Down-regulation of perlecan expression contributes to the inhibition of rat cardiac microvascular endothelial cell proliferation induced by hypoxia

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Abstract: Exposure of endothelial cells (ECs) to hypoxia leads to a decrease in EC proliferation. However, the mechanism by which hypoxia inhibits EC proliferation is unclear. Perlecan has been reported to play an important role in regulating EC proliferation. We hypothesized that perlecan was involved in the hypoxia-induced inhibition of EC proliferation. To test this hypothesis, rat cardiac microvascular ECs were cultured under normoxic or hypoxic conditions for 12 h and harvested for determination of perlecan mRNA expression using real-time reverse transcription-polymerase chain reaction (RT-PCR). The results showed that exposure of ECs to hypoxia for 12 h induced a decrease in perlecan mRNA expression (61.72%, \( P < 0.05 \)). Concomitantly, the down-regulation of endogenous perlecan induced by hypoxia or the neutralization of endogenous perlecan with anti-perlecan antibody significantly inhibited EC proliferation and responsiveness to basic fibroblast growth factor (bFGF), and decreased focal adhesion kinase (FAK) expression and extracellular signal-regulated kinase 1/2 (ERK1/2) activation. These data indicate that down-regulation of perlecan expression contributes to hypoxia-induced inhibition of rat cardiac microvascular EC proliferation by suppressing FAK-mediated and ERK1/2-dependent growth signals.

Key words: hypoxia; endothelial cell; cell proliferation; perlecan

Endothelial cells (ECs) occupy a strategic position in the vasculature as a barrier between the intravascular compartment and underlying tissues. They are often exposed to diverse stresses. Among stresses, hypoxia stress often occurs under pathological condition, controlling many properties of ECs.
It has been well established that hypoxia regulates EC proliferation. The growth rate in cultured vascular ECs was decreased under both acute and chronically hypoxic conditions, although these cells conserved their capacity to divide\[1\]. The proliferation of human dermal microvascular ECs was inhibited under the influence of hypoxia, and was re-initiated after reoxygenation\[2\]. Hypoxia-conditioned medium decreased the retinal EC proliferation\[3\]. These data indicate that hypoxia exerts an inhibitory effect on EC proliferation. However, the mechanisms involved in hypoxia-inhibited EC proliferation are less well understood.

Several studies have been shown that perlecan, an angiogenic factor, is involved in regulating EC proliferation. Perlecan appears early in tissues of vasculogenesis including the heart, pericardium, and major blood vessels. Its early expression coincided with the development of the cardiovascular system\[4\]. Increased perlecan levels were detected in metastatic melanomas that correlated with a more aggressive phenotype\[5\]. Antisense targeting of perlecan blocked fibroblast growth factor-2 (FGF-2) function in human melanoma cells\[6\], and inhibited tumor growth and angiogenesis in vivo\[7\]. These observations suggest that there is a tight association between perlecan expression and cell proliferation. This led us to consider whether hypoxia induced the decrease of perlecan expression, resulting in an inhibition of EC proliferation. However, so far there are no reports on the expression change of perlecan in ECs when exposed to hypoxia.

In the present study, the effects of hypoxia on perlecan mRNA expression were analyzed employing real-time reverse transcription-polymerase chain reaction (real-time RT-PCR). The results showed that hypoxia inhibited the expression of perlecan mRNA and that the inhibition of endogenous perlecan decreased EC proliferation and inhibited the expression of focal adhesion kinase (FAK) and extracellular signal-regulated kinase 1/2 (ERK1/2) activation. These data indicate that the down-regulation of perlecan expression is possibly involved in hypoxia-induced reduction of EC proliferation by suppressing FAK-mediated and ERK-dependent growth signals.

1 MATERIALS AND METHODS

1.1 Animals
Male Sprague-Dawley rats weighting 80-100 g (1 month old) were used for EC culture. All animals were from the Experimental Animal Center, PLA General Hospital, Beijing, China. All procedures were carried out in accordance with the Guidelines for the Care of Animals (the Council for Animal Research, Health Center, Peking University).

1.2 Isolation and culture of rat cardiac microvascular ECs
Rats were anesthetized with 20% urethane by abdominal injection (1 mL/100 g). The cultures of rat cardiac microvascular ECs were prepared as described by Nishida et al\[8\] with modification. Briefly, the left ventricles were fully minced and digested with 0.1% collagenase I (Sigma, USA) for 6 min at 37 °C in a shaking water bath. Then, 0.1% trypsin (Difco, USA) was added and incubated for 4 min at 37 °C. The digested solution was filtered through 100 μm mesh filter, and the filtrate was collected and suspended in standard M199 medium (Difco, USA) containing 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 25% fetal calf serum (Hongzhou Biological Research Institute, China), 40 U/mL heparin and 100 μg/mL endothelial cell growth supplement (ECGS, Sigma, USA). Then, the suspension was cultured in a humidified atmosphere with 5% CO₂ at 37 °C and medium was changed every 3 d. The cells were characterized on the basis of their typical cobblestone appearance and CD31, a surface marker of microvascular ECs. All studies were performed on the cells between passages 3 and 5.

