EndoPDI, a Novel Protein-disulfide Isomerase-like Protein That Is Preferentially Expressed in Endothelial Cells Acts as a Stress Survival Factor*®

Received for publication, July 25, 2003, and in revised form, September 2, 2003
Published, JBC Papers in Press, September 8, 2003, DOI 10.1074/jbc.M308124200

Dianne C. Sullivan‡, Lucasz Huminiecki§, John W. Moore‡, Joseph J. Boyle‡, Richard Poulsom, Daniel Creamer**, Jonathan Barker††, and Roy Bicknell‡‡§§

From the §Molecular Angiogenesis Laboratory, Cancer Research UK, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DS, United Kingdom, the ¶Department of Genetics, Trinity College Dublin, Dublin 2, Ireland, the ¶¶Department of Histology, Imperial College Medical School, University of London, Hammersmith Hospital, London W12 ONN, United Kingdom, the ‡In Situ Hybridization Laboratory, Cancer Research UK, The London Research Institute, London WC2A 3PX, United Kingdom, the **School of Medicine and Dentistry, King’s College Hospital, Denmark Hill, London SE5 9RS, United Kingdom, and the §§St John’s Institute of Dermatology, St Thomas’s Hospital, King’s College London, London SE1 7EH, United Kingdom

We have identified a novel protein-disulfide isomerase and named it endothelial protein-disulfide isomerase (EndoPDI) because of its high expression in endothelial cells. Isolation of the full-length cDNA showed EndoPDI to be a 48 kDa protein that has three APWGCHE thioredoxin motifs in contrast to the two present in archetypal PDI. Ribonuclease protection and Western analysis has shown that hypoxia induces EndoPDI mRNA and protein expression. In situ hybridization analysis showed that EndoPDI expression is rare in normal tissues, except for keratinocytes of the hair bulb and syncytiotrophoblasts of the placenta, but was present in the endothelium of tumors and in other hypoxic lesions such as atherosclerotic plaques. We have compared the function of EndoPDI to that of PDI in endothelial cells using specific siRNA. PDI was shown to have a protective effect on endothelial cells under both normoxia and hypoxia. In contrast, EndoPDI has a protective effect only in endothelial cells exposed to hypoxia. The loss of EndoPDI expression under hypoxia caused a significant decrease in the secretion of adrenomedullin, endothelin-1, and CD105; molecules that protect endothelial cells from hypoxia-initiated apoptosis. The identification of an endothelial PDI further extends this increasing multigene family and EndoPDI, unlike archetypal PDI, may be a molecule with which to target tumor endothelium.

Protein-disulfide isomerase (PDI) is a ubiquitously expressed multifunctional protein found in the endoplasmic reticulum (ER). It constitutes around 0.8% of total cellular protein and can reach near millimolar concentrations in the ER lumen of some tissues. PDI plays a role in protein folding because of its ability to catalyze the formation of native disulfide bonds and disulfide bond rearrangement. Proteins targeted for secretion by the cell are inserted into and translocated across the ER membrane and enter the ER lumen in an unfolded state. PDI, together with a variety of other folding factors and molecular chaperones resident in the ER correctly fold the proteins ready for secretion. The accumulation of misfolded proteins in the ER, known as the Unfolded Protein Response, results in increased transcription of chaperones and folding catalysts. Proteins that fail to fold correctly are relocalized to the cytosol for proteasomal degradation.

PDI is a modular protein consisting of a, b, b’, a’, and c domains. The a and a’ domains show sequence and structural homology to thioredoxin (Trx) and both contain the active site WCGHK motif, constituting two independent catalytic sites for thiol-disulfide bond exchange reactions. A rate-limiting step in the folding of many newly synthesized proteins is the formation of disulfide bridges and the presence of WCGHKC motif in PDI is essential for this process, as confirmed by the loss of PDI activity following mutation of the cysteine residues within these motifs. The b and b’ domains also have the thioredoxin structural fold but lack the active site motif. Thus, PDI contains both redox active and inactive thioredoxin modules. The C-terminal c domain, a putative Ca++ binding region, is rich in acidic amino acids and contains the -KDEL motif, which is necessary and sufficient for the retention of a polypeptide within the lumen of the ER. The C-terminal domain is, however, not necessary for the enzymatic, chaperone (see below) or disulfide isomerase activities of PDI.

In addition to its disulfide isomerase activity, PDI also shows chaperone activity, for example it can function as the β-subunit of prollyl-4 hydroxylase, preventing the misfolding and aggregation of the α-subunit. This function is similar to that of some molecular chaperones such as Hsp90 in other proteins. Furthermore, PDI is able to interact with and correctly fold type X collagen polypeptides that contain no cysteine residues.

There is now an increasing family of protein-disulfide isomerases, each having two or more thioredoxin or catalytically inactive b domains. Sequence homology between members is poor, their relatedness lying in the structural similarity of the thioredoxin-like fold. It has been proposed...
that different PDIs may show different substrate specificities (1) and support for this has been provided in studies showing that ERp57 is specific for the folding of N-glycosylated proteins (13, 14).

