Hypoxia-induced expression of complement receptor type 1 (CR1, CD35) in human vascular endothelial cells

Charles D. Collard, Cuneyt Bukusoglu, Azin Agah, Sean P. Colgan, Wende R. Reenstra, B. Paul Morgan, and Gregory L. Stahl. Hypoxia-induced expression of complement receptor type 1 (CR1, CD35) in human vascular endothelial cells. Am. J. Physiol. 276 (Cell Physiol. 45): C450–C458, 1999.—Reoxygenation of hypoxic human umbilical vein endothelial cells (HUVECs) increases protein expression of the complement regulators CD46 and CD55. As the receptor for C3b is known to be present on injured bovine endothelial cells, we investigated whether hypoxia or inflammatory mediators induce complement receptor type 1 (CR1; CD35) expression on HUVECs. CR1 protein expression increased 3.7 ± 0.6-fold as measured by ELISA on HUVECs following hypoxia (48 h, 1% O2). Colocalization of CD35 and von Willebrand factor by confocal microscopy confirmed that CD35 was predominantly intracellular. Lipopolysaccharide or tumor necrosis factor-α also significantly increased HUVEC CR1 protein expression. Western blot analysis of neutrophil or hypoxic HUVEC lysates revealed a 221-kDa CR1 band under nonreducing conditions. RT-PCR of hypoxic HUVEC mRNA revealed a single band that, after sequencing, was identified as CD35. In situ hybridization of hypoxic HUVECs, but not normoxic HUVECs or fibroblasts, demonstrated increased CD35 mRNA. Hypoxic HUVECs bound immune complexes and acted as a cofactor for factor I-mediated cleavage of C3b. Thus hypoxia induces functional HUVEC CR1 expression.

Methods

Cell Culture

HUVECs were obtained as previously described (3). HUVECs were harvested using 0.1% collagenase (Worthington Biochemical, Freehold, NJ) and suspended in medium 199 containing 20% heat-inactivated bovine calf serum (GIBCO Life Technologies, Grand Island, NY). The cells were initially seeded in 75-cm² flasks (Corning Costar, Cambridge, MA) and incubated at 37°C in 95% air and 5% CO₂. When confluent, the endothelial cells were passaged using 0.5% trypsin-EDTA. Endothelial cell purity was assessed by phase microscopic "cobblestone appearance," von Willebrand factor (vWF) expression, and uptake of fluorescent acetylated low-density lipoprotein. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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density lipoprotein. All experiments were conducted on HUVECs cultured for three or fewer passages.

**CR1 ELISA**

A CR1-specific ELISA was developed using monospecific rabbit polyclonal or mouse monoclonal anti-CR1 antibodies [monoclonal antibody 3C6.D11 specific for the long homologous repeat (LHR)-D domain of human CR1; T Cell Sciences, Needham, MA] and confluent HUVECs grown on 0.1% gelatinized 96-well plastic plates (Corning Costar). CR1 protein expression was measured on HUVECs subjected to 0, 24, or 48 h of hypoxic stress in a humidified sealed chamber (Coy Laboratory Products, Grass Lake, MI) maintained at 37°C and gassed with 1% O2, 5% CO2, and balance N2 as previously described (3). After removal of the HUVECs from the hypoxia chamber or incubator, the cells were washed, lightly fixed and permeabilized with 1% paraformaldehyde (Sigma Chemical, St. Louis, MO), and then washed. The HUVECs were then incubated (4°C for 105 min) with monocalc or polyonal anti-CR1 antibody. The HUVECs were then washed and incubated (4°C for 1 h) with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Cappel, Durham, NC) or HRP-conjugated goat anti-mouse IgG antibodies. The HUVECs were then washed, developed with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and read (Molecular Devices, Sunnyvale, CA) at 405 nm. Binding of a monoclonal anti-human major histocompatibility complex (MHC) class I antibody [clone W6/32, IgG2a, hybridoma HB95, obtained from the American Type Culture Collection (ATCC), Manassas, VA (1)] was also measured at each experimental time point. In additional experiments, polyclonal anti-CR1 antibody was competed off hypoxic HUVECs by addition of 4, 40, and 400 pmol/l recombinant soluble complement receptor type 1 (scCR1; T Cell Sciences) to further confirm the specificity of the CR1 ELISA. All ELISA experiments were performed in triplicate, using six wells per experimental group. An isotype-matched monoclonal antibody to human C5a and rabbit anti-rodion and polyclonal antibody (Jackson Immunobiochemicals) were used as isotype species-matched controls to detect background optical density. Background optical density was subtracted from all values. Data are presented as means ± SE of optical density at 405 nm (OD405).

