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Increased Responsiveness of Hypoxic Endothelial Cells to FGF2 is Mediated by HIF-1 Alpha-Dependent Regulation of Enzymes Involved in Synthesis of Heparan Sulfate FGF2-Binding Sites

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Increased responsiveness of hypoxic endothelial cells to FGF2 is mediated by HIF-1α-dependent regulation of enzymes involved in synthesis of heparan sulfate FGF2-binding sites

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Summary

Binding of basic fibroblast growth factor (FGF2) to its high affinity receptors requires the presence of specific heparan sulfate (HS) moieties on the cell surface that act as co-receptors. To determine the contribution of cell-surface HS to modulation of FGF2-dependent cell growth, we studied the changes in the cell mass and FGF2 binding of endothelial cell HS under normoxic and hypoxic conditions in vitro. Both large vein and cardiac microvascular endothelial cells cultured under hypoxic conditions demonstrated an increase in the ratio of cell-surface HS to chondroitin sulfate (CS), as well as an increase in the number of low affinity (HS-associated) binding sites for FGF2 with no change in the apparent K_d. This increase in the number of HS-FGF2 binding sites, in the absence of a significant change in FGF receptor expression, resulted in enhanced responsiveness of hypoxic, compared with normoxic, endothelial cells to FGF2 stimulation.

Gene expression studies demonstrated increased expression of the key regulatory enzyme responsible for HS chain synthesis, 1,4 GlcNAc transferase (GlcNAcT-I), as well as increased expression of 2-O sulfotransferase (HS2ST), the enzyme responsible for sulfation of IdoA, a crucial part of the HS-FGF2 binding site. Transduction of cells with an adenovirus encoding a HIF-1α expression construct resulted in a similar increase in GlcNAcT-I and HS2ST expression. We conclude that hypoxia increases endothelial cell responsiveness to FGF2 by promoting preferential synthesis of HS rather than CS chains and increasing the number of FGF2-binding sites on HS chains. Both of these events are mediated by a HIF-1α-dependent increase in expression of the enzymes GlnNAcT-I and HS2ST. This shift in cell-surface HS composition results in enhanced cell sensitivity to FGF2-induced growth stimulation.

Key words: FGF2, Signaling, Hypoxia, Angiogenesis, Heparan sulfate, Endothelial cells

Introduction

Hypoxia is known to alter many endothelial cell properties by increasing cellular levels of hypoxia-inducible factor (HIF)-1α, which in turn amplifies expression of angiogenesis-related genes such as vascular endothelial growth factor (VEGF) and its receptor flt-1 (Carmeliet et al., 1998; Iyer et al., 1998), angiopoietin-1 (Mandriota and Pepper, 1998) and endothelial nitric oxide synthase (Arnet et al., 1996) among others. In addition, hypoxia has a profound effect on the cell-surface-associated matrix, and its efffects include altering the expression of heparan-sulfate-carrying core proteins (Humphries et al., 1986; Karlinsky et al., 1992) and integrins (Rusnati et al., 1997). These changes in cell-surface-associated matrix and, particularly, its heparan sulfate content, can have profound effects on the ability of heparin-binding growth factors such as FGF2 to stimulate endothelial cell growth and migration.

Heparan sulfate proteoglycans can mediate both the heparin-binding growth-factor–receptor interaction at the cell surface and accumulation and storage of these growth factors in the extracellular matrix (Rosenberg et al., 1997; Tumova et al., 2000). The presence of HS is required for FGF2-dependent activation of cell growth in vitro (Rapraeger et al., 1991; Yayon et al., 1991), and removal of HS chains from the cell surface by enzymatic digestion greatly impairs FGF2 activity and inhibits neovascularization in vivo (Sasisekharan et al., 1994). In the case of FGF2, a specific heparan-binding domain including Asp^{28}, Arg^{121}, Lys^{126} and Gln^{135} has been identified (Faham et al., 1996; Ornitz et al., 1995) that interacts with a specific oligosaccharide sequence in HS (Schlessinger et al., 2000; Stauber et al., 2000). In addition, HS also interact with FGF receptor-1 (Kan et al., 1993; Schlessinger et al., 2000). FGF2 binding to the cell surface HS leads to dimerization of bFGF, which facilitates growth-factor binding to FGFR1, which is, in turn, followed by receptor dimerization and activation of intracellular signaling cascades. The interactions between HS and both FGF2 and FGF receptor are primarily in the form of hydrogen bonds between sulfate groups in 2-O (FGF2) and 6-O (FGF receptor) positions (Schlessinger et al., 2000; Stauber et al., 2000). Therefore any alteration in HS

chain composition, the extent of its sulfation or the position of sulfate groups on the cell surface by means of altered synthesis, degradation or modification of glycosaminoglycan (GAG) chains can, conceivably, affect growth-factor signaling. The importance of HS in growth-factor-dependent signaling is emphasized by findings in *Drosophila*, where several major signaling pathways including FGF, Wnt, Hedgehog and TGF-β, have been shown to depend on the presence of sulfated HS chains in glypican and syndecan (Baeg and Perrimon, 2000; Lin et al., 1999; Tsuda et al., 1999).

Glycosaminoglycan chain biosynthesis involves a number of enzymes. An enzyme 1,4-N-acetylhexosaminyl transferase (GlcNAcT-I) initiates the HS biosynthetic pathway by transferring glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc) to the core protein tetrasaccharide linkage region (Fritz et al., 1994; Kitagawa et al., 1999). The elongating HS chain can then be modified by several enzymes, including GlcNAc N-deacetylase/N-sulfatransferase (NDST1 and 2), which mediates N-sulfation, C5-epimerase, an enzyme mediating conversion of glucuronic to iduronic acid (Rosenberg et al., 1997) and 2-O and 6-O sulfotransferases (HS2ST and HS6ST, respectively), which are involved in the generation of the FGF2-binding site (HS2ST) and FGF2 signaling (HS6ST) (Bai and Esko, 1996; Pye et al., 1998).

