18C) hybridizes to not the 3'-end but to an internal site. The nucleotide sequence of the fragment CA27 was found to be 99% identical to a 3'-end of human NAD-dependent methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase (NMDMC, GenBank accession no. X16396) (20). The AC27 sequence (482 bp) was identical to a part of CA27 (493 bp). The difference in length between AC27 and CA27 was caused by the different hybridizing site of oligo(dT) primers T11AC and T11CA, respectively. The CC1 sequence completely matched to bases 1837–2013 of human DNA-binding protein, TAXREB67 (GenBank accession no. D90209) (21), and bases 1063–1239 of human cAMP-responsive element regulatory protein, CREB-2 (GenBank accession no. M86842) (22), both of which were originally designated as activating transcription factor 4 (ATF-4) (23). The CA13 fragment was identical to the 3'-end of HUMORF12 (GenBank accession no. D14695), which is one of the randomly sampled human cDNA clones reported by Nomura et al. (24).

To date we have found no significant homology with any published genes for the sequences obtained from fragments GC4, GC5, and GC13, each of which contained a typical polyadenylation signal (25) at the near 3'-end. There, however, existed a number of human cDNA-expressed sequence tags that were identical or nearly identical to those clones (data not shown).

cDNA Library Screening and Nucleotide Sequence of One Novel Gene—To consider the biological significance of gene expressivity alterations, it is necessary to obtain more information of novel genes. In the present study, we have tried to identify a full-length cDNA corresponding to the fragment GC4 and succeeded in isolating three independent clones from HUVEC cDNA library. The nucleotide sequence of the longest clone, pGC4-c (3056-bp insert), is shown in Fig. 2. Identical sequences were observed from the other clones, pGC4-a (3000-bp insert) and pGC4-b (3006-bp insert), though each of them was slightly shorter than pGC4-c. The 3'-terminal region of each clone contained the sequence of the fragment GC4 obtained by differential display analysis. Its open reading frame of 1185 bp runs from ATG start codon to a TAG stop codon, with a deduced protein sequence of 394 amino acid residues. We termed it as RTP, reducing agents and tunicamycin-responsive protein, although the meaning will be discussed below. The presumed initiating ATG was assigned to the first methionine codon. It had a purine residue 3 bases upstream, which is characteristic of favorable translation initiation sites (26). The cDNA clone contained a 1710-nucleotide 3'-untranslated region with a typical polyadenylation signal (25) at the near 3'-end.
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bases upstream of the poly(A) tail. Although the full-length nucleotide sequence was analyzed by searching for homologies with the GenBank and the EMBL data bases using both BLAST and FASTA, no significant homology with any published genes was found.

Amino Acid Sequence of RTP—The deduced protein RTP showed a calculated molecular mass of 42,835 Da. This protein had a novel 10-amino acid unit, GTR5RSHTSE, which was tandemly repeated three times in the C-terminal region. The hydropathy analysis suggested that there were no significant hydrophobic transmembrane domains (Fig. 3). A motif search with PROSITE (27) displayed that the residues 128–143, QFGLKSIIGMGTGAGA, satisfied the consensus sequence of the phosphopantetheine attachment site. Other consensus motifs were not found. A search of protein data bases using BLAST and FASTA indicated that the amino acid sequence of RTP was slightly homologous to that of ZK1073.1, which is encoded within Caenorhabditis elegans cosmid ZK1073 (EMBL accession no. Z68135) (28). An alignment between RTP and ZK1073.1 is shown in Fig. 4. There was a 28% sequence identity between residues 22–356 of RTP and 1–325 of ZK1073.1.

RTP and GRP78/BiP mRNA Expression in HUVEC—To determine a time course of RTP mRNA expression, total RNA was isolated from HUVEC at various times after addition of 10 mM homocysteine, and the mRNA expression levels were analyzed by Northern blot analysis (Fig. 5A). The RTP mRNA was expressed to some extent without stimulation of homocysteine. However, a dramatic increase was observed after 4 h incubation with homocysteine and continued at least after 12 h incubation. A maximal level was detected at 8 h. Time course of homocysteine-induced mRNA expression of GRP78/BiP was also examined (Fig. 5B), and the induction was observed slightly faster than RTP. Then, to determine the effects of homocysteine concentration, various concentrations of homocysteine (0–10 mM) were added to the culture medium, and the expressions of RTP and GRP78/BiP mRNA were analyzed after 4 h incubation (Fig. 5, C and D). Significant increase of RTP mRNA was observed at a concentration of 3 mM, and the increase was much more strengthened with 10 mM homocysteine (Fig. 5C). These observations were consistent with the findings that the band intensity of 434-bp PCR products (GC4 in Table I) on differential display analysis derived from 10 mM homocysteine-treated HUVEC was more intensive than those derived from HUVEC incubated with 0 or 1 mM homocysteine (data not shown). GRP78/BiP mRNA abundance was also increased at a concentration of >3 mM (Fig. 5D).