Expression of intercellular adhesion molecule-1 (ICAM-1) and CR1 was also quantified by ELISA in normoxic HUVECs (2) and CR1 was also quantified by ELISA in normoxic HUVECs (3) was also measured at each experimental time point. In additional experiments, polyclonal anti-CR1 antibody was competed off hypoxic HUVECs by addition of 4, 40, and 400 pmol/l recombinant soluble complement receptor type 1 (scCR1; T Cell Sciences) to further confirm the specificity of the CR1 ELISA. All ELISA experiments were performed in triplicate, using six wells per experimental group. An isotype-matched monoclonal antibody to human C5a and rabbit anti-rodion and polyclonal antibody (Jackson Immunobiochemicals) were used as isotype species-matched controls to detect background optical density. Background optical density was subtracted from all values. Data are presented as means ± SE of optical density at 405 nm (OD405).

**Confocal Microscopy Studies**

Immunohistochemistry. Cells grown on Labtek (NUNC) tissue culture slides were subjected to 0 or 48 h of hypoxia, washed in ice-cold PBS, and then fixed in 4% paraformaldehyde-PBS for 10 min. After a wash in PBS, the tissues were permeabilized with 0.1% Triton X-100 for 5 min. After another wash in PBS, the cells were incubated for 10 min in 10% normal goat serum (GIBCO) to block nonspecific secondary antibody staining.

A monoclonal antibody specific for CR1 (YZ-1, gift from Dr. Lloyd Klickstein) was diluted 1:100 in 3% normal goat serum-PBS. YZ-1 binds to LHR-A, LHR-B, and LHR-C within short consensus repeats 5–7 of each LHR (10). A polyclonal antibody specific for vWF was diluted 1:1,000 in 3% normal goat serum-PBS. The cells were incubated overnight at room temperature. Excess primary antibody was removed by washing in PBS. The CR1 and vWF primary antibodies were visualized using tetramethylrhodamine isothiocyanate-IgG goat anti-mouse and FITC-IgG goat anti-rabbit secondary antibodies, respectively (Jackson Immunoresearch, West Grove, PA). After three 10-min washes in PBS, the slides were coated with anti-fade mounting medium, covered, and analyzed with a Leica confocal microscope.

Controls with secondary antibody only were processed as above, omitting the primary antibody to determine nonspecific binding. To determine if FITC fluorescent probes were not recorded on the rhodamine photomultiplier tube, crossover controls were analyzed as follows. Single-labeled material for each fluorescent tag was scanned at the same confocal microscope settings with the opposite filter set and photomultiplier tube. Human dermal fibroblast cells were utilized as a negative control (i.e., non-CR1 containing cells). This experiment was repeated four times.

In situ hybridization. Escherichia coli HB101 strain containing pCR1 plasmid was obtained from ATCC (no. 73730). The cells were grown in Luria broth containing ampicillin (50 µg/ml) and tetracycline (12.5 µg/ml) for 15 h at 37°C. The plasmid DNA was isolated using Qiagen-tip 500 (Qiagen, Chatsworth, CA). The purified plasmid DNA was digested with EcoRI to release the 0.79-kb fragment of human CR1 cDNA. The cDNA fragment was then purified from a low-melting agarose gel (11) and labeled with biotin-16-dUTP, and the probes were separated with spin columns. Samples of the biotinylated probes were used for Southern blot analysis, and the incorporated biotin was analyzed with a colorimetric reaction product with a DNA detection system [Bethesda Research Laboratories (BRL)]. Biotin-labeled phage fX174 DNA HinI fragments (BRL) were used as DNA size markers.