1.3 Assessment of cell proliferation
DNA synthesis assays were performed to test the role of hypoxia in cell proliferation. The cells were plated in 24-well plates at a density of 5×10⁴ cells/well in M199 supplemented with 10% FBS and 100 μg/mL ECGS, and allowed to accommodate for 24 h and cultured in serum-deprived M199 medium for 24 h. Then the cells were stimulated with basic fibroblast growth factor (bFGF, 5 ng/mL) and/or anti-perlecan antibody (10 µg/mL) under either normoxic (95% air and 5% CO₂) or hypoxic (95% N₂ and 5% CO₂) conditions at 37 °C in the presence of 1 μCi ³H-thymidine (³H-TdR) (Amersham, USA) for 12 h. At the end of incubation the cells were washed twice with PBS, incubated with 0.5 mL of 0.2 mol/L perchloric acid for 3 min, washed with 1 mL of PBS, and lyed in 0.3 mL of 1% SDS and 0.1 mol/L NaOH. Samples were harvested and mixed with liquid scintillation mixture (Ecoscint H, National Diagnostics, Atlanta, GA), and incorporated radioactivity was counted (cpm/min) in a liquid scintillation counter (Beckman LS 6500).

1.4 Western blot
The cells were lysed at 4 °C in a lysis buffer (20 mmol/L HEPES, pH 7.7, 2.5 mmol/L MgCl₂, 0.1 mmol/L EDTA, 20 mmol/L β-glycerophosphate, 0.5 mmol/L DTT, 0.1 mmol/L sodium orthovanadate, 75 mmol/L NaCl, 0.05%
Triton X-100) containing a protease inhibitor cocktail. The samples were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes (Millpore Corporation, Bedford). Equal protein loading was controlled by Ponceau Red staining of membranes. Blots were probed using anti-vascular endothelial growth factor (VEGF) antibody, anti-FAK antibody and anti-phospho-ERK1/2 antibody, followed by a peroxidase-conjugated secondary antibody. Antigen-antibody complexes were visualized by enhanced chemiluminescence.

1.5 Real-time RT-PCR for perlecan and β-actin
Total RNA was extracted from cardiac microvascular ECs using TRIzol (Invitrogen). RNA was treated with DNase I to minimize genomic DNA contamination. RT was carried out with the extracted RNA for 1 cycle at 65 °C for 5 min, 37 °C for 1 h, and 90 °C for 5 min. Oligonucleotide primers and TaqMan probes for rat perlecan and β-actin were designed from the GenBank database using Primer Express version 1.0. For the rat perlecan gene, the forward primer was 5'-5192TGGTTCGGGCCACATTCTT209-3', the reverse primer was 5'- 5234CACTTCTAGGCTCACAGCACTG 5256-3', and the TaqMan probe was FAM-5'-5211CTCAGTGCCACGGGCAGCCA5230-3'-TAMRA. For the rat β-actin as an internal control, the forward primer was 5'-1073CTCCTCCTGAGCGCAAGTACTC1094-3', the reverse primer was 5'- 1105CGG ACTCGTCA TACTCCTGCTT1126-3', and the TaqMan probe was FAM-5'-1099TCC A TCCTGGCCTCGCTGTCCA1120-3'-TAMRA. To prepare template DNA standards, a DNA fragment spanning nucleotide position +5 129 to +5 332 of perlecan gene was fused into plasmid, amplified and refined. The amount of construct per well was adjusted to 4×10^8 copies, and then serially diluted, yielding samples containing 4×10^8, 4×10^7, 4×10^6, 4×10^5, 4×10^4 copies, which were then used to construct standard curve. Real-time RT-PCR was carried out in an ABI Prism 7000 Sequence Detection System using 96 samples per assay. The amplifications were performed in 20-µL reaction volume containing 3 mmol/L MgCl₂, 0.2 µmol/L of each primer, 0.15 µmol/L of probe, and 1.5 U TaKaRa Taq. The amplification parameters were as follows: initial denaturation of one cycle of 2 min at 94 °C, followed by 40 cycles of 5 s at 94 °C and 30 s at 60 °C. All reactions were performed three times. Real-time data were analyzed by using ABI Prism 7000 SDS version1.1. To verify that the amplified products were the target genes, the products from one sample tube were sequenced.

1.6 Statistical analysis
Data were expressed as mean±SD. Statistical analyses were performed using one-way ANOVA. P<0.05 was considered significant.