We designed the present study to analyze the effect of hypoxia on the composition of heparan sulfates on the surface of two different populations of primary endothelial cells - large-vein-derived human endothelial cells (HUVEC) and primary rat cardiac microvascular endothelial cells (CMEC). In addition, we also examined the expression of key enzymes involved in HS synthesis in hypoxic as well as HIF-1α-encoding adenovirus-transducted endothelial cells. We found that hypoxia leads to a significant shift from CS to HS chain synthesis owing to increased expression of HS synthesis enzyme GlcNAcT-I, as well as an increase in the number of HS-FGF2 binding sites owing to increased expression of 2-O sulfotransferase HS2ST. The increase in expression of both of these enzymes is mediated by HIF-1α. These changes in the HS cell-surface composition resulted in a significant increase in FGF2-induced cell proliferation.

Materials and Methods

Endothelial cell cultures

Cardiac microvascular endothelial cells (CMEC) were harvested from neonatal rats by enzymatic dissociation as previously described (Edelberg et al., 1998). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) supplemented with 20% fetal bovine serum (HyClone, Logan, UT), penicillin (100 units/ml) and streptomycin (100 mg/ml). All experiments were performed on cells three to four passages from the primary culture. Human umbilical vascular endothelial cells (HUVEC) were purchased from Clonetics (San Diego, CA). Cells were cultured in M199 medium (Gibco BRL, Grand Island, NY) supplemented with 20% fetal bovine serum, 10 units/ml heparin (Elkins-Sinn, Inc. MA), 30 μg/ml endothelial mitogen (Biomedical technologies Inc., MA) and streptomycin/penicillin mixture. Cells in the third to fifth passage were used for the experiments. Exogenous HS for cell growth studies was obtained from Sigma and the amount calculated to double the total HS mass was used.

For hypoxia studies, cell cultures were incubated in a sealed plastic chamber containing 5% CO_2/95% nitrogen mixture in a 37°C incubator. The oxygen level in the chamber was monitored using an oxygen analyzer (Vascular Technology, Inc., MA), and it remained at 1-3% O_2 for up to 72 hours of the incubation. Control cultures were maintained in a 95% air/5% CO_2 tissue culture incubator.

Transduction of HUVEC with a HIF-1α-encoding adenovirus

A recombinant adenovirus construct expressing the HIF1α-VP16 hybrid (Vincent et al., 2000) was a kind gift from K. Vincent (Genzyme Corp, Cambridge, MA). Briefly, the full length *HIF-1α* gene was isolated from a HeLa cell cDNA library and cloned into the expression vector, pcDNA3 (Invitrogen, Carlsbad, CA). The HIF1α-VP16 hybrid was constructed by truncating HIF1α and then joining the transactivation domain of HSV VP16 downstream of HIF-1α. The media in which cells were transfected contained adenovirus-encoding HIF1α. For HUVEC and CMEC transfection, the cells were incubated in 5 ml of adenovirus-containing media containing ~2×10^{10} IU/ml virus with a particle/infectious unit ratio of ~50 at 37°C. Five hours later, an equal amount of fresh medium was added, and the cells were incubated continually overnight. Successful transduction was confirmed by showing increased expression of HIF-1α-VP16 and VEGF in transducted cell but not in control cells by western blotting. Transduction of HUVEC and CMEC with the Ad2 vector was used as a control.

Metabolic labeling of proteoglycans

Determination of the total mass of cell-surface-associated glycosaminoglycans was carried out as previously described (Shworak et al., 1994). Briefly, the endothelial cell cultures were incubated for 24 hours with sulfate-free DMEM supplemented with 1% Neutrodoma-SP (Boehringer Mannheim, Indianapolis, IN) in hypoxic and normoxic condition then trypsinized with Trypsin/EDTA (Gibco BRL, Grand Island, NY) followed by centrifugation at 15,000 ***g*** for 10 minutes at 4°C. Total proteoglycans (PG) were isolated from the supernatant by DEAE chromatography. GAGs were cleaved from the total PG pool by β-elimination. After phenol extraction and ethanol precipitation of GAGs, the relative contents of HS and CS were determined by appropriating enzyme digests with chondroitinase ABC or *Flavobacterium* heparatinase 1 and 3 (Sigma). Digested HS or CS was collected by ethanol precipitation and dissolved in water containing 0.0005% triton X-100. Quantification of HS and CS was done spectrophotometrically using Alcian blue solution containing 4 M guanidine HCL, 27 mM H_2SO_4, 0.25% Triton X-100 and 0.25% alcian blue (Fluka, Ronkonkoma, NY) at A_{600} wavelength. A concentration scale of dilutions of heparan sulfate (Fluka, Ronkonkoma, NY) was used as a standard. Using this approach, the sum of HS and CS accounted for >98% of the total PG sulfate.

Scatchard analysis of FGF2-binding sites

For determination of the number and affinity of FGF2-HS binding sites, endothelial cells were grown to near confluence in 24-well dishes (Gibco BRL, Grand Island, NY) in 10% FBS-DMEM. After two washes with cold PBS, 200 μl of binding buffer (25 mM HEPES, pH 7.4, 0.1% BSA, 0.05% gelatin in M199 medium), 4000 CPM (0.5 ng/ml) ^{125}I-FGF2 (New England Nuclear, Boston, MA specific activity 2000 C/mmol) and increasing amounts (0-500 ng/ml) cold rFGF2 (Chiron Corp, Sunnyvale, CA) were added to each well, and the cells were incubated at 4°C for 2 hours with gentle agitation. Binding in the presence of 500 ng/ml rFGF2 (1000-fold excess) was considered nonspecific. To determine total ^{125}I-FGF2 binding, the cells were washed three times with 1 ml PBS containing 0.1% BSA and then incubated for 5 minutes with 1 ml 1 M NaOH at room temperature with vigorous shaking. Following this, 0.4 ml aliquots were counted in a 1272 CliniGamma counter (LKB). Low affinity binding of FGF2 was determined specifically by salt extraction (5 minutes in 1 ml cold 2 M NaCl) before treatment with NaOH as