The complete in situ hybridization protocol has been published (19). Briefly, isolated HUVECs were subjected to 0 or 48 h of hypoxia. The cells were then fixed immediately with 4% paraformaldehyde-PBS (10 min) and washed in PBS-3% BSA (5 mmol/l). All materials were kept RNase-free throughout the procedure.

Before hybridization, the cells were hydrated in 0.2 mol/l Tris-HCl (pH 7.4) and 0.1 mol/l glycine for 10 min and then changed to 50% formamide-2× SSC (SSC contains 0.15 mol/l NaCl, 0.015 mol/l sodium citrate, pH 7.0) at 65°C for 15 min. While the HUVECs were being hydrated, the probe (80–100 µl probe), 4 µl E. coli tRNA (Sigma), and 4 µl salmon sperm DNA (Sigma) were melted in 10–30 µl of 100% formamide (Sigma) at 90°C for 10 min. An equal volume of hybridization mix was added for a final concentration of 50% formamide, 2× SSC, 0.2% BSA, 10 nmol/l vanadyl sulfate-ribonucleoside complex (BRL), 10% dextran sulfate, 1 µg/ml E. coli tRNA, and 1 µg/ml salmon sperm DNA. The final concentration of the probe was 80–100 ng/30 µl hybridization mix (19). The probe and hybridization mix were added to the tissue culture slides, the covers were replaced, and the mixture was incubated at 37°C (4–16 h) in a closed, 2× SSC-saturated chamber. After hybridization, the cells were washed with 2× SSC-50% formamide for 30 min at 37°C, then in 1× SSC-50% formamide for 30 min at 37°C, and twice in 1× SSC at room temperature for 30 min.

The cells were incubated in 4× SSC-1% BSA with avidin-FITC (2 µg/ml) for 30 min and then washed three times in 2× SSC at room temperature on a rotating shaker. The cells were then mounted in antifade mounting medium, covered, and...
viewed on a Leica confocal scanning microscope. Human fibroblasts served as negative controls.

Additional control hypoxic HUVECs were incubated in RNaseA (100 µg/ml in 2× SSC for 1 h at 37°C) to determine specificity of the probe for RNA. After incubation in RNaseA, the cells were hybridized as described above. Incubated with avidin-FITC, washed, and viewed by confocal microscopy.

A third control preparation was used to determine avidin-binding specificity. This control consisted of hypoxic cells incubated in the hybridization mixture without the probe, washed, reacted with FITC-avidin, and then viewed on the confocal microscope. All in situ hybridization studies were done in triplicate.

Western Blot

Confluent HUVEC cultures grown in 60-mm petri dishes were incubated under hypoxic conditions for 48 h. The HUVECs were then washed twice and solubilized with ice-cold lysis buffer (1% Nonidet-P40, 0.1% SDS, 3 mmol/l EDTA, 2 mmol/l phenylmethylsulfonyl fluoride, 3 mmol/l aprotinin, 29 µmol/l pepstatin, and 37 µmol/l leupeptin in PBS, pH 7.4). Polymorphonuclear neutrophils (PMNs; CR1-positive cells) were isolated from freshly collected human blood and solubilized (6). HUVEC and PMN lysates were then resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were then blocked with 10% nonfat dry milk in PBS buffer containing 0.1% Tween 20 and 0.1% BSA and incubated with murine monoclonal anti-CR1 antibody (10 µg/ml; YZ-1; gift of Dr. Richard Jackson) for 1 h at 20°C. The membranes were then washed five times with PBS buffer and incubated with a 1:5,000 dilution of HRP-conjugated rabbit anti-mouse IgG antibody (Sigma) for 1 h at 20°C. The enhanced chemiluminescence (ECL) system (Amersham) was used to develop the Western blots. Western blot analysis was performed in triplicate.