2 RESULTS

2.1 Hypoxia inhibited EC proliferation and responsiveness to bFGF
EC proliferation was analyzed by 3H-TdR incorporation under normoxia and hypoxia in the absence or presence of bFGF (Fig.1). Exposure of ECs to hypoxia significantly decreased the cell proliferation compared to that in the control group. Under the normoxic condition, 3H-TdR incorporation of ECs treated by bFGF was 1.33-fold higher than that in the control group. However, when ECs were exposed to hypoxia, bFGF could not greatly stimulate EC proliferation.

Fig.1. Inhibition of EC proliferation and responsiveness to bFGF by hypoxia. ECs were cultured in the absence and presence of 5 ng/mL bFGF under normoxia and hypoxia as indicated. The number of viable cells was determined after 12 h of incubation as described in "materials and methods". *P<0.001 vs control, #P<0.001 vs bFGF.

2.2 Hypoxia promoted the expression of VEGF in cultured ECs
It has been reported that hypoxia increases VEGF expression in ECs, leading to the stimulation of EC proliferation. As shown in Fig.2, a thicker band of VEGF in ECs exposed to hypoxia was detected by Western blot as compared to that in ECs exposed to normoxia.

2.3 Hypoxia decreased the expression of perlecan mRNA in cultured ECs
We employed real-time RT-PCR to quantify perlecan mRNA expression in ECs exposed to hypoxia. The linear range of the standard curve for target gene mRNA of perlecan (Fig.
3.4) was determined. The threshold cycle (CT) was plotted as a function of initial template copy concentration to generate the standard curve. Figure 3B shows the level of perlecan mRNA expression in ECs after 12 h of exposure to hypoxia. Perlecan mRNA expression was decreased by 61.72% in ECs exposed to hypoxia (P<0.05).

2.4 Inhibition of endogenous perlecan decreased EC proliferation and responsiveness to bFGF
To delineate the effect of inhibition of endogenous perlecan on EC proliferation and responsiveness to bFGF, ECs were treated with anti-perlecan antibody. This antibody neutralized the effect of perlecan on the proliferation of smooth muscle cells[9]. As shown in Fig.4, there was a significant decrease in the proliferation in anti-perlecan antibody-treated ECs compared with that in untreated ECs (P<0.05). Also, the responsiveness to bFGF in anti-perlecan antibody-treated ECs was greatly decreased compared with that in untreated ECs (P<0.05).

Fig.2. Western blot analysis of the expression of vascular endothelial growth factor (VEGF) in hypoxia-treated ECs. ECs were exposed to either normoxia or hypoxia for 12 h. The cells were harvested for preparation of total protein. Immunoblotting was performed with anti-VEGF antibody.

Fig.3. Analysis of perlecan mRNA expression in ECs under hypoxia by real-time RT-PCR. A: The standard curve for perlecan using TaqMan PCR. The graph shows the typical amplification pattern of the perlecan template standards obtained by serial dilution. Threshold cycle (CT) is the PCR cycle in which an increase in reporter fluorescence above the baseline signal is first detected. B: The inhibitory effect of hypoxia on the expression of perlecan mRNA. ECs were exposed to either normoxia or hypoxia for 12 h. Values were expressed as mean±SD of perlecan/β-actin mRNA ratios. *P<0.05 vs normoxia.

Fig.4. Inhibition of endogenous perlecan decreased EC proliferation and responsiveness to bFGF. ECs were neutralized with anti-perlecan antibody (10 µg/mL) in the absence and presence of 5 ng/mL bFGF as indicated. The number of viable cells was determined after 12 h of incubation as described in “materials and methods”. *P<0.05 vs control, #P<0.05 vs bFGF.

2.5 Hypoxia and anti-perlecan antibody inhibited ERK1/2 activation in ECs
Phospho-ERK1/2 protein levels were tested in anti-perlecan antibody-treated and untreated cells in the absence or presence of bFGF under normoxic and hypoxic conditions. As shown in Fig.5A, anti-perlecan antibody and hypoxia led to a decrease of phospho-ERK1/2 expression in ECs in the absence or presence of bFGF. To quantify phospho-ERK1/2 expression levels, Image-Pro Plus software was used to analyze the integrated optical density (IOD) of ERK1/2 bands. As shown in Fig.5B, phospho-ERK1/2 expression was reduced in anti-perlecan antibody-treated ECs in the absence (84.19%, P<0.05 vs control group) or presence of bFGF (83.43%, P<0.05 vs bFGF group) under normoxic condition. Similarly, under hypoxic condition the expression levels of phospho-ERK1/2 were decreased in the absence (73.77%, P<0.05 vs control group) or presence of bFGF (73.87%, P<0.05 vs bFGF group).
2.6 Hypoxia and anti-perlecan antibody inhibited the expression of FAK in ECs

