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Hypoxia Stimulates the Autocrine Regulation of Migration of Vascular Smooth Muscle Cells Via HIF-1α-Dependent Expression of Thrombospondin-1

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Abstract The migration of vascular smooth muscle cells from the media to intima and their subsequent proliferation are critical causes of arterial wall thickening. In atherosclerotic lesions increases in the thickness of the vascular wall and the impairment of oxygen diffusion capacity result in the development of hypoxic lesions. We investigated the effect of hypoxia on the migration of human coronary artery smooth muscle cells (CASMCs) via HIF-1α-dependent expression of thrombospondin-1 (TSP-1). When the cells were cultured under hypoxic conditions, mRNA and protein levels of TSP-1, and mRNA levels of integrin β_3 were increased with the increase in HIF-1α protein. DNA synthesis and migration of the cells were stimulated under the conditions, and a neutralizing anti-TSP-1 antibody apparently suppressed the migration, but not DNA synthesis. The migration was also inhibited by RGD peptide that binds to integrin β_3. Furthermore, the migration was completely suppressed in HIF-1α-knockdown cells exposed to hypoxia, while it was significantly enhanced in HIF-1α-overexpressing cells. These results suggest that the hypoxia induces the migration of CASMCs, and that the migration is elicited by TSP-1 of which induction is fully dependent on the stabilization of HIF-1α, in autocrine regulation. Thus we suggest that HIF-1α plays an important role in the pathogenesis of atherosclerosis. J. Cell. Biochem. 104: 1918–1926, 2008.

Key words: hypoxia; migration; thrombospondin-1; hypoxia-inducible factor-1; integrin beta3

Since in atherosclerotic lesions the arterial wall thickens and blood-diffusion capacity is impaired, oxygen consumption is augmented, and an energy imbalance may occur. Direct measurements of oxygen in vitro and in situ indicated that pO_2 is decreased in the more deeply situated parts of the media, and atherosclerotic plaques are also hypoxic [Bjornheden et al., 1999]. Therefore, hypoxia would be an important factor for the development of atherosclerosis.

Hypoxia up-regulates the expression of a number of genes including those of growth factors, with activation of the transcription factor hypoxia-inducible factor-1 (HIF-1) [Osada-Oka et al., 2006]. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β, the latter known as aryl hydrocarbon receptor nuclear translocator (ARNT) [Wang and Semenza, 1995; Wang et al., 1995]. Although the ARNT protein is readily found in cells, HIF-1α is virtually undetectable in normal conditions, as it HIF-1α is degraded through prolyl hydroxylation which permits the binding of the von Hippel-Lindau protein (pVHL), a component of the E3 ubiquitin ligase [Ivan et al., 2001; Jaakkola et al., 2001]. However, under hypoxic conditions, HIF-1α evades prolyl hydroxylation, and is stabilized. We have previously found that HIF-1α signal transduction during hypoxia was mediated by NADPH-cytochrome P-450 reductase (NPR) [Osada et al., 2002].

Thrombospondin-1 (TSP-1) is also associated with atherosclerotic lesions, acute vascular injury, hypercholesterolemia, and hypertension

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[van Zanten et al., 1994; Roth et al., 1998]. Moreover, antibody against TSP-1 has been shown to significantly accelerate re-endothelialization and reduce neointimal formation in balloon-injured rat arteries [Chen et al., 1999]. TSP-1 has been reported to be a potent inducer of the migration of bovine pulmonary artery smooth muscle cells [Yabkowitz et al., 1993] and human vascular smooth muscle cells (VSMCs) [Patel et al., 1997; Ichii et al., 2001]. However, TSP-1, which is used in these experiments, is commercially available or purified from platelets, and thus the role of endogenous TSP-1 in autocrine regulation under hypoxic conditions remains unexplored.