Table 1. Specific gene primers used for PCR analysis

Gene	Accession No.	Sense	Antisense	Location	Size
GlcNAcT-I	AF000416	CTAAGCTGCAGGGAAATAAA	TTGCTGTCTGTTGTTTGAAG	127-692	546
HS2ST	AB024568	AGGATTTTATCATGGACACG	TCTTTCCTGTGCGATAGAGT	762-1204	443
CS/DS2ST	AB020316	GCACCCAATATGAAACATCT	CCTTAGGACAGTGAGACTGC	2305-2775	451
HS6ST1	NM_004807	CTACATGAGCCACATCATTG	GAGTTAAGAGCGAGTGCTGT	1314-1753	440
HS6ST2	AB024565	CAAAGTCCTTCTGGAAAGTG	TGTCTCTACGCTCCCTATGT	925-1513	589
NDST1	NM_001543	ATCTTCTGCCTGTTCAGCGT	CTCATTGGCCTTGAAGAAGC	496-965	450
NDST2	NM_003635	CATGAAGGTGGCTGATGTTG	CGGATTAAGCAGCACTGTCA	1011-1610	580
Epimerase	AF003927	AGGTGGTTAGGTTGATTGCG	GCAGTTGATTGATGTGGGTG	689-1332	624

described (Bono et al., 1997). Cell counts determined by Coulter Counter were employed to establish the number of cells per well. Background counts were subtracted from all samples. Scatchard analysis of the specifically bound material versus the molar amount of cold competitor was carried out using GraphPad Prism 3.0 software. All experiments were done in triplicate and repeated at least twice.

RNA isolation and northern analysis

For the RNA analysis of the time course of expression of syndecans, glypican, perlecan and FGF receptor 1, total RNA was made from cultured cells using the Tri-Reagent solution (Sigma, MO). The 10 μg RNA pellet was dissolved in RNase-free water, fractionated on a 1.3% formaldehyde-agarose gel and transferred to GeneScreen Plus (Du Pont) filter. The cDNA probes of interest were labeled with α^{32}P-dCTP (New England Nuclear) with a random-priming labeling kit (Boehringer, Indianapolis, IN) and purified from unincorporated nucleotides using G-50 Quick Spin Columns (Boehringer, Indianapolis, IN). The typical specific activity of the probes used in the experiments was $1\text{-}2\times10^9$ cpm/μg. The blots were hybridized at 68°C for 3 hours in QuikHyb solutions (Stratagene, La Jolla, CA). After the hybridization, blots were washed twice in 2×SSC, 0.1% SDS for 15 minutes at room temperature and then twice in 0.1×SSC, 0.1% SDS for 15 minutes at 60°C. Autoradiography was carried out with Kodak XAR film at –80°C for 16-20 hours.

Cell growth assay

CMEC or HUVEC were plated at 10-12,000 cells per well in 24-well plates and cultured either in the hypoxic chamber (average O_2 content 2% over 72 hours) or in the normal oxygen environment for 16 hours in 0.25% FBS-DMEM (growth arrest medium) prior to initiation of FGF2 stimulation. At that time, 10 ng/ml FGF2 (Chiron Corp) was added, and the cell counts were determined at 24, 48 and 72 hours, respectively, in a Coulter Counter (Coulter Corp. Cortland, NY). Cell growth data are shown as a percentage increase from the baseline. All experiments were carried out in triplicate.

RT-PCR analysis of HS biosynthetic enzyme expression

The reverse transcription reaction was performed on 2 μg of total RNA from normoxic, hypoxic and HIF1α-transfected cells with oligo dT primer (Gibco BRL custom primer) at 37°C for 90 minutes. Specific gene primers of each interested enzyme for PCR analysis were designed by using Biology WorkBench 3.2 software on the basis of the published sequences (Table 1).

Primers for 18S RNA, which produce a 300 bp fragment (Amicon Inc. Beverly MA), were used with amplification of each sample to control for RNA loading. The PCR conditions were as follows: 94°C denaturing for 1 minute, 58°C annealing and 72°C extending for a total of 32 cycles. The PCR products were subjected to electrophoresis on a 1% agarose gel, visualized by ultraviolet activation of incorporated ethidium bromide and photographed. The photos were scanned and quantified using Image-Quant software (Molecular Dynamic). Statistical analysis used the ratio of the PRC product for the enzymes we were studying and that of18S. All experiments were repeated three times.

The quantitation of gene expression was confirmed by Real Time PCR technique using the GeneAmp 5700 system with SYBR Green PCR core reagents (PE Applied Biosystems, Foste City,CA). The primers were designed and selected as optimal primer pairs using Primer Express™ software (PE Applied Biosystems) as shown in Table 2, and PCR reactions were run under PE Applied Biosystems universal thermal cycling conditions.

Quantitative analysis of gene expression was carried out using the GeneAmp 5700 system software. To eliminate the effect of variation in the amount of total RNA among the various samples, 18S RNA was amplified for each RNA sample in a specific PCR reaction. The ratio of the specific RNA transcript was then adjusted for the amount of 18S RNA to allow quantitative comparisons among the samples.