We now report the identification of a novel PDI-like protein, which we have called EndoPDI because of its high expression in endothelial cells. This tissue-specific expression is unusual among members of the PDI family (12). Previous work has shown that PDL, the archetypal member of the PDI family, can protect endothelial cells from stress-induced apoptosis and compared its activity to that of PDI. In contrast to PDI, which is essential for the survival of endothelial cells in the resting as well as the stressed state, EndoPDI protects endothelial cells only under conditions of stress. We have sought a possible protective role of EndoPDI under hypoxia and found that loss of EndoPDI results in reduced secretion of adrenomedullin and endothelin-1 together with a reduction in membrane-bound CD105. While EndoPDI is essential for folding of CD105, PDI is not, illustrating differences at the molecular level in the mechanisms of protection afforded by two molecules.

**EXPERIMENTAL PROCEDURES**

Bioinformatic Methods—EndoPDI was initially found as a gene preferentially expressed in vascular endothelial cells by analysis of expression data deposited in SAGEmap (15). Briefly, the SAGEmap data set was downloaded from the project website (www.ncbi.nlm.nih.gov/ SAGE/) in February 2001 and deposited in a MySQL database. Only normal tissue libraries (total = 37) were used in the analysis. There were two libraries representing vascular endothelium; SAGE Duke HMVEC and SAGE Duke HMVEC+vEGF. The Preferential Expression Measure (PEM) was used to identify genes preferentially expressed in vascular endothelium. PEM = log(e/T), where e is the observed SAGE tag count in vascular endothelium, and T is the expected tag count if the distribution was uniform across the libraries. PEM > 0.847.

Cultivation of Endothelial Cells—Human dermal microvascular endothelial cells (HDMEC) and human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics BioWhittaker (Wokingham, Berkshire, UK) and were cultured in MCDB131 medium (Invitrogen) containing 20% fetal calf serum (Sigma-Aldrich), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 5 IU/ml heparin, and 50 μg/ml endothelial cell growth supplement (Sigma, Dorset, UK). Cells were routinely split 1:3 and were used up to the 8th passage.

Isolation of Full-length cDNA for EndoPDI—Total RNA was extracted from HDMEC and 1 mg was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen). The first 250 nucleotides at the 5'-end were then amplified by PCR using the upstream primer 5'-CCGGTACCCTGGCGGGCAAGACGCCCTTCCTCC-3', designed to include a KpnI restriction endonuclease site, and the downstream primer 5'-GAGCATCCAGTTTCCAGTG-3' and the PCR product cloned into the topo II PCR vector (Invitrogen) and sequenced. An IMAGE clone (3356029) containing partial EndoPDI cDNA sequence was identified from the Unigene data base. The clone is complete at the 3'-end to give a full-length cDNA of EndoPDI.

Production of Polyclonal Antibodies to EndoPDI and Western Blotting—The peptide of sequence ADGEDGDQDHPCK was synthesized by the Protein and Peptide Chemistry Department of Cancer Research UK, using standard solid-phase techniques. This sequence corresponds to amino acids 52–63 of the human EndoPDI protein sequence with an additional cysteine residue added to the C terminus to enable coupling to a carrier protein. The peptide was coupled to Imject Maleimide-activated mcKLH (Pierce) following the manufacturer’s instructions and diluted with Freund’s adjuvant before injection into rabbits. A standard immunization protocol was followed with 200 μg of immunogen used for the first injection and 100 μg of immunogen for subsequent boosts. The test bleeds were screened against pre-bleeds by ELISA to identify the presence of antibodies to EndoPDI in the rabbit serum. Serum that was found to contain EndoPDI antibodies by ELISA was used at 1:100 dilution in Western blotting experiments for the detection of EndoPDI protein by standard Western blotting techniques. Antibodies to PDI were purchased from Bioquote Ltd. (North Yorkshire, England) for use in Western blotting.

Preparation of a Recombinant EndoPDI Construct for Riboprobe Synthesis—A 300-bp region of the 3'-UTR of EndoPDI was amplified by PCR from plasmid DNA containing the EndoPDI clone described above using 5'-TCTGGCTTCCTGATTTCAAGC-3' as the upstream forward primer and 5'-ACTCAGGCAAGTTCGAAATG-3' as the reverse downstream primer. The Basic Local Alignment Search Tool (BLAST) (16) was used to ensure that the chosen region of EndoPDI did not have homology to other sequences. The PCR product was cloned into pcRII-TOPO (Invitrogen) following the manufacturer’s instructions and sequenced to confirm identity and orientation. Riboprobe were transcribed (MAXiscript in vitro transcription kit, Ambion AMS Ltd, Witney, Oxford, UK) from linearized plasmids in the presence of [32P]UTP (Amersham Biosciences) to give radioactively labeled probe.

**RNase Protection Assays**—Total RNA was extracted from cells in culture using TRI reagent (Sigma). RNA protection assays were performed in duplicate using 10–30 μg of total RNA as described (16). To attenuate the signal strength of the highly abundant loading control, U6 small nuclear RNA (accession no. X01366), a riboprobe of significantly lower specific activity was prepared by addition of unlabeled CTP to the labeling reaction. The protected fragment size for EndoPDI was 300 nucleotides. In each assay, a positive and negative (RNA only) control and three digested probes were analyzed. Intensity of signal, quantified on a PhosphorImager (Molecular Dynamics, Chesham, Buckinghamshire, UK) was calculated as the ratio of the signal of control and undigested riboprobes were analyzed. Intensity of signal, quantified on a PhosphorImager (Molecular Dynamics, Chesham, Buckinghamshire, UK) was calculated as the ratio of the signal of control and undigested riboprobes were analyzed.