The identification of receptors for TSP-1 on human VSMCs is complicated by the presence of multiple domains, which interact with distinct cellular receptors, within the sequence of TSP-1. The N-terminal heparin-binding domain binds to heparin sulphate proteoglycans [Feitsma et al., 2000], while the type I receptors, containing the peptide sequence CSVTCG, bind to CD36 [Dawson et al., 1997]. The Arg-Gly-Asp (RGD) sequence in the type III calcium-binding repeat binds to integrin receptors of the β_3 subclass [Lawler et al., 1988], while the C-terminal cell-binding domain binds to a 52 kDa receptor identified as integrin-associated protein (CD47) [Gao et al., 1996]. Patel et al. [1997] showed that TSP-1-induced chemotaxis is dependent on RGD and associated with the activation of tyrosine kinases, whereas TSP-1-induced DNA synthesis is independent of RGD but dependent on tyrosine kinase. Likewise, Lymn et al. [2002] have demonstrated that cell chemotaxis is suppressed by anti-$\alpha_v\beta_3$ antibody, TSP-1-induced DNA synthesis is suppressed by anti-$\alpha_3\beta_1$ antibody in human VSMCs. The importance of integrin β_3 to the formation of neointimal lesions was indicated by Choi et al. [2004], who demonstrated that neointimal thickening, which is associated with the migration of VSMCs from the media into the intima after carotid ligation, was reduced in integrin β_3-deficient mice.

The development of atherosclerosis is characterized by two events: the migration of VSMCs from the media into the intima and the proliferation of VSMCs. Under hypoxic conditions, growth factors such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) are implicated in the latter event. However, the factors that regulate the migration of VSMCs under hypoxic conditions are yet to be completely identified. It has been reported that hypoxia enhances the expression of TSP-1 in both human and bovine endothelial cells [Phelan et al., 1998]. Therefore, we examined in this study the effect of hypoxia on cell migration and an increase in TSP-1 expression via a HIF-1α-dependent pathway, and investigated the autocrine regulation of migration via TSP-1 in human coronary artery smooth muscle cells.

MATERIALS AND METHODS

Materials

MCDB131 medium, FGFB, EGF, Insulin, G418, and MG132, were purchased from Sigma (St. Louis, MO). Opti-MEM medium and Lipofectamine2000 were from Invitrogen (Carlsbad, CA). Isogen was from Nippon Gene (Toyama, Japan). ReverTra Ace was from Toyobo (Osaka, Japan). Anti-TSP-1 IgG and anti-Hsp90 IgG were from Santa Cruz Biotech (Santa Cruz, CA) and BD Biosciences (San Jose, CA). [^{3}H]thymidine was from MP Biomedicals, Inc. (Irvine, CA). pGL and pRL-TK were from Promega (Madison, WI). Anti-HIF-1α antibody was prepared as described previously [Osada et al., 2002].

Cell Culture

Human coronary artery smooth muscle cells (CASMCs) obtained from Cambrex Co. (Walkersville, MD) were maintained in MCDB 131 medium containing 5% fetal calf serum, 1 μg/L of FGFB, 500 ng/L of EGF, and 5 ng/L of insulin. After being incubated in serum-free medium for 24 h, the cells were incubated under hypoxic conditions (1% O_2, 5% CO_2). In the experiments with hypoxia, the cells were incubated in a sealed 2.5-L box with an Anero Pack (oxygen absorber) for cell (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan).

Western Blot Analysis

Whole cell lysates were prepared with 20 mM Tris–HCl (pH7.4) containing 1% Triton X-100, 150 mM NaCl, 5 mM EGTA, 10 μM MG132, and 100 μM PMSF. Proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) with a 7.5 or 10% gel. Immunoblot analysis was performed with antibodies against TSP-1 and HIF-1α, which had been prepared

previously [Osada et al., 2002]. Blotted proteins were visualized using horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG and enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK).