Results

Hypoxia alters glycosaminoglycan expression on endothelial cells

To explore the effect of hypoxia on HS chain composition in cultured endothelial cells, the cell-surface-associated GAG content and the HS mass were measured by steady-state incorporation of radioactively labeled sulfate into CMEC and HUVEC. After 48 hours of hypoxia, there was a slight decline in the total GAG content in both cell types (HUVEC, 65.8±2.4 versus 53.8±5.2; CMEC, 56.4±0.3 versus 47.1±0.1 CPM/μg protein normoxia versus hypoxia) (Fig. 1A). At the same time,

Table 2. Optimal primer pairs for Real Time PCR

Gene	Accession No.	Sense	Antisense	Location	Size
GlcNAcT-I	AF000416	CCTTGTGGCCCTGCCA	GTTACATCAAAACCATGCTGGACT	1782-1844	62
HS2ST	AB024568	ATTATGATGCCGCCCAAGTT	AAGAGCATCGCCACCGC	370-431	61
CS/DS2ST	AB020316	GTCAGCAAGCCCCCCT	TCGATTGGCTCCTCGGTTT	1205-1272	67
HS6ST1	NM_004807	GCGTGGAGGTGGATGAAGAC	TGCATGTCCAGGTCGTTGAG	1085-1145	60
HS6ST2	AB024565	CCTGGTGCGCAACATCC	TGCATTTCTTCTGCCCCAC	298-359	61
NDST1	NM_001543	TCCTGGAGTCCAGCCGC	CGTGGGCATGTCACCCTT	731-795	64
NDST2	NM_003635	AGACCGGTACTGCGTGGAGT	GGCTGTGCTGCTGGGCT	498-562	64
Epimerase	AF003927	CCATCTATGACCTCCGGCAC	AGTCCCAGCGGGCCAG	1226-1285	59

Table 3. Characterization of high and low affinity FGF2 binding sites on endothelial cells under normoxic and hypoxic conditions

		High affinity Kd ($\times10^{-11}$ M)	FGF R sites per cell ($\times10^{3}$)	Low affinity Kd ($\times10^{-9}$M)	FGF2-HS sites/cell ($\times10^{5}$)
CMEC	Normoxia	3.9±1.1	2.5±0.6	2.5±0.8	1.3±0.2
	Hypoxia	3.3±1.4	2.7±1.3	2.5±1.3	2.7±0.2
HUVEC	Normoxia	2.1±0.8	2.1±0.7	1.9±0.5	1.6±0.8
	Hypoxia	2.5±0.9	3.0±0.3	2.0±0.4	2.5±0.7

CMEC, cardiac microvascular endothelial cells; FGF R high affinity binding site (FGF receptor); HUVEC, human umbilical vein endothelial cells, HS, Heparan sulfate.

there was a significant increase in the HS fraction of the total GAG content (37.5±1.4% versus 51.9±0.4% in CMEC and 19.7±3.2% versus 39.4±4.3% in HUVEC; $P<0.05$, normoxia versus hypoxia for both) signifying preferential synthesis of HS rather than CS chains on core proteins. The extent of the increase in the cell-surface-associated HS mass was more prominent in HUVEC compared with CMEC (Fig. 1B). However, in both cell types hypoxia was associated with a significant increase in the HS/CS ratio (1.8 fold for CMEC and 2.6 fold for HUVEC, Fig. 1C).

To test whether these changes in the GAG content on the endothelial cell surface altered binding properties of HS-binding proteins, we have measured the number of low affinity binding sites for FGF2, a prototypical HS-binding growth factor. In both HUVEC and CMEC cells, there was nearly a doubling in the number of low affinity binding sites for FGF2, whereas the apparent Kd of this interaction remained the same (Table 3). At the same time, there was no change in either the Kd or the number of high affinity (FGF receptor) binding sites (Table 3). Thus in the case of both HUVEC and CMEC, hypoxia induced a significant increase in the number of HS-FGF2 binding sites per cell (Fig. 1D).

Effect of hypoxia on expression of HS-carrying core proteins and FGF receptor

The change in the cell surface GAG population and the HS cell mass under hypoxia can result from altered expression of HS-carrying core proteins (syndecans or glypican), from changes in glycanation of mature cores (i.e., a shift from HS to CS chain attachment), increased frequency of FGF2 binding sites per HS chain or a combination of all three. To explore these possibilities, we carried out northern blot analysis of syndecan-1, 2 and 4 and glypican-1 core gene expression under normal cell culture conditions and following 24 hours of hypoxia. In both endothelial cell types, hypoxia induced a reduction in syndecan-1, 2 and 4 and glypican-1 expression (and this was more pronounced in HUVEC) (Fig. 2). At the same time, there was no change in expression of extracellular matrix proteoglycan perlecan. Thus a change in expression of HS-carrying proteins could not have contributed to increased HS cell mass and the increased number of FGF2 low affinity (HS) binding sites.

To confirm the results of the Scatchard analysis, which suggested no significant change in the number of FGF-receptor-binding sites on the cell surface after 24 hours of

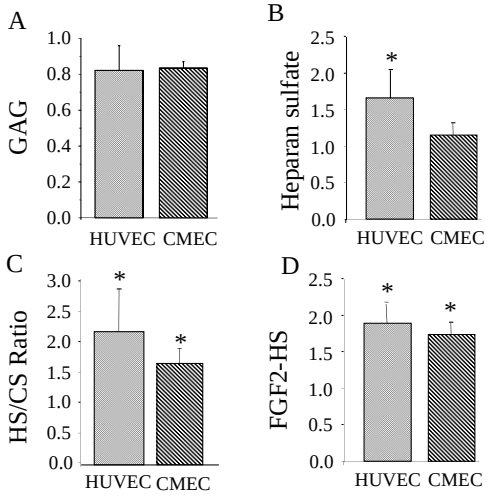

Fig. 1. Hypoxia-induced changes in endothelial cell HS matrix. Changes in the cell-surface-associated GAG content (A), heparan sulfate (B), HS/CS ratio (C) and FGF2-HS binding sites (D) are shown as a ratio between hypoxic and normoxic states for cardiac microvascular endothelial cells (striped bars) and human umbilical vein endothelial cells (gray bars). * denotes a significant change in the index under hypoxic versus normoxic conditions ($P<0.05$). Data are shown as mean±s.d.