**In Situ Hybridization**—**In situ** hybridization analysis was performed

| Cell/Tissue type | Tags per million |
|------------------|-----------------|
| SAGE Duke HMVEC + VEGF (vascular normal endothelium cell line) | 1224 |
| SAGE Duke HMVEC (vascular normal endothelium cell line) | 741 |
| Forebrain fibroblasts | 204 |
| SAGE T SU (prostate normal cell line) | 175 |
| SAGE PR317 (prostate normal SAGE microdissected) | 117 |
| SAGE H126 (pancreas epithelial ductal normal cell line) | 91 |
| SAGE 293 CTRL (kidney normal cell line) | 90 |
| SAGE IOSE2911 (ovary epithelial normal cell line) | 82 |
| SAGE 293 IND (kidney normal cell line) | 63 |
| SAGE HOSE 4 (ovary epithelial normal) | 61 |
| SAGE Chen Normal Pr (prostate normal bulk) | 60 |
| SAGE mammary epithelium (mammary gland epithelium ductal normal) | 60 |
| SAGE NHA (5th) brain normal SAGE astrocyte | 57 |
| SAGE breast myoepithelial (mammary gland myoepithelial) | 51 |
| SAGE NC2 (epithelial normal colon) | 40 |
| SAGE NC1 (epithelial normal colon) | 39 |
| SAGE normal gastric body epithelium (stomach normal) | 39 |
| SAGE PERITO 13 (normal peritoneum) | 36 |
| SAGE normal pediatric cortex H1571 (normal cortex fetal) | 25 |
| SAGE normal lung | 22 |
| SAGE Duke thalamus (normal bulk thalamus) | 20 |
| SAGE normal spinal cord | 18 |
| SAGE normal pool (6th) (brain normal) | 15 |
| SAGE normal liver | 14 |
| SAGE normal heart | 11 |
| SAGE BS542 whitematter (brain normal) | 10 |
with radioactively labeled probes as described in Poulsom et al., 1998 (17). The EndoPDI-specific probe used for in situ hybridization was the same as that used for the RNase protection assay described above. Human tissue was collected with full ethical approval during routine pathology, fixed in formalin, and embedded in paraffin.

**Multiple Tissue Array Analysis**—Human multiple tissue expression arrays (Clontech, Oxford, UK) with poly(A)/H11001 RNA from different tissues were used for analysis of the distribution of EndoPDI mRNA in human tissues. DNA from the same region of EndoPDI used for riboprobe production was used for preparation of a cDNA probe. The cDNA was labeled with \( ^{32}P \)dCTP (Rediprime random primer labeling kit, Amersham Biosciences) and hybridized at 65 °C overnight in ExpressHyb (Clontech) solution following the manufacturer's instructions.

**Transfection of Microvascular Endothelial Cells with siRNA**—EndoPDI specific (5'-GAAGCTGTGAGTACCAGGT-3') and PDI-specific (5'-GACCTCCCCTCAATGTTTT-3') siRNA oligos were synthesized using the Silencer\textsuperscript{TM} siRNA Construction kit (Ambion, Huntingdon, UK) following the manufacturer's instructions. HDMEC (5 \times 10\(^5\) cells) were plated onto 0.2% gelatin coated 10-cm Petri dishes and incubated for 24 h. Cells were then transfected with 10 nm EndoPDI and/or PDI siRNA using oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. The cells were then incubated for 24 h prior to hypoxia for 16 h (0.1% O\(_2\), 5% CO\(_2\), and 94.9% N\(_2\)) or continuation of normoxic exposure for 16 h before performing FACS analysis as described.

**FACS Analysis**—To distinguish apoptotic from necrotic cells, double staining for exposed phosphatidylserine and propidium iodide (PI) exclusion was performed as follows: Cells were harvested, washed twice with PBS, and resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl\(_2\)). 5 \mu l of annexin V-FITC antibody (BD Pharmingen, San Diego) and 10 \mu l of PI (50 \mu g/ml) were added to the cells. After incubation for 15 min in the dark at room temperature, the cells were analyzed by a FACSscan. Controls of unstained cells, cells stained with annexin V-FITC only, and cells stained with PI only were

---

**Fig. 1. Sequence homology and structural organization of EndoPDI.**

A, homology alignment of human EndoPDI with other species. Comparison of the human EndoPDI sequence with genome databases for other species identified homologues of EndoPDI in the rat, mouse, *Xenopus*, *Drosophila*, and mosquito. B, structural comparison of EndoPDI with other members of the PDI family. In the diagram, PDIs are classified according to the presence of the PDI CXXC or Trx motif, together with the KDEL endoplasmic reticulum retention sequence. The domains containing the CXXC motif are also known as a domains and the domains containing no active site but containing the thioredoxin structural fold are known as b domains. Unlike, the other members of the family, EndoPDI appears to contain no b domain, rather it has the structural organization a', a, a', c.
used to set up compensation and quadrants.

The cell surface expression of CD105 was quantified by incubating 10⁵ cells per tube with 50 μl (10 μM) in phosphate-buffered saline of monoclonal antibody to CD105 on ice for 1 h and washed twice with cold phosphate-buffered saline. After incubation with FITC-labeled rabbit anti-mouse F(ab)₂ (1/40 DAKO) for 30 min on ice, the cells were washed and resuspended in 0.3 ml of 2% buffered formalin and analyzed on a FACScan.

