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FAST TRACK

Gene Transfer of Human Heme Oxygenase Into Coronary Endothelial Cells Potentially Promotes Angiogenesis

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Abstract Heme oxygenase (HO-1) is a stress protein that has been suggested to participate in defense mechanisms against agents that induce oxidative injury such as hemoglobin/heme, hypoxia-ischemia and cytokines. Overexpression of HO-1 in endothelial cells (EC) might, therefore, protect against oxidative stress produced under these pathological conditions, by generation of CO, a vasodilator, and bilirubin, which has antioxidant properties that enhance blood vessel formation to counteract hypoxia-induced injury. A plasmid containing the cytomegalovirus promoter (pCMV) neomycin human HO-1 gene complexed to cationic liposomes, lipofectin, was used to transfect rabbit coronary microvessel EC. Cells transfected with human HO-1 gene demonstrated a twofold increase in HO activity and maintained a similar phenotype as in the nontransfected cells. Cell number in transfected cells with human HO-1 gene increased by about 45%, as compared to nontransfected or those transfected with control pCMV. Transfected and nontransfected EC revealed a similar response to basic fibroblast growth factor (bFGF) in capillary formation. However, transfected cells with the human HO-1 gene exhibited a twofold increase in blood vessel formation. The angiogenic response of EC to overexpression of HO-1 gene provides direct evidence that the inductive form of HO-1 following injury represents an important tissue adaptive mechanism for moderating the severity of cell damage produced in inflammatory reaction sites of hemorrhage, thrombosis and hypoxic-ischemia. Thus, HO-1 may participate in the regulation of EC activation, proliferation and angiogenesis. J. Cell. Biochem. 68:121–127, 1998.

Key words: heme oxygenase; stress protein; overexpression; oxidative injury; endothelial cells

Heme oxygenase (HO) controls the initial and rate-limiting step in heme catabolism. The enzyme cleaves heme to biliverdin, which is subsequently converted to bilirubin by biliverdin reductase. Iron is released when the heme ring is opened, while carbon monoxide is liberated. The heme molecule plays a central role in biological processes as the prosthetic moiety of heme proteins and is involved in cell respiration, energy generation, oxidative biotransformation, growth differentiation processes, and the generation of inflammatory mediators such as eicosanoids and nitric oxide (NO) [Abraham et al., 1996]. Two HO isozymes (HO-1 and HO-2), the products of distinct genes, have been described [Shibahara et al., 1993; McCoubrey et al., 1992]. HO-1, which is distributed ubiquitously in mammalian tissues, is induced strongly and rapidly by many compounds that elicit cell injury; the natural substrate of HO, heme, is itself a potent inducer of the enzyme. HO-2, which is believed to be constitutively expressed, is present in high concentrations in such tissues as brain and testis and is thought to be noninducible [McCoubrey et al., 1992]. HO-1 activity is increased in whole animal tissues and in cultured cells following treatment with heme, metals, and inflammatory cytokines, as well as in hypoxic and oxidative conditions [Lee et al., 1997; Matz et al., 1996; Applegate et al., 1991; Rizzardini et al., 1994]. HO-1 is also induced by hyperthermia and the enzyme belongs to a class of macromolecules known as stress proteins, which are responsive

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to various types of acute cellular injuries [Abraham et al., 1995; Stocker, 1990].

Induction of HO-1 is thought to be of considerable importance in the initiation of cellular protective mechanisms following exposure to various forms of cell stressful stimuli [Abraham et al., 1995; Stocker, 1990; Nakagami et al., 1993; Vogt et al., 1996]. This idea derives, in part, from the fact that increased HO activity enables the removal of heme, a lipid-soluble, transmissible form of the potent prooxidant iron, CO, and results in the generation of bilirubin and biliverdin, with significant antioxidant and anti-complement properties [Nakagami et al., 1993; Vogt et al., 1996]; upregulation of HO activity provides resistance to cell injury in glomerular inflammation [Vogt et al., 1996]. Indeed, a study by Nath et al. [1992] provides evidence that induction of HO-1 coupled to ferritin synthesis to sequester iron released from heme degradation protects against tissue injury [Nath et al., 1992; Eisenstein et al., 1991]. CO, a by-product derived from HO reaction, shares some properties of NO such as increasing cGMP [Furchgott and Jothiandan, 1991; Morita et al., 1995] and is being considered a putative neural messenger [Verma et al., 1993] and regulator of the Na^+ K^+ ATPase pump [Nathanson et al., 1995].