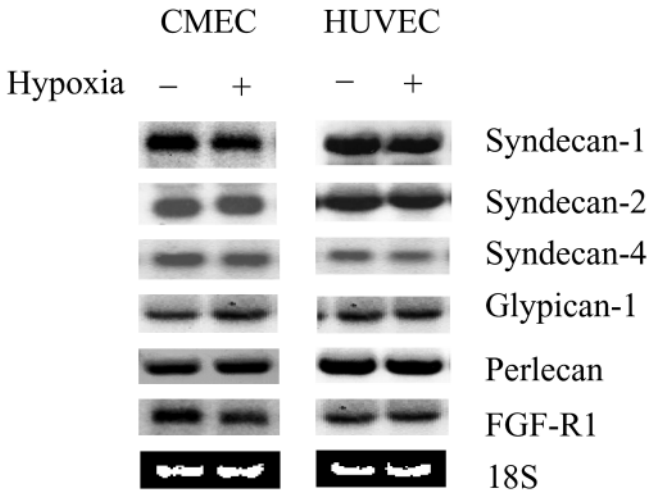

Fig. 2. Northern blot analysis of HS core protein and FGF receptor expression. Total RNA blots of CMEC and HUVEC cultured under hypoxic (+) or normoxic (–) conditions probed for expression of syndecans 1,2 and 4, glypican-1, perlecan and FGF receptor 1 (FGF R1). Ethidium bromide staining of the 18S RNA band is shown as the loading control. Note the decreased expression of syndecan-1, 4 and glypican-1 under hypoxic conditions and the lack of significant change in expression with other tested genes.