**Measurement of Endothelin-1 Secretion by ELISA**—The secretion of endothelin-1 by endothelial cells was measured using a human endothelin-1 ELISA (R&D Systems, Abingdon, UK) following the manufacturer’s instructions. Briefly, 50,000 cells were treated with RNAi oligos as before, the medium collected and diluted 1/25 for use in the ELISA.

**Measurement of Adrenomedullin by Radioimmunoassay**—The secretion of adrenomedullin by endothelial cells was measured using an adrenomedullin radioimmunoassay (Peninsula Laboratories Europe, Ltd, Merseyside, England) following the manufacturer’s instructions. Briefly, 50,000 cells were treated with RNAi oligos as before, the medium collected and used in the radioimmunoassay.

**RESULTS**

**Identification of EndoPDI using Bioinformatic and cDNA Sequence Analysis**—We utilized serial expression of gene analysis (SAGE) libraries (www.ncbi.nlm.nih.gov/SAGE) to find genes that are highly expressed in endothelial cells. Using this approach we identified a novel gene, which we subsequently called EndoPDI, that is highly expressed in both VEGF-stimulated and quiescent microvascular endothelial cells (HMVEC) with counts of 1224 and 741tags per million, respectively (Table I). The library with the next highest tag count for EndoPDI was the foreskin fibroblast library with 204 tags per million, i.e., less than a third of that for HMVEC. Homologues of EndoPDI have been identified in the mouse, rat, *Xenopus*, *Drosophila*, and mosquito (Fig. 1A). While PDI has only two APWCCHGC thioredoxin motifs, EndoPDI has three (Fig. 1B); however, both have in common a C-terminal KDEL sequence, which is characteristic of proteins that are retained within the endoplasmic reticulum. We used the Neural Network program, SignalIP (18) to analyze the protein sequence of EndoPDI and found that it contains a signal peptide, MPARPORLPLLARPAALTALLLHGGGGRW at the N terminus with the most likely cleavage site being located between positions 32 and 33 (GGG-RW). All other members of the PDI family contain a domain that has the thioredoxin structural fold, also called the b domain, but contain no thioredoxin active site. Using the structure prediction program VAST (19) we found that EndoPDI has the structure α₃, a, a, a, c, with no b domain (Fig. 1B).

**Genomic Organization, Chromosomal Localization, and Tissue Distribution of EndoPDI**—The putative full-length gene encoding EndoPDI has been found in genome databases (accession no. BD127641) and mapped to human chromosome 6 at position 6p25.2. This region of chromosome 6 also encodes another molecule containing thioredoxin-like domains called PICOT (20).

We performed RNase protection analysis to examine the expression of EndoPDI in vitro in ten different cell types (Fig. 2). As expected, EndoPDI expression was highest in endothelial cells. The other cell lines tested were MRC-5 (fibroblast), SY-SH-SY (neuroblastoma), SK23 (skin fibroblast), MDA468 (breast carcinoma), NCIH520 (squamous cell lung carcinoma), ZR75 (estrogen-dependent breast carcinoma), HL60 (promyelocytic leukemia), HMME (immortalized endothelial cell line), HUVEC (human umbilical vein endothelial cells), and HMVEC (human dermal microvascular endothelial cells) by RNase protection assay. Quantitation was performed using a phosphorimager and quantitation software. The relative abundance of EndoPDI in each cell type tested is shown in the bar chart.

The level of EndoPDI mRNA expression was determined in 10 different cell types; MRC-5 (fibroblast), SY-SH-SY (neuroblastoma), SK23 (skin fibroblast), MDA468 (breast carcinoma), NCIH520 (squamous cell lung carcinoma), ZR75 (estrogen-dependent breast carcinoma), HL60 (promyelocytic leukemia), HMME (immortalized endothelial cell line), HUVEC (human umbilical vein endothelial cells), and HMVEC (human dermal microvascular endothelial cells) by RNase protection assay. Quantitation was performed using a phosphorimager and quantitation software. The relative abundance of EndoPDI in each cell type tested is shown in the bar chart.

**FIG. 2. RNase protection analysis of EndoPDI expression by cell lines in vitro.** The level of EndoPDI mRNA expression was determined in 10 different cell types; MRC-5 (fibroblast), SY-SH-SY (neuroblastoma), SK23 (skin fibroblast), MDA468 (breast carcinoma), NCIH520 (squamous cell lung carcinoma), ZR75 (estrogen-dependent breast carcinoma), HL60 (promyelocytic leukemia), HMME (immortalized endothelial cell line), HUVEC (human umbilical vein endothelial cells), and HMVEC (human dermal microvascular endothelial cells) by RNase protection assay. Quantitation was performed using a phosphorimager and quantitation software. The relative abundance of EndoPDI in each cell type tested is shown in the bar chart.