The effects of other related agents on RTP and GRP78/BiP mRNA expression were investigated, and the results are shown in Fig. 6. Incubating HUVEC with methionine, which is a methylated form of homocysteine, did not induce the expression of RTP mRNA (Fig. 6A, lane 3). On the other hand, a sulfhydryl group-containing amino acid, cysteine, induced the expression (lane 4), although its activity was slightly less than homocysteine (lane 2). Considering that 2-mercaptoethanol had great activity in inducing RTP mRNA expression (lane 5) like homocysteine, the free sulfhydryl group should be an effective stimulant on the induction. Similar expression patterns were observed when a GRP78/BiP probe was used (Fig. 6B, lanes 2–5). Since GRP78/BiP is known to be induced by treatment with tunicamycin (29), an inhibitor of N-linked glycosylation, we also examined its effect on RTP mRNA expression. Interestingly, incubation of HUVEC with 10 μg/ml tunicamycin for 4 h showed a greater increase of RTP mRNA (Fig. 6A, lane 6) than homocysteine treatment (lane 2). Heat shock of HUVEC had no effect on RTP mRNA expression (lane 7), although GRP/BiP mRNA was enhanced (Fig. 6B, lane 7).

RTP mRNA Expression in Human Tissues—We next used the Northern blot analysis to examine the tissue distribution of RTP mRNA (Fig. 7). A single band of human RTP mRNA was approximately 3.3 kilobases in size, and its expression was found in all human tissues tested, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas.

DISCUSSION

Recently, it has become clear that homocysteinemia is a major independent risk factor for vascular diseases (1–4). For this reason, the mechanism by which altered gene expression occurs in response to homocysteine is an important topic for
investigation. To date, induction of tissue factor mRNA expression (6) and slight increase of thrombomodulin mRNA (9) by homocysteine were reported in HUVEC, and increased mRNA levels of cyclin D1 and cyclin A in rat smooth muscle cells were demonstrated (15). In the present study, we identified seven genes (GRP78/Bip, NMDMC, ATF-4, HUMORF12, and three novel genes) whose mRNA levels were altered after 4 h of homocysteine treatment of HUVEC, although changes in mRNA expression of other genes, which return to the basal level within 4 h or are induced after 4 h, might be missed.

GRP78/Bip is an ER-resident molecular chaperone binding to a wide repertoire of proteins traversing through the ER, where proteins are post-transitionally modified and then destined for transport to the Golgi apparatus, the plasma membrane, lysosomes, or the cell exterior. Since GRP78/Bip mRNA abundance in cultured HUVEC is under such a stressful condition. Up-regulation of ATF-4 observed in this study also may reflect the stress-inducing effects of homocysteine, because ATF-4 mRNA is known to be induced by increasing intracellular Ca\(^{2+}\) concentration (21) and by anoxia (34).

We identified three previously uncharacterized genes whose mRNA levels were altered by homocysteine treatment and succeeded in isolating one almost full-length cDNA clone, pGC4-c. Its open reading frame encoded a novel polypeptide, termed RTP. Three-tandem repeats of GTRSRSHTSE in its C-terminal region was a novel motif. RTP did not have an apparent signal sequence, a transmembrane domain, or an ER-retention sequence. The amino acid sequences showed a 28% identity to ZK1073.1, a predicted protein of C. elegans (Fig. 4). In addition, Northern blot analysis showed the ubiquitous tissue distribution of RTP mRNA (Fig. 7). These two characteristics of RTP suggest that this protein might serve an essential, housekeeping function.

We investigated the effects of various conditions on RTP mRNA expression. The expression was increased by not only homocysteine but also other sulfhydryl reagents, cysteine, and 2-mercaptoethanol (Fig. 6A). The induction was also observed by treatment with tunicamycin. These observations in mRNA induction of RTP were quite similar to those of GRP78/Bip (Fig. 6B). This may relate to functional properties of RTP.