Angiogenesis is a fundamental process by which new blood vessels are formed [Folkman, 1992]. Angiogenesis is increased during embryogenesis and in pathological events, such as hypoxia, ischemia, inflammation, tumor growth, and wound healing, in response to angiogenic factors. These factors increased vascular permeability, EC activation, migration, proliferation, and capillary formation [Folkman, 1992]. These circumstances lead simultaneously to the production of a number of soluble mediators, including cytokines, and acute-phase proteins such as HO-1 [Abraham et al., 1996]. Hypoxia-ischemia, but not hypoxia alone, induces HO-1 in the rat brain [Nimura et al., 1996] and that, surviving EC following hemorrhage-ischemia, continue to synthesize stress proteins [Nimura et al., 1996]. We recently characterized a model of hemoglobin-induced-EC injury and demonstrated that adenovirus or liposome-cDNA mediated HO-1 gene transfer markedly reduced heme/hemoglobin toxicity [Abraham et al., 1995, 1996]. However, the link between overexpression of HO in EC and blood vessel formation was not established. The present study was undertaken to examine the relationship of HO-1 gene expression in EC and the formation of blood vessels.

MATERIALS AND METHODS

Methods

In vitro angiogenesis and capillary EC formation. EC were isolated from the midportion of the rabbit myocardium by collagenase digestion, filteration homogenization, and centrifugation as described [Gerrittsen et al., 1988]. Permanent transfection with human HO-1 gene was performed as previously described [Abraham et al., 1996]. Cell proliferation in nontransfected and transfected cells was determined as described [Laniado-Schwartzman et al., 1994]. Assessment of in vitro capillary formation used growth factor reduced basement membrane Matrigel matrix. The Matrigel matrix 10 mg/ml was thawed, mixed to homogeneity, and transferred using cooled pipettes to 12-well plates, which were incubated at 37°C, to induce gelling. EC (HO-1 transfected or nontransfected) were diluted to a final concentration of 50,000 cells/ml in DMEM (Gibco Laboratories, Grand Island, NY) with 0.5% fetal bovine serum (FBS) and were pipetted onto the 1:2 Matrigel.

Cultures were photographed and tubular network growth area was quantitated using an OPTIMAS video imaging analysis program (Bioscan, Edmonds, WA) by calculating the percentage of growth area selected in a given field of view based on different threshold ranges for the image. The data were compiled and treated by computer with Microsoft EXCEL 5 program. Statistical analyses were performed using a one-way analysis of variance (ANOVA), followed by a modified t-test for multiple comparison.

Materials

Hemin was obtained from Sigma (St. Louis, MO). Matrigel was obtained from collaborative Research (Bedford, MA). Basic-FGF was obtained from Gibco BRL (Gaithersburg, MD). The plasmid was a pRc/CMV neomycin-human HO-1 construct; the *Xho* I/*Xba* I fragment (−63/924) of HHO-1 cDNA was in the correct orientation under the promoter/enhancer of cytomegalovirus (CMV). The same plasmid without the human fragment was used as control [Nakagami et al., 1993]. Heme oxygenase activity was measured as previously described [Abraham et al., 1995].

RESULTS AND DISCUSSION

Formation of branching and anastomosing capillary-like cords of EC were experimentally induced by embedding EC within a Matrigel matrix in the presence or absence of bFGF 10 ng/ml [Laniado-Schwartzman et al., 1994]. A distinguishable pattern of growth from control or bFGF stimulated culture was observed within the first 8 and 12 h of plating which was maximal by 24 h (Fig. 1). The amount of tubular growth was significantly decreased in the absence of bFGF; and EC tubules were less pronounced, shorter and less numerous (Fig. 1A). When transduced EC were added to Matrigel matrix, a marked augmentation of capillary-like formation in the absence of bFGF was observed (Fig. 1C). Addition of bFGF caused twofold increase in capillary and tube formation in transfected cells as compared to nontransfected cells (Fig. 1D, as compared to Fig. 1B).