RT-PCR for Human CR1

RNA was purified according to the manufacturer's procedure from normoxic and hypoxic (48 h) HUVECs using the RNeasy total RNA purification kit (Qiagen). The reaction was set up according to Promega's reverse transcription system (Promega, Madison, WI). Briefly, 1 µg of RNA was added to the reaction cocktail consisting of 4 µl of 25 mmol/l protocol (Promega, Madison, WI). Briefly, 1 µg of RNA was set up according to Promega's reverse transcription system (Promega, Madison, WI). The reaction was performed for 35 cycles at 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min. The PCR products were then analyzed and visualized on a NUSIEVE GTG low-melting-temperature (FMC BioProducts, Rockland, ME) 1% agarose gel containing 5 µg/ml ethidium bromide. The resulting 514-bp product was excised from the gel using the Gene Clean II kit (Bio 101, Vista, CA), purified, and submitted for sequencing (Brigham and Women's Hospital Sequencing Facility).

Immune Complex Binding

Immune complex deposition on hypoxic HUVECs was measured by ELISA to demonstrate that endothelial CR1 was functional. HUVECs were grown to confluence on 0.1% gelatinized 96-well plastic plates (Corning Costar) and then subjected to 0 or 48 h of hypoxia. After hypoxia, the cell medium was replaced with 100 µl of 30% human serum treated with peroxidase-conjugated goat anti-human IgG antibody (1:100 dilution; Jackson Immunoresearch) and 0, 4, 40, or 400 pmol/l sCR1 (T Cell Sciences). The cells were then incubated for 1 h at 37°C in 95% air and 5% CO2. The plates were then washed, developed with ABTS, and read. Background controls consisted of cells incubated with 100 µl of Hanks' balanced salt solution treated with peroxidase-conjugated goat anti-human IgG antibody (i.e., no human serum). Background optical density was subtracted from all groups. All ELISA experiments were performed three times using six wells per experimental group.

Western Blot of CR1-Mediated Cleavage of C3b

Factor I-mediated cleavage of C3b in the presence of hypoxic HUVECs was analyzed by Western blotting to demonstrate cofactor activity. HUVECs were grown to confluence on 0.1% gelatinized 96-well plastic plates (Corning Costar) and then subjected to 0 or 48 h of hypoxia. After hypoxia, the cell medium was replaced with 50 µl of the following complement components (Advanced Research Technologies, San Diego, CA): 1) 10 µg/ml of factor I with or without 0.1% Triton X-100; 2) 200 µg/ml of C3b with or without 0.1% Triton X-100; or 3) 10 µg/ml of factor I and 200 µg/ml of C3b with or without 0.1% Triton X-100. As a positive CR1 control, 50 µl of 10 µg/ml factor I and 200 µg/ml C3b were added to 7.3 × 105 red blood cells (RBCs). The cells were then incubated for 1 h at 37°C in 95% air and 5% CO2. The supernatants were then resolved by SDS-PAGE (9%), transferred to nitrocellulose membranes (Bio-Rad), and blocked with 10% nonfat dry milk in PBS buffer containing 0.1% Tween 20 and 0.1% BSA. The membranes were then incubated with peroxidase-conjugated goat IgG fraction to human complement C3 (Cappel, West Chester, PA) for 1 h at 20°C. The ECL system (Amersham) was used to develop the Western blots. This experiment was performed in triplicate.

Statistical Analysis

All data are expressed as means ± SE. Data analysis was performed using SigmaSTAT (Jandel Scientific, San Rafael, CA). CR1 expression and immune complex binding, as measured by ELISA, were analyzed by two-way ANOVA. ELISA data obtained by competing bound anti-human CR1 antibody off hypoxic HUVECs with sCR1 were analyzed by one-way ANOVA. All pairwise multiple comparisons were made using the Student Newman-Keuls test.

RESULTS

CR1 ELISA

We have previously shown increased HUVEC CD46 and CD55 protein expression following hypoxia (3). In
In the present study, we investigated whether 48 h of hypoxia induced HUVEC expression of the complement receptor CD35 (i.e., CR1). CR1 protein expression (Fig. 1) was significantly increased (3.7 ± 0.6-fold) following 48 h of hypoxia compared with normoxic HUVECs (P < 0.05). A small yet significant increase in CR1 was also observed following 24 h of hypoxia using monoclonal anti-human CR1 antibody. Hypoxia-induced CR1 expression was specific, since similar conditions of hypoxia did not influence HUVEC MHC class I antigen expression (OD405 0.28 ± 0.03, 0.31 ± 0.03, and 0.23 ± 0.04 for conditions of normoxia, 24 h hypoxia, and 48 h hypoxia, respectively; not significant). Thus the increase in CR1 expression is not a generalized increase in protein expression.