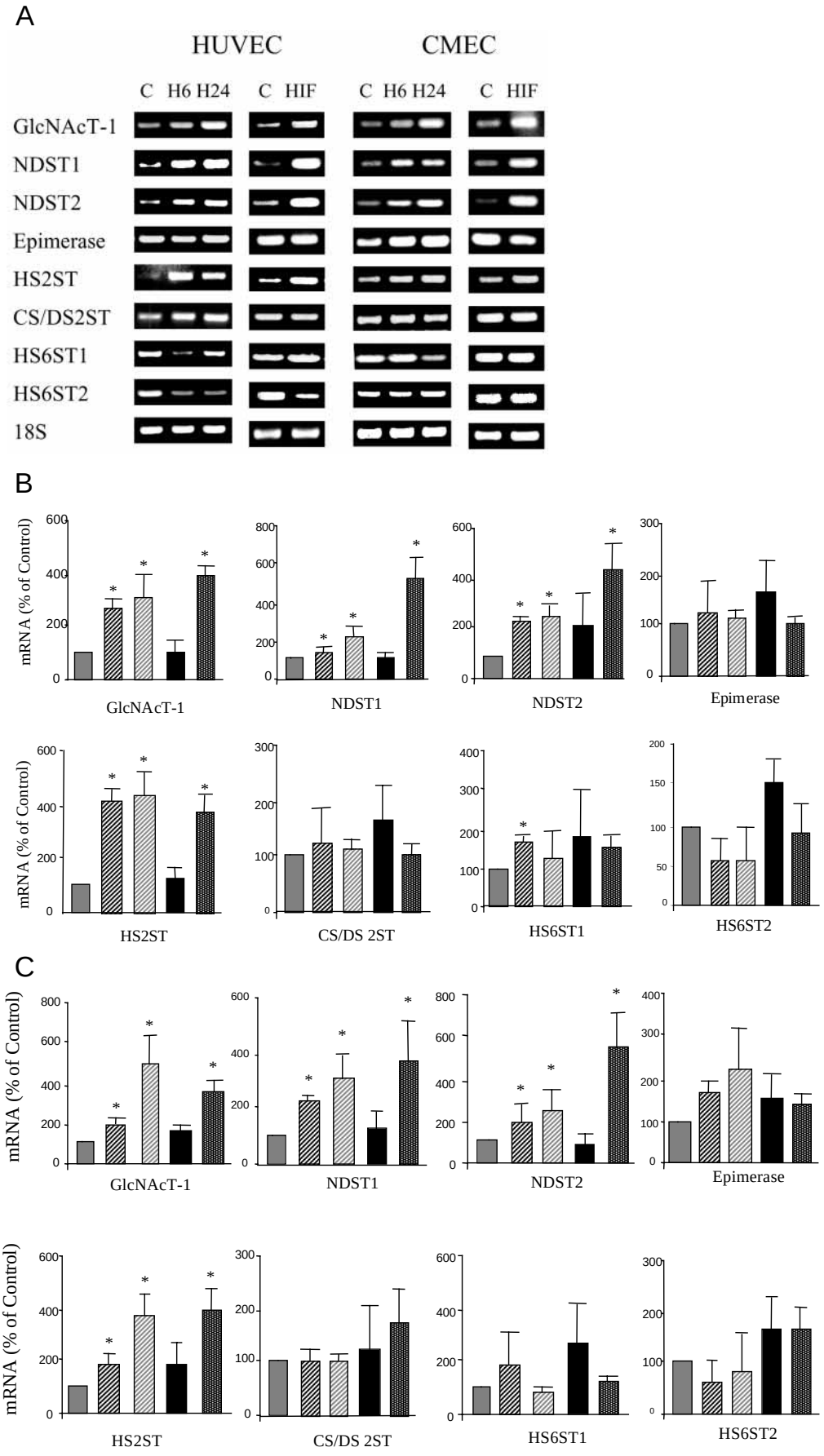

Fig. 3. Effect of hypoxia and HIF-1α gene expression on mRNA levels of HS biosynthetic enzyme expression. (A) RT-PCR analysis of mRNA levels of enzymes involved in GAG chain biosynthesis was carried out on HUVEC (left panels) and CEMC (right panel) cultured under normoxic (C) or hypoxic conditions for 6 hours (H6) or 24 hours (H24). In addition, expression of the same enzymes was measured in cells transduced with an empty adenoviral vector (C) or an adenoviral vector carrying the HIF1α-VP16 construct (HIF). The internal PCR loading control with 18S ribosomal RNA is show on the bottom of each panel. (B,C) Quantitative analysis of GAG chain biosynthesis enzymes expression in HUVEC (B) and CMEC (C) cells. The levels were determined after cell culture under normoxic conditions (gray bars), after 6 hours (striped bars) or 24 hours (light striped bars) of hypoxia or following infection with a control (empty vector) adenovirus (black bars) or a HIF1α-VP16 adenovirus (stippled bars). Densitometric analysis of RT-PCR-determined expression levels of enzymes shown in (A) adjusted for loading are shown as mean±s.d. of three independent experiments. Note the increased expression of GlcNAcT-I, NDST1 and HS2ST in both HUVEC and CMEC cultured under hypoxic conditions or in cells transduced with HIF1α-VP16 adenovirus. GlcNAcT-I, N-Acetyl glucosamine transferase; NDST, N-deacetyl/N-sulfotransferase; HS2ST, heparan sulfate 2-O sulfotransferase; CS/DS2ST, chondroitin sulfate/dermatan sulfate 2-O sulfotransferase; HS6ST, heparan sulfate 6-O sulfotransferase. * P<0.05 by Student's t-test.

hypoxia, we performed northern analysis of FGF receptor 1 (FGFR1) expression. In both cell types, 24 hours of hypoxia did not alter mRNA levels of FGFR1 (Fig. 2).

Effect of hypoxia on expression of enzymes involved in HS and FGF2-HS chain synthesis

To determine whether the increase in the HS/CS ratio and in the number of HS-FGF2 binding sites in hypoxic endothelial cells is caused by an alteration in the expression of enzymes involved in the biosynthesis of HS chains, we determined the expression of various HS and CS biosynthesis enzymes in HUVEC and CMEC cultured under normoxic conditions or after 24 hours of hypoxia.

RT-PCR analysis demonstrated a significant increase in the mRNA level of 1,4-N-acetylhexosaminyl transferase (GlcNAcT-I), the enzyme responsible for initiation of HS chain synthesis, N-sulfating enzymes NDST1 and NDST2, and heparan sulfate 2-O sulfotransferase (HS2ST) in both cell types under hypoxic conditions (Fig. 3A-C). To confirm these results, we carried out quantitative real time PCR analysis of the expression of these genes. In each case, real time PCR confirmed the observed changes in expression, and the extent of expression changes as determined by band densitometry correlated well with real time PCR results. In particular, HS2ST levels increased 3.9-fold in HUVEC at 6 hours, 4.6-fold at 24 hours and 5.2-fold after transduction with HIF-1α, whereas in CMEC, levels increased 2.2-fold at 6 hours, 3.3-fold at 24 hours and 4.3-fold following HIF-1α transduction.

At the same time there were no significant change in the expression of chondroitin/dermatan sulfate 2-O sulfotransferase (CS/DS2ST) or C5 epimerase (Fig. 3A-C), which was also confirmed by real time PCR analysis (data not shown). In HUVEC, hypoxia also led to a somewhat decreased expression of 6-O sulfotransferase isoforms HS6ST2 (0.63±0.34-fold, Fig. 4B, 0.75±0.1-fold by real time PCR), whereas the expression of HS6ST1 was unchanged. The expression of both 6-O sulfotransferase isoforms was unchanged in CMEC (Fig. 3C).

Alterations in gene expression under hypoxic conditions are frequently mediated by the HIF-1α transcription factor. To examine whether HIF-1α is involved in modulation of HS biosynthetic enzymes expression, we transduced HUVEC and CMEC with an adenoviral vector carrying the HIF-1α–VP16 hybrid cDNA construct. An empty adenoviral vector was used as control. In the cases of both HUVEC and CMEC, transduction with an empty (control) adenoviral vector had no significant effect on mRNA levels of any of the enzymes assayed, as measured by densitometry of routine PCR (Fig. 3) or by real time PCR.

Similar to findings in hypoxic cells, HIF-1α expression was associated with increased expression of GlcNAcT-I, HS2ST, NDST1 and NDST2 in both cell types (Fig. 3). Furthermore, expression of HS6ST2, decreased by hypoxia in HUVEC but not in CMEC, was similarly affected by adeno-HIF-1α construct transduction (Fig. 3B,C). In the hypoxia studies, real time PCR confirmed the magnitude of changes in enzyme expression.

Hypoxia increases the ability of endothelial cells to respond to bFGF-induced proliferation

The increased HS content of cell surface GAGs and an increase in the number of low affinity (HS-FGF2) binding sites following exposure to hypoxia suggest that the cells may demonstrate altered sensitivity to FGF2 stimulation. To test this possibility, the growth rate of both CMEC and HUVEC cells in response to FGF2 was studied following 48 hours of culture under normoxic or hypoxic conditions. The hypoxic cells demonstrated significantly greater proliferative response to FGF2 both in terms of cell number and ^{3}H-thymidine uptake (Fig. 4).

To evaluate whether this increased responsiveness was caused by increased synthesis of 2-O-enriched HS on cell-surface-associated proteins or a general increase in the HS mass, we added exogenous HS chains to CMEC and HUVEC cells cultured under both normoxic and hypoxic conditions at amounts calculated to double the total HS mass. The addition of HS chains by themselves had little effect on cell proliferation, and the extra HS did not enhance the ability of FGF2 to stimulate cell growth under both sets of conditions (Fig. 4).