As shown in Fig.5A, there was a significant decrease in FAK expression induced by hypoxia and the neutralization by anti-perlecan antibody in the absence or presence of bFGF. To quantify FAK expression levels, Image-Pro Plus software was used to analyze the IOD of FAK bands. As shown in Fig.5B, anti-perlecan antibody could reduce FAK expression in the absence (59.69%, \( P<0.05 \) vs control group) or presence of bFGF (86.47%, \( P<0.05 \) vs bFGF group) under normoxic condition. Also, under hypoxic condition the expression levels of FAK were decreased in the absence (68.56%, \( P<0.05 \) vs the control group) or presence of bFGF (79.21%, \( P<0.05 \) vs bFGF group).

3 DISCUSSION

Growing evidence shows that hypoxia inhibits EC proliferation. However, little information has been known about the mechanisms by which hypoxia elicits its inhibitory effect on EC proliferation. In the present study, we found that hypoxia reduced the expression of perlecan mRNA in cultured rat microvascular ECs, inhibited EC proliferation and responsiveness to bFGF, and decreased FAK expression and ERK1/2 activation. The study suggested that perlecan might play a role in hypoxia-induced inhibition of EC proliferation by suppressing FAK-mediated and ERK1/2-dependent growth signals.

Our findings were consistent with reports suggesting that hypoxia induced the release of VEGF, whereas concomitantly hypoxia inhibited EC proliferation[3,4]. Eichler et al. reported that hypoxia increased the release of VEGF from retinae or retinal glial cells, whereas supernatants from hypoxic retinae or retinal glial cells failed to stimulate EC growth more than media conditioned under normoxic conditions. When the final VEGF concentration in hypoxia-conditioned media was adjusted to that in normoxia-conditioned media, EC proliferation was inhibited. Also, it was true for our study. Hypoxia increased the protein level of VEGF in ECs. However, hypoxia-induced VEGF release failed to cause an increase in EC proliferation. In addition, we found that hypoxia largely decreased the stimulatory effect of bFGF on EC proliferation. It seemed that there was an important factor that regulated the effects of VEGF and bFGF on EC proliferation.

Several lines of evidence indicate a tight interrelationship between perlecan and VEGF or bFGF. For example, the reduction of perlecan expression in Kaposi’s sarcoma cells resulted in an inhibited responses of these cells to VEGF[10]. The overexpression of an antisense perlecan cDNA in fibroblasts and carcinoma cells significantly reduced perlecan expression, and these cells were also unable to respond to bFGF[6,7]. And the response could be rescued by addition of exogenous perlecan[6]. Therefore, perlecan might be the important factor that led to a reduced response of ECs to VEGF and bFGF under hypoxic condition.

Perlecan is a major heparan sulfate proteoglycan secreted by ECs. It functions as an essential co-receptor for VEGF and bFGF, enabling them to bind and activate their receptors, and to thereby promotes cell growth and differentiation[11]. Perlecan interacts with VEGF and bFGF mainly through its heparan sulfate side chains. When complexed with heparan sulfate side chains, VEGF and bFGF are protected from proteolytic degradation[12,13], and maintain a long-term stimulation of EC proliferation[16]. Therefore, we speculated that hypoxia might cause the inhibition of EC proliferation by the following ways. Firstly, as a co-receptor for VEGF and bFGF, the reduction of perlecan inevitably led to a decrease in the bound and activated receptors for VEGF and bFGF. Secondly, the reduction of perlecan might
inhibit the formation of perlecan-VEGF complexes or perlecan-bFGF complexes, leading to an increase in the amount of degraded VEGF and bFGF.

The signaling events underlying perlecan-mediated EC proliferation suppression under hypoxia is unclear. In the present study, we showed that the inhibition of endogenous perlecan by hypoxia or the neutralization of endogenous perlecan with anti-perlecan antibody inhibited FAK expression and ERK1/2 activation. FAK is a major non-receptor tyrosine kinase. It is implicated in regulating cell proliferation by stimulation of a cell-signaling cascade that ultimately activates the Ras/MAPK/ERK pathway [14]. ERK1/2 is thought to be involved in transmitting signals to the nucleus to regulate gene transcription and protein synthesis, leading to proliferation [15-16]. We proposed that perlecan contributed to EC proliferation suppression under hypoxia by inhibiting FAK-mediated and ERK1/2-dependent growth signals.

In summary, our results demonstrate that hypoxia decreases the expression of perlecan mRNA. And the reduction of perlecan mRNA contributes to the inhibition of EC proliferation under hypoxia by suppressing FAK-mediated and ERK1/2-dependent growth signals.

REFERENCES