Recombinant sCR1 was used to compete with HUVEC CR1 for anti-CR1 antibody binding to confirm further the specificity of the CR1 ELISA (Fig. 2). Anti-CR1 antibody binding decreased 68, 75, and 100% following addition of 4, 40, and 400 pmol/l recombinant sCR1 to hypoxic HUVECs (P < 0.05), respectively. These data demonstrate that the ELISA is specific for CR1 and not a result of nonspecific antibody binding.

To rule out the possibility that the increase in CR1 expression was due to cellular contamination of other cell types, we used confocal microscopy. Endothelium-specific CR1 was confirmed by dual labeling (Fig. 3) for CR1 (red) and vWF (green). Figure 3 clearly demonstrates increased CR1 staining within hypoxic (Fig. 3B) HUVECs (i.e., vWF-positive cells) compared with normoxic cells (Fig. 3A). Although some CR1 staining was observed on the extracellular membrane, the majority of CR1 staining was intracellular.

We also investigated whether the inflammatory mediators LPS and TNF-α increased CR1 protein expression. Stimulation of normoxic HUVECs with LPS or TNF-α (18 h of stimulation) increased CR1 expression in a dose-dependent manner (Fig. 4). TNF-α (1–10 ng/ml) or LPS (10–1,000 ng/ml) significantly increased CR1 protein expression (Fig. 4, A and B). Similarly, ICAM-1 expression (Fig. 4, A and B) significantly (P < 0.05) increased following incubation (18 h) with TNF-α (1–10 ng/ml) or LPS (10–1,000 ng/ml). Thus, in addition to hypoxia, inflammatory mediators may also increase HUVEC CR1 protein expression.

Western Blot Analysis

Western blotting of hypoxic HUVEC lysates, PMN lysates, and recombinant sCR1 was performed (Fig. 5). Monoclonal anti-human CR1 antibody, under nonreducing conditions, recognized a high-molecular-mass band (~221 kDa) in the HUVEC and PMN lysates that was of a slightly higher molecular mass than the sCR1 band. The HUVEC CR1 band was similar in molecular mass to that of the PMN lysate. A similar protein band profile was observed using the rabbit polyclonal anti-human CR1 antibody (data not shown). Biotinylation of cell surface membrane proteins and subsequent immunoprecipitation failed to demonstrate cell surface CR1, thus confirming the confocal findings that CR1 is primarily intracellular (data not shown). Hypoxic HUVECs stimulated with thrombin (10 U/ml) or histamine (10 mmol/l) failed to induce CR1 expression on the cellular surface or in cellular supernatants (data not shown).

RT-PCR

To confirm further the presence of CR1, RT-PCR was used to identify CR1 mRNA in normoxic and hypoxic HUVECs. Amplification of the RT template from normoxic and hypoxic HUVECs EXPRESS CR1 by 10.2 ± 0.3 on November 6, 2017 http://ajpcell.physiology.org/ Downloaded from http://ajpcell.physiology.org/
moxic HUVECs failed to give an observable (i.e., ethidium bromide-stainable) CR1 product (data not shown). Amplification of the RT template from hypoxic HUVECs yielded the expected 514-bp product (Fig. 6). Blast alignment of the sequenced 514-bp product confirmed the product to be CR1 cDNA ($P < 10^{-123}$).

In Situ Hybridization

In situ hybridization studies were performed to confirm the RT-PCR findings, to further demonstrate CR1 mRNA, and to rule out cellular contamination (Fig. 7). HUVECs were identified by positive vWF staining (green). HUVECs grown under hypoxic (Fig. 7B) conditions demonstrated an increase in cytoplasmic staining and negative nuclear staining compared with normoxic cells (Fig. 7A). RNase-treated hypoxic cells (data not shown) and human dermal fibroblasts (Fig. 7D) were used as negative controls and to demonstrate the specificity of the staining. Furthermore, HUVECs did not demonstrate collagen type X expression, as an additional control (Fig. 7C). These data further confirm the presence of CR1 mRNA in hypoxic HUVECs.