Discussion

Hypoxia and ischemia trigger a multitude of responses designed to compensate for reduced oxygen availability; these responses include increased expression of angiogenic growth

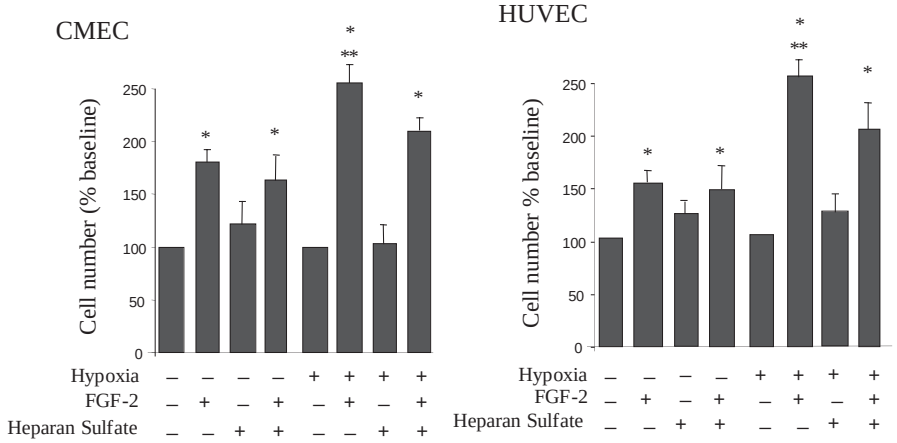

Fig. 4. Effect of hypoxia on FGF2-induced cell growth. Growth of CMEC and HUVEC in 0.25% serum in the presence (+) or absence (–) of FGF2 (10 ng/ml) was determined after 48 hours of hypoxia (+) or normoxia (–). For each condition, the cells counts are normalized to the baseline number taken as 100%. Note increased growth response to FGF2 for both cell types cultured under hypoxic conditions. *P<0.05 hypoxia versus normoxia.

factors, their receptors and specific integrins capable of promoting these factors' signaling (Semenza, 2000; Ware and Simons, 1997). In this study we demonstrate that another set of responses to hypoxia includes alterations in the cell-surface-associated HS matrix that results in increased endothelial cell responsiveness to FGF2. In particular, we have shown that hypoxic exposure of primary endothelial cells derived from large veins or cardiac microvasculature results in a preferential shift towards HS chains on the cell surface and an increase in the number of FGF2-binding sites on HS chains.

Although both endothelial cell types demonstrated an increase in the relative proportion of HS in the total cell-surface-associated GAG content (increased HS/CS ratio) and an increased HS-FGF2 binding capacity, there were interesting differences. Ischemic HUVEC had a much higher increase in the HS content (per mg cell protein) than CMEC (1.6- versus 1.15-fold, respectively) and a higher increase in the HS/CS ratio (2.6-fold versus 1.8-fold). However, the cell-surface-associated HS mass was nearly identical in both cell types (21.1±0.2 versus 24.4±0.1 CPM per μg protein, respectively). This shift towards HS chains under hypoxic conditions is similar to results observed in bovine aortic and pulmonary endothelial cells (Karlinsky et al., 1992).

Endothelial cell-surface-associated heparan sulfates are carried on two major classes of core proteins - syndecans and glypicans (Rosenberg et al., 1997; Tumova et al., 2000). The four members of the Syndecan family of transmembrane proteins are capable of carrying both HS and CS chains (Bernfield et al., 1999; Zimmermann and David, 1999) and, under normal conditions, less then 50% of these chains are HS (Shworak et al., 1994). In contrast, glypican-1, the sole member of the glypican family found in endothelial cells, is a GPI-anchor-linked protein that carries essentially only HS chains (Aviezer et al., 1994). Although both syndecans and glypican can support FGF2-FGF receptor-1 interactions (Rosenberg et al., 1997), syndecan-4 appears to be the principal core protein involved in FGF2 signaling (Simons and Horowitz, 2001; Volk et al., 1999).

In the absence of changes in syndecan and glypican expression in both HUVEC and CMEC following 24 hours of hypoxia, preferential synthesis of HS chains on syndecan cores must account for the increase in the HS mass and the HS/CS ratio. The observed increase in expression of GlcNAcT-I (EXTL2) is in accord with this conclusion. EXTL2 is closely related to the human *EXT2* gene, a member of the hereditary multiple exostoses (EXT) family of tumor suppressors, which also encode HS polymerase of uncertain function (Lind et al., 1998; McCormick et al., 1999). Although EXTL2 has been purified on the basis of their ability to catalyze additions of glucuronic acid and GlcNAc to the HS chain in vitro (Kitagawa et al., 1999), to our knowledge this is the first demonstration in an in vivo study of the effect of modulation of its expression on the extent of HS chain synthesis.

In addition to the increase in the ratio of HS to CS, both cell types demonstrated increased numbers of low affinity FGF2 binding sites per cell, which are thought to represent FGF2 binding by HS chains. Binding of FGF2 to heparan sulfate chains requires the presence of iduronic acid 2 sulfate (IdoA(2S)), with the affinity of oligosaccharides for FGF2 increasing with increasing number of IdoA(2S) residues (Nugent and Iozzo, 2000; Rapraeger et al., 1994). The appearance of IdoA(2S) sites requires transformation of D-GlcA into IdoA, which is carried out by GlcA C5 epimerase followed by sulfation of IdoA in the 2-O position (Shworak and Rosenberg, 1999), which in HS chains is carried out by the enzyme HS2ST. The importance of HS2ST in formation of FGF2-binding sites is confirmed by a marked reduction in FGF2 binding in a mutant cell line lacking this enzyme (Bai and Esko, 1996). In the current study, hypoxia was associated with induction of expression of HS2ST in both HUVEC and CMEC cells, whereas C5 epimerase levels remained unchanged. Interestingly, there was no change in the expression level of a related 2-O sulfotransferase involved in formation of IdoA(2S) sites on CS and DS chains (CS/DS2ST).