**Measurement of EndoPDI mRNA Expression in Human Tissues**—We utilized serial analysis of gene expression (SAGE) libraries (www.ncbi.nlm.nih.gov/SAGE) to find genes that are highly expressed in endothelial cells. Using this approach we identified a novel gene, which we subsequently called EndoPDI, that is highly expressed in both VEGF-stimulated and quiescent microvascular endothelial cells (HMVEC) with counts of 1224 and 741 tags per million, respectively (Table I). The library with the next highest tag count for EndoPDI was the foreskin fibroblast library with 204 tags per million, i.e., less than a third of that for HMVEC. Homologues of EndoPDI have been identified in the mouse, rat, *Xenopus*, *Drosophila*, and mosquito (Fig. 1A). While PDI has only two APWCCHGC thioredoxin motifs, EndoPDI has three (Fig. 1B); however, both have in common a C-terminal KDEL sequence, which is characteristic of proteins that are retained within the endoplasmic reticulum. We used the Neural Network program, SignalIP (18) to analyze the protein sequence of EndoPDI and found that it contains a signal peptide, MPARPORLPLLARPAALTALLLHGGGGRW at the N terminus with the most likely cleavage site being located between positions 32 and 33 (GGG-RW). All other members of the PDI family contain a domain that has the thioredoxin structural fold, also called the b domain, but contain no thioredoxin active site. Using the structure prediction program VAST (19) we found that EndoPDI has the structure α₃, a, a, c, with no b domain (Fig. 1B).

**Genomic Organization, Chromosomal Localization, and Tissue Distribution of EndoPDI**—The putative full-length gene encoding EndoPDI has been found in genome databases (accession no. BD127641) and mapped to human chromosome 6 at position 6p25.2. This region of chromosome 6 also encodes another molecule containing thioredoxin-like domains called PICOT (20).

We performed RNase protection analysis to examine the expression of EndoPDI in vitro in ten different cell types (Fig. 2). As expected, EndoPDI expression was highest in endothelial cells. The other cell lines tested were MRC-5 (fibroblast), SY-SH-SY (neuroblastoma), SK23 (skin fibroblast), MDA468 (breast carcinoma), NCIH520 (squamous cell lung carcinoma), ZR75 (estrogen-dependent breast carcinoma), and HL60 (promyelocytic leukemia). We found that EndoPDI expression was greatest in large vessel endothelial cells (HUVEC) compared with microvascular endothelial cells (HMVEC), with the immortalized cell line HMME displaying the lowest expression among the endothelial cells. The promyelocytic leukemia cell line, HL60 displayed a 2.5-fold higher expression of EndoPDI than MDA468, the next highest expressing cell line. Expression of EndoPDI in HL60 may reflect the common cell lineage of hematopoietic and endothelial cells in that both originate from hemangioblasts in the embryonic blood islands. The expression of EndoPDI in the remaining 6 cell lines was at least 4-fold lower than in endothelial cells.

**Tissue Expression Array Studies**—Human multiple tissue expression arrays were used to determine the relative expression of EndoPDI mRNA in human tissues (Fig. 3A). The blots contained poly(A)⁺ RNA from 72 different human tissues. EndoPDI was detected in 20 of 72 tissue spots. The highest levels were found in lymph node, followed by stomach then heart. The high expression in the stomach was unexpected since the stomach is not a particularly well vascularized organ, and in light of the hypoxic induction of EndoPDI described later, not known to be hypoxic. Arrays containing tissue from matched tumor and normal tissue samples were used to determine whether EndoPDI is up-regulated in human tumors (Fig. 3B). Up-regulation of endoPDI was found in tumors of the cervix, uterus, stomach, and lung.

**In Situ Hybridization Studies**—In situ hybridization studies were performed in order to define expression of EndoPDI in human tissues in vivo (Fig. 4). Expression was found to be rare and seen only in the vasculature of human melanoma (A–D), the syncytiotrophoblasts of placenta (E and F), in macrophages and the microvasculature of the atherosclerotic plaque (G and H) and in the keratinocytes of a hair follicle (I and J).

**EndoPDI Is Up-regulated by Hypoxia in HMVEC**—We next investigated whether EndoPDI is regulated by hypoxia. Using RNase protection analysis we found that EndoPDI is 2-fold up-regulated after 1 h hypoxia in HMVEC with a maximal 2.5-fold induction after 16 h hypoxia (Fig. 5A). Western analysis confirmed the protein to be 3-fold up-regulated by hypoxia (Fig. 5B).

**Loss of EndoPDI Causes Increased Apoptotic Cell Death in Microvascular Endothelial Cells in Hypoxia but Not Normoxia**—Since the up-regulation of PDI has been previously...
shown to have a protective effect against apoptotic cell death induced by hypoxia in neuronal cells (21), we investigated whether EndoPDI has a role in protecting endothelial cells from apoptosis under hypoxia. The approach we used was to down-regulate EndoPDI expression using specific siRNA oligos. The siRNA efficiently down-regulated EndoPDI mRNA

**Fig. 3.** *Multiple tissue array analysis of EndoPDI expression.* A, relative abundance of EndoPDI mRNA in normal tissues. Normal tissue blots were used to determine the level of EndoPDI expression in human tissues. The bar chart shows the relative expression above background for each tissue. The tissues have been grouped into cardiac, gastrointestinal and others. B, relative abundance of EndoPDI in matched normal versus tumor tissue. Matched tumor and normal tissue blots were used to examine the relative expression of EndoPDI in tumor and normal tissues. The number of patient samples in each group were as follows: cervix, n = 1; uterus, n = 3; stomach, n = 8; lung, n = 6.
Cells were treated with either transfection reagents alone, EndoPDI-specific siRNA, or scrambled siRNA. Scrambled siRNA is siRNA that contains the same overall nucleotide composition as the gene-specific siRNA but has no homology to any known genes according to BLAST search results. We found that under normoxia, HDMEC that had been treated with transfection reagents alone (control) or with scrambled oligos expressed the same level of EndoPDI mRNA and protein, and this expression was completely blocked after transfection with EndoPDI-specific siRNA (Fig. 6). Similar results were observed when the cells were subjected to 16 h hypoxia (0.1% O2) in that the expression level of EndoPDI mRNA and protein was unaffected by treatment with scrambled siRNA (Fig. 6). Again under hypoxia, there was loss of EndoPDI expression after transfection with siRNA specific to EndoPDI.