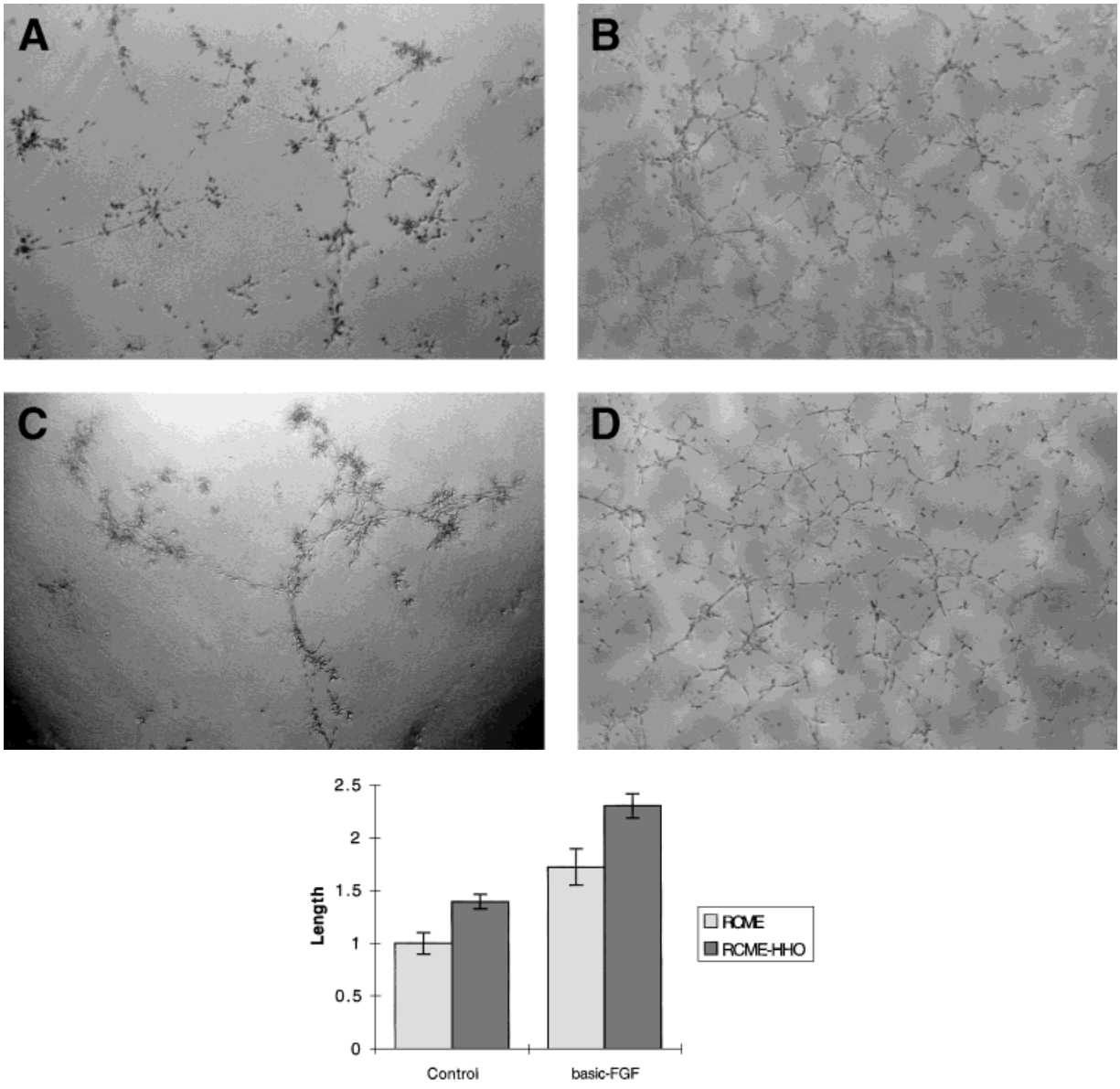

Fig. 1. In vitro angiogenesis of endothelial cells. **A:** Nontransfected cell. **B:** Nontransfected and culture medium supplemented with bFGF. **C:** Transfected EC in absence of VEGF. **D:** In presence of bFGF. Photos are representative of three separate experiments, each performed in quantrate. Quantitative analysis of tubular network area were performed with an OPTIMAS video images software analyzer. Data plotted are given in the lower panel. Results are presented as percentage increase in vessels tube size over control. (*) significantly different ($P < 0.01$) from control.