Functional Effects of HUVEC CR1

Immune complex binding. To identify a possible functional effect of HUVEC CR1 protein expression, we investigated the ability of normoxic and hypoxic (48 h, 1% $O_2$) HUVECs to bind immune complexes. Hypoxic HUVECs bound significantly more immune complexes than normoxic HUVECs (Fig. 8). Recombinant sCR1 inhibited immune complex binding in a dose-dependent manner. Furthermore, sCR1 also inhibited C3b-labeled chicken RBCs from binding to hypoxic HUVECs (data not shown). These data demonstrate that extracellular HUVEC CR1 binds C3b and immune complexes.

Western blot of CR1-mediated cleavage of C3b. Factor I cleaves C3b into iC3b in the presence of the cofactor CR1 or MCP (present on HUVECs). Cleavage of iC3b into C3dg and C3d requires factor I and the cofactor CR1 or CR2 (present on B cells) (17). Thus factor I-mediated cleavage of C3b was investigated in isolated RBCs (i.e., CR1-positive cells) or permeabilized and nonpermeabilized hypoxic HUVECs by Western blotting (Fig. 9). Incubation of RBCs (lane 8) or permeabilized hypoxic HUVECs (lane 3) with C3b and factor I increased C3dg and C3d formation compared with nonpermeabilized HUVECs (lane 2). C3d and C3dg were not observed when permeabilized or nonpermeabilized hypoxic HUVECs were incubated with C3b or factor I alone (lanes 4–7). These results demonstrate that HUVEC CR1 functionally acts as a cofactor for factor I-mediated cleavage of C3b to C3dg. These results further demonstrate that HUVEC CR1 is mainly...
intracellular, as incubation of permeabilized hypoxic HUVECs with C3b and factor I led to increased formation of C3dg compared with nonpermeabilized HUVECs.

DISCUSSION

We present the novel finding that HUVECs contain CR1 mRNA and are capable of increasing CR1 protein expression in response to hypoxia or inflammatory stimuli (i.e., LPS and TNF-α). Although CR1 has been previously described on a wide variety of cell types (16), this is the first demonstration of CR1 in human endothelial cells. Furthermore, we demonstrate that HUVEC CR1 binds immune complexes and acts as a cofactor for the cleavage of iC3b to C3c and C3dg. Thus the ability to experimentally control endothelial CR1 expression may have important implications in complement-mediated disease states.

Although endothelial expression of CR1 might be considered protective against complement-mediated injury, the time course of hypoxia-induced CR1 upregulation (24–48 h) is not conducive for cellular protection during acute ischemia-reperfusion injury or acute inflammatory conditions in vivo. However, the time course of hypoxia-induced endothelial CR1 expression in vitro may not mimic that of endothelial cells in vivo because of the lack of stimulation by cytokine and other inflammatory mediators. We have previously demonstrated that combined cytokine and hypoxia stimulation augments E-selectin and ICAM-1 expression on endothelial cells compared with hypoxia alone (24, 25). In the present study, TNF and LPS increased CR1 expression in a dose-dependent manner following 18 h of stimulation. We have not examined whether shorter periods of hypoxia increase CR1 mRNA levels. Furthermore, the combined effects of cytokines (or other inflammatory stimuli) and hypoxia on CR1 expression have not yet been investigated. Thus further studies investigating
the mechanisms regulating expression of endothelial CR1 are warranted.