We used the same approach to down-regulate the expression of PDI using siRNA oligos specific to PDI. As with EndoPDI, we found that HDMEC treated with siRNA to PDI had lost PDI protein expression under both normoxia and hypoxia (Fig. 6C).

We used siRNA to examine the effect of down-regulation of EndoPDI on HDMEC survival under hypoxia. We found that down-regulation of EndoPDI under normoxia had no effect on HDMEC survival (Fig. 7A). However, when HDMEC were treated with EndoPDI siRNA under hypoxia there was a significant increase in the apoptotic and necrotic cell populations (Fig. 7B).

**FIG. 4.** Demonstration of EndoPDI in human tissues by in situ hybridization. Moderate expression (arrows) of EndoPDI is seen in the endothelium of a melanoma (A and C, bright field; B and D, dark field). High expression (arrows) is seen in the syncytiotrophoblast cells of placenta (E, bright field; F, dark field). There is a detectable signal (arrow) in the endothelial cells and macrophages of an atherosclerotic plaque (G, bright field; H, dark field). Very strong signal was detected in the keratinocytes of a human skin hair follicle (I, bright field; J, dark field).

**FIG. 5.** Induction of EndoPDI expression by hypoxia. RNase protection and Western blotting analysis were used to measure the expression of EndoPDI mRNA (A) and protein (B) after 1, 4, 8, 16, and 24 h of exposure to 0.1% oxygen. Induction of EndoPDI mRNA was seen after 1 h and during 16 h of hypoxia. Induction of EndoPDI protein was seen after 4 h and during at least 24 h of hypoxia.

**Loss of PDI Causes Increases Apoptotic Cell Death in Microvascular Endothelial Cells in Both Normoxia and Hypoxia—** The effect of EndoPDI on endothelial cell behavior was compared with that of PDI. Using PDI-specific siRNA to down-regulate PDI, we performed FACS analysis to determine the extent of apoptosis resulting from the loss of PDI (Fig. 8). In contrast to EndoPDI, we found that loss of PDI caused a high level of apoptosis in normoxia as well as under hypoxia. In fact, under the same conditions, PDI down-regulation resulted in 55 and 48% of the cell population being apoptotic in normoxia and hypoxia, respectively, whereas 12% of the cell population was apoptotic under hypoxia after down-regulation of EndoPDI and only 4.5% apoptotic under normoxia.
The Effect of Lack of EndoPDI and PDI Expression on the Secretion or Cell Surface Expression of Hypoxically Induced Endothelial Survival Factors—Under hypoxia, endothelial cells produce a number of molecules that act as hypoxia survival factors. Examples of such molecules are endothelin-1 (22) adrenomedullin (23) and CD105 (24). We compared the expression of these molecules by endothelial cells under hypoxia after knockout of EndoPDI, PDI, or both EndoPDI and PDI together. There was a significant decrease in endothelin-1 expression after both PDI siRNA treatment and EndoPDI siRNA treatment (Fig. 9A). The reduction in endothelin-1 secretion after treatment with either EndoPDI siRNA alone or PDI siRNA alone was statistically the same as secretion under normoxia. Treatment with combined EndoPDI and PDI siRNA caused a larger, but not significant reduction in the secretion of endothelin-1 compared with that of EndoPDI siRNA alone or PDI siRNA alone. These results suggest that both PDI and EndoPDI have a role in the folding and secretion of endothelin-1 under hypoxia with neither protein having greater specificity for endothelin-1 than the other.

In contrast, while loss of EndoPDI expression significantly reduced adrenomedullin secretion under hypoxia, loss of PDI expression had little effect (Fig. 9B). Furthermore, there was no significant difference in adrenomedullin secretion after both PDI and EndoPDI siRNA treatment compared with EndoPDI siRNA alone. These results suggest that PDI has little or no role in the folding and secretion of adrenomedullin under hypoxia, but that EndoPDI has greater specificity for this molecule. That there is still adrenomedullin production after the knockout of both PDI and EndoPDI suggests that there are other chaperones that function in the folding and secretion of this peptide.

CD105 (also called endoglin) is an endothelial specific gene whose expression is up-regulated by hypoxia and has been shown to protect endothelial cells from apoptosis under hypoxia (24). Fig. 9C shows that hypoxia caused up-regulation of CD105 expression on HDMEC, corresponding to a 64% increase in cell surface expression as determined using the geometric mean fluorescent intensities. While siRNA to PDI had little effect on CD105 expression, that to EndoPDI caused a marked reduction in expression of 50% of the population (two peaks apparent) and that the two siRNAs administered together completely ablated CD105 expression. The effect of EndoPDI specific siRNA on CD105 expression is similar to that previously reported for treatment with antisense CD105 where a similar CD105 mixed cell expression population results (24). We conclude that while EndoPDI may complement CD105 folding in the absence of PDI, the reverse is not the case, arguing strongly for a role for EndoPDI in folding this molecule under hypoxia.