As shown in Figure 1 (lower panel), quantitation of the tubular network growth area, using an imaging analysis and plotting the compiled data, revealed that transfection of human HO-1 gene doubled the length of the tubes formed on Matrigel. bFGF added to culture cells caused a further stimulatory effect on capillary formation and tube network area in both cultures. Nontransfected EC exhibited lesser number of tubes per cell (Fig. 1, lower panel).

Although transfected EC with the human HO-1 gene exhibited similar AC-LDL uptake and factor VIII antigen to nontransfected cells, transfected EC seemed to grow faster and to stay closer to each other. The ability of the human HO-1 gene transfer to stimulate cell growth (increase in cell number) would implicate this gene expression in the regulation of cell cycle and in the modulation of angiogenesis. We therefore, examined the rate of cell proliferation in both nontransfected and transfected cells; quiescent EC were plated at the same cell number, and cell proliferation was measured as previously described [Laniado-Schwartzman et al., 1994]. As shown in Figure 2, transfection of the human HO-1 gene significantly stimulated cell growth of quiescent EC at 24 h ($P < 0.001$). The observed effect required a background serum concentration of 0.5% FBS and suggested that one of the HO products may be involved in the regulation of cell cycle. The observed effect was not seen in the pCMV-transfected cells without human HO-1 (data not shown).

Heme is a natural substrate of HO known to cause elevation of HO-1 mRNA and activity [Abraham et al., 1996]. Heme caused 40- to 60-fold elevation in HO-1 mRNA, which significantly increased HO activity within 2 h [Lutton et al., 1993]. Experiments were performed to measure the effect of heme (10 μM) on HO activity in transfected and nontransfected EC. Results are depicted in Table I. The basal level of HO activity in nontransfected EC was 650+77 pmol bilirubin/mg/h, as compared with 1,566+230 pmol bilirubin formed/mg/hr after human HO-1 cDNA transfection ($P < 0.01$). Addition of 10 μM heme to cell cultures for 6 h increased HO activity by 340% and 132% in both nontransfected and transfected cells. The rate of induction of HO in transfected cells with human genes was less pronounced than in nontransfected cells, suggesting that overexpression of human genes may interfere with heme activation of endogenous HO promoters due to the interaction of human HO-1 protein with added heme at the promoter binding site. Heme does not activate human HO transgene in EC (unpublished observation).

The ability of heme to upregulate HO activity in both transfected and nontransfected cells prompted us to examine the effect of heme on in

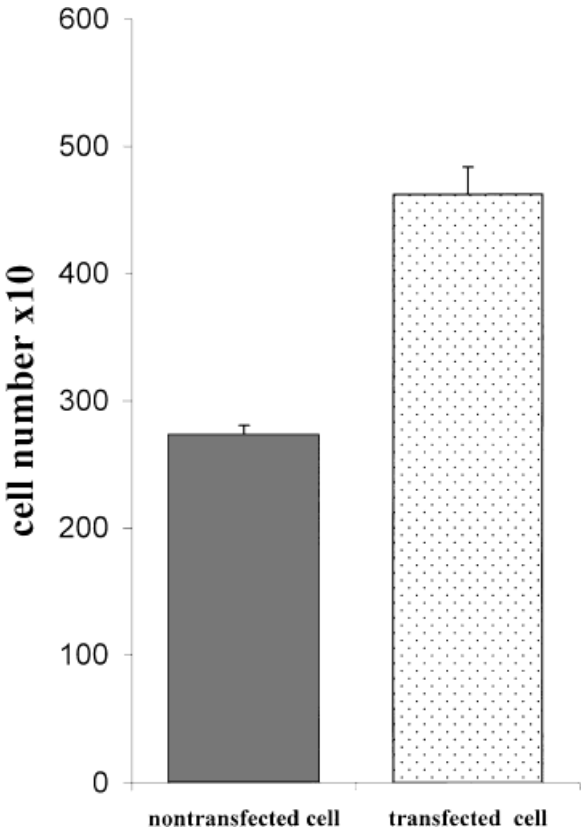

Fig. 2. Influence of human HO-1 gene transfer on endothelial cell proliferation. Nontransfected and transfected cell numbers were determined after plating the same number of cells in 96 well flasks in the presence of bFGF and 0.5% FBS. Results are average +SEM; n = 3 in triplicates $P < 0.001$.