In addition to 2-O sulfation, 6-O sulfation, which is carried out by 6-O sulfotransferase, and, in particular, the presence of IdoA(2S)alpha1,4GlcNSO3(6S) disaccharide also appear to be important for FGF2 signaling (Pye et al., 1998) since 6-O sulfates are involved in hydrogen bonding between the HS chain and the FGF receptor (Schlessinger et al., 2000). The relative specificities of the two known 6-O HS sulfotransferase isoforms (HS6ST-1 and HS6ST-2) have not been established. Neither isoforms' expression was altered by hypoxia in microvascular endothelial cells whereas only HS6ST2 was decreased by hypoxia in HUVEC.

We have also observed the increase in expression of both NDST1 and NDST2 genes.

The N-sulfation of HS chains, carried out by NDST enzymes, plays an important role in development. Of the four NDST isoforms, only NDST1 and 2 show a widespread pattern of expression (Aikawa and Esko, 1999). Disruption of the *NDST2* gene results in a severe mast cell defect while not significantly affecting HS composition in other organs (Forsberg et al., 1999). On the other hand, disruption of the *NDST1* gene causes structural alterations in HS in most basement membranes, leading to neonatal mortality (Ringvall et al., 2000). Although little is known regarding the physiological consequences of increased expression of these enzymes, increased NDST-1 activity results, in addition to increased N-sulfation, in increased chain length (Pikas et al., 2000). This increase in the HS chain length may result in an increased number of FGF2-binding sites per HS chain even in the absence of increased 2-O sulfation activity.

Hypoxia induces a variety of cellular responses, ranging from upregulation of VEGF and VEGF receptor 1 to increased expression of glucose transporters (Semenza, 2000). Most of these are thought to be mediated by the transcription factor HIF-1α (Carmeliet et al., 1998; Semenza, 2000) although its recently described analog, HIF-2α, may also be involved (Blancher et al., 2000). To investigate the potential role of HIF-1α in hypoxia-mediated induction of GAG biosynthetic enzymes, we analyzed their expression in HUVEC and CMEC transduced with an adenovirus carrying a HIF-1α cDNA construct with a deleted oxygen-dependent degradation domain fused with a VP16 activation domain (Vincent et al., 2000). The native form of HIF-1α is inherently unstable under normal conditions owing to the presence of the oxygen-dependent degradation domain, which facilitates ubiquitination and the subsequent degradation of the protein. The removal of this domain or selective mutations within it result in stabilization of the HIF-1α protein (Huang et al., 1998; Sutter et al., 2000).

The adenovirus-mediated expression of the HIF1α-VP16 fusion construct in both HUVEC and CMEC produced a GAG enzyme expression pattern that closely mimicked findings in the hypoxic cells. This was equally applicable to enzymes that were upregulated (GlcNAcT1, HS2ST, NDST1 and NDST2), downregulated (HS6ST2- in HUVEC only) or unchanged (HS6ST1, CS/DS2ST and epimerase) by hypoxia. We conclude, therefore, that the expression of these genes is most probably controlled by HIF-1α.

The alteration in the HS cell surface composition, which is more pronounced in HUVEC than CMEC, had a profound impact on cell responsiveness to FGF2 in a proliferation assay; both cell types demonstrated faster growth rate in the presence of FGF2 under hypoxic conditions. The magnitude of this increase in FGF2 responsiveness closely paralleled the magnitude in increased HS mass. In keeping with the larger increase in the number of low affinity FGF2 binding sites on HUVEC than CMEC, the former demonstrated a more pronounced increase in the FGF2-induced growth rate than the latter. This increased FGF2 responsiveness could not be attributed to changes in the FGF receptor levels or changes in the affinity (FGF R1) as both of these parameters were largely unchanged.

Furthermore, it is unlikely that a simple increase in HS chains (rather then an alteration in HS surface composition) is responsible for this effect, since the addition of HS chains by themselves did not stimulate cell proliferation, and the extra HS had little effect on the ability of FGF2 to stimulate cell growth under normoxic or hypoxic conditions. Rather, we hypothesize that the increase in cell-associated HS chains enriched in 2-O sulfates is responsible for this effect. In support of this thesis is an observation of hypoxia-induced increase in the total cell-associated HS mass and in HS2ST levels (and, by implication, an increase in the 2-O fraction of the total HS mass).

It is likely that the cell-surface-associated HS and not the extracellular HS is responsible for enhanced FGF2 signaling. As already noted, the addition of HS to the cell culture medium under either normoxic or hypoxic conditions had no effect on response to FGF2 in either HUVEC or CMEC cells. Although this HS was not selectively enriched in the 2-O sulfated fraction, the overall amount of 2-O HS was doubled, mimicking overall changes in HS composition observed in hypoxic HUVEC cells. The difference from the latter situation, however, is that this additional total HS (and its 2-O HS fraction) is not on cell-membrane-associated core proteins. Of these, syndecan-4 is likely to be particularly important with regard to FGF2 signaling, since its overexpression but not that of glypican-1 or syndecan-1 enhances FGF2 signaling (Simons and Horowitz, 2001).

In summary, hypoxia induces alterations in HS composition on the endothelial cell surface that enhances cell responsiveness to FGF2. This effect is mediated by an increase in total cell surface HS chains as well as by an increase in the number of HS-FGF2 binding sites. Both of these changes are mediated by a HIF-1α-dependent increase in expression of enzymes responsible for preferential HS versus CS chain synthesis (GlcNAcT-I) and generation of FGF2 binding sites (HS2ST). This novel mechanism may constitute an important adaptive response to hypoxia and may play a fundamental role in control of angiogenesis by regulating cellular interactions of heparin-binding growth factors.

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