Although hypoxia modulates HUVEC CR1 protein expression, its functional significance has yet to be fully characterized. The majority of HUVEC CR1 protein is intracellular, and a role for intracellular CR1 has not been described. We demonstrate that sCR1 inhibits C3b and immune complex binding on hypoxic HUVECs. Furthermore, we demonstrate that factor I-mediated cleavage of C3b in the presence of HUVEC CR1 forms C3dg. Although HUVEC CR1 acted as a cofactor for factor I-mediated cleavage of C3b into C3c and C3dg, permeabilization of the HUVECs increased the cleavage of C3b. These data further support the confocal

**Fig. 7.** In situ hybridization of HUVECs for CR1. HUVECs were grown under normoxic (A) or hypoxic (B) conditions and hybridized with a CR1 cDNA probe. Confocal microscopy revealed diffuse cytoplasmic staining and nuclei devoid of staining. Hypoxia (B) increased level of fluorescent staining, indicating an increase in CR1 mRNA. C: hypoxic HUVECs do not express collagen type X mRNA (hybridization control). D: dermal human fibroblasts are negative for CR1. Green staining in these panels represents vWF, which is positive for HUVECs and negative for fibroblasts. Images are representative of 3 experiments.

**Fig. 8.** Endothelial immune complex deposition. Immune complex deposition on hypoxic HUVECs was significantly greater than on normoxic cells. Furthermore, addition of sCR1 (4, 40, or 400 pmol/l) to human serum experimentally treated with goat anti-human IgG antibody competitively inhibited endothelial immune complex deposition in a dose-dependent manner. Experiments were performed in triplicate using 6 wells per experimental group. Error bars are SE. *P < 0.05 compared with respective normoxic cells.

**Fig. 9.** Western blot of factor I-mediated cleavage of C3b in presence of HUVEC CR1. Factor I-mediated cleavage of C3b in presence of HUVEC CR1 was analyzed by Western blotting. An increased density of C3dg (band A; molecular mass ~40 kDa) and C3d (band B; ~35 kDa) bands was observed in red blood cells (lane B) or permeabilized (0.1% Triton X-100) hypoxic (48 h) HUVECs (lane 3) incubated with C3b (10 µg) and factor I (0.5 µg) compared with nonpermeabilized hypoxic HUVECs (lane 2). C3d and C3dg were not observed when permeabilized or nonpermeabilized hypoxic HUVECs were incubated with C3b or factor I alone (lanes 4–7). Lane 1, C3d and C3dg standards (1 µg); lane 2, nonpermeabilized HUVECs incubated with C3b and factor I; lane 3, permeabilized HUVECs incubated with C3b and factor I; lane 4, nonpermeabilized HUVECs incubated with C3b only; lane 5, permeabilized HUVECs incubated with C3b only; lane 6, permeabilized HUVECs incubated with factor I only; lane 7, permeabilized HUVECs incubated with factor I only; lane 8, red blood cells incubated with C3b and factor I.
findings that the majority of HUVEC CR1 is intracellular. The immune complex binding and confocal studies demonstrate that a portion of HUVEC CR1 is expressed on the extracellular membrane. Although CR1 and sCR1 are potent complement regulators, recent evidence suggests that the LHR-D region of CR1 is a high-affinity receptor for C1q (9, 23). As endothelial C1q receptors have been reported (14), we cannot rule out the possibility that endothelial CR1 may also bind C1q. Studies investigating the regulation of HUVEC CR1 expression are clearly needed.

The RCA proteins MCP, DAF, CR1, CR2, and C4 binding protein are encoded in an 800-kb span of the 1q32 region on chromosome 1 (21). DAF and MCP promoter regions contain cAMP response elements, which may regulate their expression (4). Along these lines, we have shown that hypoxia induces MCP and DAF protein expression and decreases intracellular cAMP in endothelial cells (3, 24). Although the promoter of CR1 has not been fully elucidated (20), it is possible that decreased intracellular cAMP concentrations following hypoxia may regulate CR1, DAF, and MCP expression. Further characterization of the entire CR1 5′ flanking region is needed to address this speculation.

In summary, hypoxia, LPS, or TNF-α induces HUVEC CR1 protein expression. Hypoxia also increases CR1 mRNA transcript levels. Sequencing of the RT-PCR product confirmed the existence HUVECR1. Although the in vivo role of endothelial CR1 has yet to be characterized, we demonstrate that HUVEC CR1 binds immune complexes and acts as a cofactor for factor I-mediated cleavage of C3b to C3dg. Controlling endothelial CR1 expression may have important therapeutic implications in disease states in which complement contributes to vascular endothelial cell injury.

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REFERENCES