Homocysteine treatment of HUVEC also increased NMDMC mRNA. Human NMDMC is a mitochondrial bifunctional enzyme that has activities of both 5,10-methylenetetrahydrofolate dehydrogenase and 5,10-methylenetetrahydrofolate cyclohydrolase (35). The pathway of homocysteine catalolysis is either transsulfuration to form cystathionine catalyzed by cystathionine β-synthase or remethylation to form methionine catalyzed by methyltransferase. Because the latter requires 5-methyltetrahydrofolate as a methyl donor, the increase of NMDMC mRNA would contribute to activation of the remethylation pathway. This speculation is supported by the previous reports of existence of the homocysteine-uptake system (36) and homocysteine metabolic enzymes (37) in cultured EC. It would be a reasonable assumption that the same remethylation pathway would be activated in the cells of homocysteinemia patients.

The major clinical manifestations of homocysteinemia include mental retardation, ectopia lentis, osteoporosis, skeletal abnormalities, fatty liver, and vascular disease. We suppose that such various symptoms are caused by the elevated concentration of intracellular, rather than extracellular (plasma), changes created by homocysteine would affect the proper disulfide formation of certain proteins, these observations could be explained by binding of misfolded proteins with GRP78/Bip and their retention in the ER. Therefore, the increase of GRP78/Bip mRNA indicates that homocysteine-stimulated HUVEC is under such a stressful condition. Up-regulation of ATF-4 observed in this study also may reflect the stress-inducing effects of homocysteine, because ATF-4 mRNA is known to be induced by increasing intracellular Ca\(^{2+}\) concentration (21) and by anoxia (34).

We identified three previously uncharacterized genes whose mRNA levels were altered by homocysteine treatment and succeeded in isolating one almost full-length cDNA clone, pGC4-c. Its open reading frame encoded a novel polypeptide, termed RTP. Three-tandem repeats of GTRSRSHTSE in its C-terminal region was a novel motif. RTP did not have an apparent signal sequence, a transmembrane domain, or an ER-retention sequence. The amino acid sequences showed a 28% identity to ZK1073.1, a predicted protein of C. elegans (Fig. 4). In addition, Northern blot analysis showed the ubiquitous tissue distribution of RTP mRNA (Fig. 7). These two characteristics of RTP suggest that this protein might serve an essential, housekeeping function.

We investigated the effects of various conditions on RTP mRNA expression. The expression was increased by not only homocysteine but also other sulfhydryl reagents, cysteine, and 2-mercaptoethanol (Fig. 6A). The induction was also observed by treatment with tunicamycin. These observations in mRNA induction of RTP were quite similar to those of GRP78/Bip (Fig. 6B). This may relate to functional properties of RTP.

Homocysteine treatment of HUVEC also increased NMDMC mRNA. Human NMDMC is a mitochondrial bifunctional enzyme that has activities of both 5,10-methylenetetrahydrofolate dehydrogenase and 5,10-methylenetetrahydrofolate cyclohydrolase (35). The pathway of homocysteine catalolysis is either transsulfuration to form cystathionine catalyzed by cystathionine β-synthase or remethylation to form methionine catalyzed by methyltransferase. Because the latter requires 5-methyltetrahydrofolate as a methyl donor, the increase of NMDMC mRNA would contribute to activation of the remethylation pathway. This speculation is supported by the previous reports of existence of the homocysteine-uptake system (36) and homocysteine metabolic enzymes (37) in cultured EC. It would be a reasonable assumption that the same remethylation pathway would be activated in the cells of homocysteinemia patients.

The major clinical manifestations of homocysteinemia include mental retardation, ectopia lentis, osteoporosis, skeletal abnormalities, fatty liver, and vascular disease. We suppose that such various symptoms are caused by the elevated concentration of intracellular, rather than extracellular (plasma),
homocysteine in various cells and tissues of the body. Cell functions in homocysteinemia patients may be altered through the changes in gene expression identified in the present study because intracellular homocysteine levels would be much higher than normal. In fact, de Groot et al. (38) indicated that EC derived from homocysteinemia were more susceptible to sulfur-containing amino acids than normal EC, resulting in EC dysfunction.

The present study indicates that cell injury by homocysteine leads to a stress condition probably caused by alteration of the intracellular redox potential and induces great changes in gene expression. Further investigations, including studies of novel gene transcripts such as RTP, are required to reveal the underlying mechanisms of homocysteine-induced cell injury, and the results may throw light on the pathophysiology of homocysteinemia.

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