DISCUSSION

We describe here a novel human protein-disulfide isomerase that we have called EndoPDI due to its high and preferential expression in endothelial cells. Experiment has shown that EndoPDI expression is up-regulated by hypoxia, is only expressed in vivo in hypoxic tissues and protects endothelial cells from death under hypoxia. We also show that the protective effect of EndoPDI under hypoxia could be caused by a folding and chaperone activity on hypoxic-induced anti-apoptotic molecules.

Unlike archetypal PDI, EndoPDI is a rare example of a PDI
that has three a-type domains containing the conserved AP-WCGHC thioredoxin domain but no b domains. The existence of a strong Kozak sequence and a well defined signal peptide sequence lends support to this being a full-length cDNA clone for EndoPDI. The N-terminal leader sequence and the C-terminal KDEL, provide strong evidence that like PDI, EndoPDI should be predominantly localized to the ER.

EndoPDI appears to have an unusual pattern of tissue expression. As expected we detected expression in well vascularized tissues such as heart, lung, and lymph node but unexpectedly we found higher expression in some tissues of comparatively low vascular density such as the stomach. Nevertheless, the overall pattern of tissue expression obtained for EndoPDI from the tissue expression array is fairly consistent with the SAGE expression data; for example the SAGE data suggests that the expression of EndoPDI in the stomach is around 4-fold higher than in the heart. Furthermore, the SAGE array data suggest that the expression of EndoPDI in the liver and heart
is roughly equal, a result that is confirmed by the tissue expression array data. The relative levels of the other PDI family members vary greatly between different tissue types, between different cell types within the same tissue and with the physiological state of the cell (28). The only member of the PDI family to date shown to have restricted tissue expression is...
Endothelial Protein-disulfide Isomerase

PD1P, the pancreas specific PD1 (29). PD1P may be required for the folding of pancreas specific proteins such as zymogens (30). We may similarly speculate that EndoPDI may be required for the folding of endothelial cell specific proteins.

Expression of EndoPDI in endothelial cells is at least 3-fold higher than in the carcinoma or other tumor cell lines tested, with the notable exception of HL60. A detailed array analysis recently identified EndoPDI (known as MGC5178 in that study) as the most up-regulated gene in multiple myeloma (31). Our results show that PDI is an absolute requirement of endothelial cells under hypoxic conditions due to its enabling secretion of adrenomedullin and endothelin-1, whereas PDI production in these cells is not increased above basal expression levels upon hypoxia. We therefore hypothesized that EndoPDI in concert with PDI enables endothelial cells to show a greater capacity for survival under hypoxia. To test this hypothesis, we measured the secretion of endothelin-1 and adrenomedullin together with the cell surface expression of CD105, molecules with known protective effects against hypoxia-induced apoptosis in endothelial cells. All three are known to be hypoxia-induced and to protect endothelial cells from death under hypoxia (22, 24, 27). We found that PDI and EndoPDI appear to be equally important in the secretion of endothelin-1, whereas EndoPDI may be more important than PDI in CD105 and adrenomedullin secretion from endothelial cells. In fact, knock-out of PDI had little effect on CD105 expression whereas knockout of EndoPDI and PDI together completely abolished expression.

In summary, we have identified EndoPDI as a novel protein-disulfide isomerase-like protein that is highly expressed in endothelial cells, is up-regulated by hypoxia and is expressed in the endothelium of tumors and atherosclerotic plaques. EndoPDI appears to be a requirement for endothelial cell survival under hypoxic conditions due to its enabling secretion of several endothelial cell survival factors. Further work is needed to delineate the role of PDI and EndoPDI in the hypoxic endothelial cell.

Acknowledgments—We thank Rosemary Jeffery for help with the in situ analysis and Pat Clissold for BLAST identification of mosquito EndoPDI. We also thank Steven Suchting for helpful discussions.