TABLE I. Effect of Heme on Transfected and Nontransfected Endothelial Cell HO Activity*

Control	HO activity pmol of bilirubin/mg of protein/h
Control cells	650 ± 70
Control cells + heme	2,216 ± 207
Transfected cells	1,566 ± 230
Transfected cells + heme	2,065 ± 257

*HO activity was assayed as described by Lavrovsky et al. [1994]. Heme at a final concentration of 10 μM was added to cell cultures; 6 h later, cells were harvested and activity measured. Data are presented as the means ± SEM.

vitro angiogenesis. Analysis of the growth area and capillary size in cells treated with heme revealed that formation of branching and anastmosing capillary-like cords, both in transfected cells or cells treated by heme (10 μM), was increased within 6–8 h (data not shown).

The mechanism of stimulatory effect of heme on HO-1 and angiogenesis is unclear. Heme was reported to have mitogenic properties in several cell types [Stenzel et al., 1981; Smith et al., 1997] and caused release of inflammatory cytokines [Stenzel et al., 1981]. Elevation of HO activity was associated with elevation of carbon monoxide and modulation of EC function, with vascular tone, and may promote angiogenesis. These results indicate that HO-1 induction by heme or by transfection of the HO-1 gene in EC may activate microvessel EC and accelerate their development into capillary tubes.

In the present study, we were able to demonstrate successfully that overexpression of the HO-1 gene in EC caused a significant increase in angiogenesis. The mechanism by which HO-1 gene expression increased blood vessel formation and its involvement in endothelial angiogenic network is currently being investigated. Angiogenesis can be the response of various stress conditions, tissue injury [Odekon et al., 1992; Aubrey et al., 1996], ischemia, and solid tumor growth (Polverini and Leibovich, 1984]. Upregulation of the HO-1 gene was observed by several angionic stimulating factors, such as interleukin-1 and -6 (IL-1, IL-6), tumor necrosis factor (TNF), and transforming growth factor-β (TGF-β) [Lutton et al., 1993; Neil et al., 1995]. Several transcriptional motives, such as NF-κB, STAT, AP-1, and AP-2, have been shown to activate the HO-1 promoter and upregulate HO-1 gene expression [Abraham et al., 1996; Lavrovsky et al., 1996]. Similar transcriptional factor binding sites have been identified on the promoter region of bFGF and VEGF [Ryuto et al., 1996; Gille et al., 1997], which suggests that physical interaction or competition among these factors may be involved in the activation of several genes, such as HO-1 [Lavrovsky et al., 1994], c-*fos* [Lavrovsky et al., 1996], and bFGF. Hypoxia and hypoxia ischemia causes elevation of HO-1 mRNA [Lee et al., 1997; Matz et al., 1996] and several other glycolytic enzyme genes that are hypoxia inducible [Semenza et al., 1994].

To examine the presence of different transcriptional factors whose activation is impli-