REFERENCES

1. Freedman, R. B., Hirst, T. R., and Tuite, M. F. (1994) Trends Biochem. Sci. 19, 331–336
2. Cottam, D. M., and Sambrook, J. (1992) Nature 355, 53–45
3. Edman, J. C., Ellis, L., Blacher, R. A., and Rutter, W. J. (1985) Nature 317, 267–270
4. Hawkins, H. C., and Freedman, R. B. (1991) Biochem. J. 275, 335–339
5. LaMania, M. L., and Lennarz, W. J. (1993) Cell 74, 899–908
6. Lyles, M. M., and Gilbert, H. F. (1994) J. Biol. Chem. 269, 30946–30952
7. Darby, N. J., and Creighton, T. E. (1995) Biochemistry 34, 11725–11735
8. Nan, Y., Zhou, Y., and Wang, C. (1997) EMBO J. 16, 651–658
9. Karpus, J., Pirinenkuski, A., Karponen, P., Ljung, J., Helaasikokki, T., Notbohm, H., and Kivirikko, K. I. (1999) EMBO J. 18, 65–74
10. John, D. C., Grant, M. E., and Bulleid, N. J. (1998) EMBO J. 17, 1587–1595
11. McLaughlin, S. H., and Bulleid, N. J. (1998) Biochem. J. 331, 795–805
12. Clissold, P. M., and Bicknell, R. (2003) Bioessays 25, 603–611
13. Elliott, J. G., Oliver, J. D., and High, S. (1997) J. Biol. Chem. 272, 13849–13855
14. Oliver, J. D., van der Wal, F. J., Bulleid, N. J., and High, S. (1997) Science 275, 86–88
15. Lash, A. E., Tolstoshev, C. M., Wagner, L., Schuler, G. D., Strausberg, R. L., Riggins, G. J., and Altschul, S. F. (2000) Genome Res. 10, 1051–1060
16. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
17. Paulisom, R., Longeroft, J. M., Jeffery, R. E., Rogers, L. A., and Steel, J. H. (1998) EMBO J. 17, 121–132
18. Nielsen, A., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) Proc. Natl. Acad. Sci. 10, 1–6
19. Gibart, J. F., Madej, T., and Bryant, S. H. (1996) Curr. Opin. Struct. Biol. 6, 377–385
20. Wirtz, S., Villalba, M., Bi, K., Liu, Y., Isakov, N., and Altman, A. (2000) J. Biol. Chem. 275, 1962–1969
21. Tanaka, S., Uehara, T., and Nomura, Y. (2000) J. Biol. Chem. 275, 10388–10393
22. Shichiri, M., Kato, H., Marumo, F., and Hirata, Y. (1997) Hypertension 30, 1198–1203
23. Oehler, M. K., Norbury, C., Hague, S., Rees, M. C., and Bicknell, R. (2001) Oncogene 20, 2937–2945
24. Li, C., Issa, R., Kumar, P., Hanscom, J. M., Lopez-Mejia, J. M., Benameh, C., and Kumar, S. (2003) J. Cell Sci. 116, 2677–2685
25. Keumbraban, S., Marodon, P. A., McQuillan, P. L., and Fuller, D. V. (1991) J. Clin. Invest. 88, 1054–1057
26. Sugo, S., Minamino, N., Kangawa, K., Miyamoto, K., Kitamura, K., Sakata, J., Eto, T., and Matsuo, H. (1994) Biochem. Biophys. Res. Commun. 201, 1160–1166
27. Miller, M. J., Martinez, A., Unsworth, E. J., Thiele, C. J., Moody, T. W., Causer, T., and Cuttitta, F. (1996) J. Biol. Chem. 271, 23345–23351
28. Freedman, R. B., Dunn, A. D., and Rudder, L. W. (1998) Curr. Biol. 8, 468–470
29. Desilva, M. G., Notkins, A. L., and Lan, M. S. (1997) DNA Cell Biol. 16, 269–274
30. Volkner, J., Guth, S., Nastainczyk, W., Knippe, P., Klapp, P., Gnau, V., and Zimmermann, R. (1997) FEBS Lett. 406, 291–295
31. Claudio, J. O., Nishimura, F., Hase, Y., Hasegawa, H., Voralia, M., Li, Z. H., Nadeem, V., Cukerman, E., Francisco-Pahalan, O., Liew, C. C., Woodgett, J. R., and Stewart, A. K. (2002) Blood 100, 2175–2186
32. Vazquez, P., Kallinowski, F., and Okunless, P. (1999) Adv. Exp. Med. Biol. 477, 895–905
33. Bjornheden, T., Levin, M., Evaldson, M., and Wiklund, O. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 870–876
34. Yano, K., Brown, L. F., and Detmar, M. (2001) J. Clin. Invest. 107, 409–417
35. Adams, M. D., Kerlavage, A. R., Fleischmann, R. D., Fuldner, R. A., Bult, C. J., Lasker, T., Mulligan, M., and Lipman, D. J. (1995) Nature 377, 3–174
36. Tucci, M., McDonald, R. J., Aaronson, S., Graven, K. K., and Farber, H. W. (1996) Am. J. Physiol. 271, L341–348
37. Graven, R. K., and Farber, H. W. (1994) J. Lab. Clin. Med. 123, 456–463
38. Graven, K. K., Molvær, C., Roncorati, J. S., Klahn, B. D., Lowrey, S., and Farber, H. W. (2002) Am. J. Physiol. Lung Cell Mol. Physiol. 282, L996–1003
39. Humineckie, L., and Bicknell, R. (2000) Genome Res. 10, 1796–1806
40. Ho, M., Yang, E., Mateck, G., Deng, D., Sampas, N., Tsaklenko, A., Tabibiazar, R., Zhang, Y., Chen, M., Tabb, S., Hsu, Y. T., Wang, J., Tsa, P. S., Ben-Dor, A., Yakhini, Z., Bruhn, U., and Quertermous, T. (2003) Proc. Natl. Acad. Sci. USA 100, 2347–2352
EndoPDI, a Novel Protein-disulfide Isomerase-like Protein That Is Preferentially Expressed in Endothelial Cells Acts as a Stress Survival Factor
Dianne C. Sullivan, Lucasz Huminiecki, John W. Moore, Joseph J. Boyle, Richard Poulsom, Daniel Creamer, Jonathan Barker and Roy Bicknell

J. Biol. Chem. 2003, 278:47079-47088.
doi: 10.1074/jbc.M308124200 originally published online September 8, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308124200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 16 of which can be accessed free at
http://www.jbc.org/content/278/47/47079.full.html#ref-list-1