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CTACATCCCA GGCTCAAGTG AACCTCCAGC CTCAGCCTCC CAAGTAGCTG GGACCACAGG CATGTGCCAC CATGCCCAGC TAATTTATTT TATATTTTGT -1201
                                              HIF-like
AGAGACGGGG TCTCCCTATG TTGCCCAGGC CAGTCTCGAA CTCAAAGCAA TCTTCCCACC TCGACTGGGC TCAAAGCGCT CTTCCCACCT CAACCTCCCA -1101
AAGTACTGGG ACTACAGGTG TGAGCTACCA TGCCAGGCCT GAAAGCCATC TTAAAAAAAA AATCTTAGAA TGAGATCACA GTATTGGGAA AGGACTGTAT -1001
GAATCATCTG GTCCATTCGT TTTGTCCTCT GGGTTCACCC AGTGACCCTA TTTCCCCCGA GTTCTAAGGA GTCCACCTCA TGCAGAATTG ATTCAATAGG -901
CGATCAGCAA GGGCCAGCTC TGCTCTGGGC CCTGAGCAGG CACTGAGTAT AAGTCAGACC TGAATGTGCC TGGAAGAGTG TCCCACGCAT TCCAGCAGGG -801
AAGCAGTTTG TATGACAGGT GTCCCAGTCC AGGCGGATAC CAGGTGCTGC CAGAGTGTGG AGGAGGCAGG CGGGGACTTA GTCTCCTCCC TGGGTTTGGA -701
CACTGGCATC CTGCTTTATG TGTGACACCA CTGCACCCCT CTGAGCCTCG GTTTCCCCAT CTGTAAAATA GAAGCGATCT ACCCTCACAG GTCAGTTGTA -601
                          E-Box
GGGATGAACC ATGAAAATAC TAGAGTCTCT GTTTTTTGAC AGGAACTCAA AAAACAGATC CTAAATGTAC ATTTAAAGAG GGTGTGAGGA GGCAAGCAGT -501
CAGCAGAGGA TTCCAGCAGG TGACATTTTA GGGAGCTGGA GACAGCAGAG CCTGGGGTTG CTAAGTTCCT GATGTTGCCC ACCAGGCTAT TGCTCTGAGC -401
AGCGCTGCCT CCCAGCTTTC TGGAACCTTC TGGGACGCCT GGGGTGCATC AAGTCCCAAG GGGACAGGGA GCAGAAGGGG GGGCTCTGGA AGGAGCAAAA -301
                    HSE
TCACACCCAG AGCCTGCAGC TTCTCAGATT TCCTTAAAGG TTTTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTATGTGT GTGTGTGTGT GTGTGTGTGT -201
GTGTTTTCTC TAAAAGTCCT ATGGCCAGAC TTTGTTTCCC AAGGGTCATA TGACTGCTCC TCTCCACCCC ACACTGGCCC GGGGCGGGCT GGGCGCGGGC -101
                              NF-kappa-B                                                    AP2
CCCTGCGGGT GTTGCAACGC CCGGCCAGAA AGTGGGCATC AGCTGTTCCG CCTGGCCCAC GTGACCCGCC GAGCATAAAT GTGACCGGCC GCGGCTCCGG -1
   AP2                                                  HIF-like
CAGTCAACGC CTGCCTCCTC TCGAG
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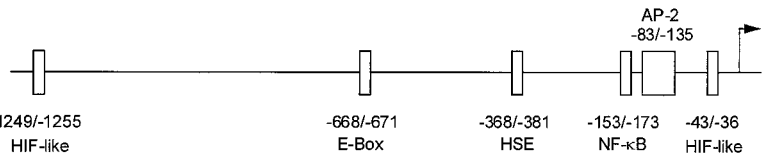

Fig. 3. Sequence of the human HO-1 gene upstream region. The sequences (lowercase letters) were found by computer search; underlined sequences were also confirmed by DNase footprint assay.

cated in response to hypoxia [Semenza et al., 1994], we devised a specific database search program using the National Center of Biotechnology information and the NIH sequence database. Two hypoxic factor transcriptional (HIF-1) sites were found by computer search using 5′ to 3′ and 3′ to 5′ directions for screening (Fig. 3). Several binding sequences (TAGCTGGG) that resemble the HIF (−1249 to −1255) and (−43 to −36) were found to share the same sequences as those described for hypoxia inducible genes [Semenza et al., 1994]. Activation of HIF-1 factors as a result of HO-1 gene overexpression may also enhance other growth factors involved in EC cell proliferation.

We have shown that heme causes AP-2 and NF-κB transcriptional activation and leads to rapid induction of HO-1 genes and c-*fos* protooncogene [Lavrovsky et al., 1994, 1996]. There is numerous evidence that NF-κB and related proteins are involved in growth control. It is therefore possible that the immediate induction of HO-1 results in stimulation of other genes involved in cell proliferation through the physical interaction or competition for certain transcriptional factors. This interaction may be responsible for stimulation of angiogenesis seen in the present report. Taken together, this information and the direct evidence provided in this report that overexpression of HO-1 genes in EC enhances angiogenesis, suggest that this enzyme could play an important role in moderating tissue damage, as that seen in ischemia. In contrast, overexpression of HO-1 genes may cause exacerbation and stimulation of tumor growth by increase in blood supply. Recently, Goodman et al. [1997] showed that HO-1 mRNA was increased by severalfold in human adenocarcinoma cells and that overexpression of HO-1 may enhance tumor growth. HO activity was also increased in several tumors and maligant cells [Schacter et al., 1986]. Therefore, pharmacological manipulation of HO-1 to further enhance or suppress angiogenesis may have important therapeutic potential in these clinical circumstances.

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