Isolation of Total RNA and Reverse-Transcription PCR

Total RNA (1 μg) extracted from CASMCs with Isogen was transcribed into cDNA using ReverTra Ace in 10 μl (total volume) according to the manufacturer's directions as follows: incubation for 10 min at 30°C and 40 min at 42°C, followed by heating for 10 min at 70°C to stop the reaction. PCR with 5 pmol of each primer, 1.0 U of Ampli Taq, and the cDNA was performed for 10 min at 94°C and then 35 cycles of 1 min at 94°C, 1 min at 56°C, and 2.5 min at 72°C. The primers for TSP-1 (accession No. NM_003246) were 5′-ATCCA AAGCG TCTTC ACCAG-3′ (sense) and 5′-CTCCGT TGTGA TAGCA TAGGG-3′ (antisense). Those for Integrin β_1 (NM_002211) were 5′-AATGA AGGGC GTGTT GGTAG-3′ (sense) and 5′-CACGT TTGCC CTTGA AACTT-3′ (antisense). Those for Integrin β_3 (NM_000212) were 5′-TTTGC CTTAT TGGCA GCTCT-3′ (sense) and 5′-GA-ATG ATGCC AAGTG GGAGT-3′ (antisense). The primers for CD36 (NM_005506) were 5′-AGGCC CGATA TCTCT CCCTA-3′ (sense) and 5′-CGACT CGCCG TCTCT TTATC-3′ (antisense). The primers for β-action (NM_001101) were 5′-GATCA TTGCT CCTCC TGAGC-3′ (sense) and 5′-CACCT TCACC GTTCC AGTTT-3′ (antisense).

Cell Proliferation Assay

DNA synthesis was evaluated by measuring the incorporation of [^{3}H]thymidine into DNA of cells, which were cultured in 35-mm dishes at a density of 1×10^4 cells/dish. The cells were exposed to hypoxia for 12 h in MCDB medium containing 0.1% FCS in the presence of anti-TSP-1 IgG (5 μg/ml) or non-immune IgG (5 μg/ml). [^{3}H]thymidine (1 μCi/ml) was added to the medium in each dish for the last 6 h under a N_2 current. The cells were washed with ice-cold phosphate-buffered saline and fixed with 10% trichloroacetic acid for 10 min. They were next washed with ethanol. Finally, the cells were solubilized with 0.1 M NaOH containing 0.1% sodium dodecyl sulfate. The radioactivity in each sample was measured with a liquid scintillation counter (Tri-Carb 2000CA, Perkin-Elmer, Boston, MA). Data are shown as the mean ± SD of three different cell preparations and as the ratio of hypoxia to normoxia.

Cell Migration Assay

The migrational activity of human CASMCs was determined using a Matrigel invasion system (BD Biosciences) as recommended by the manufacturer. Human CASMCs (1×10^4) were suspended in 0.5 ml of conditioned medium and added to the upper chamber. The upper chamber was lodged into the lower chamber containing 0.75 ml of conditioned medium. After incubating at 37°C for 12 h, the cells in the upper side of the filter membrane were removed with cotton swabs. Photos of cells that migrated to the lower side of the membranes were taken with the use of a microscope. After cells were trypsinized, the number of cells in the lower side was counted.

Transfection

HIF-1α-sense (pcDNA-sHIF-1α) or antisense (pcDNA-aHIF-1α) plasmid was transiently introduced into CASMCs using Lipofectamine-2000. After the transfection, the cells were cultured in serum-free Opti-MEM medium for 12 h. Then the cells received fresh medium containing 5% fetal calf serum, 1 μg/L of FGFB, 500 ng/L of EGF, and 5 ng/L of insulin, and the transfected cells were selected by culture in medium containing G418 (300 μg/ml). The expression of HIF-1α in CASMCs transfected with pcDNA-sHIF-1α or pcDNA-aHIF-1α was checked by immunoblot with anti-HIF-1α antibody.

RESULTS

mRNA and Protein Expression of TSP-1 Under Hypoxic Conditions

When human CASMCs were cultured under hypoxic conditions, protein levels of HIF-1α were markedly increased at 3 h, and remained high even after 12 h (Fig. 1A). We examined whether or not hypoxia induces the expression of TSP-1, which is highly expressed in atherosclerotic lesions. As shown in Figure 1B,C, the mRNA levels of TSP-1 induced under hypoxic conditions in a time-dependent manner, and the increase in the amount of TSP-1 in the medium was greater than that under normoxic conditions.

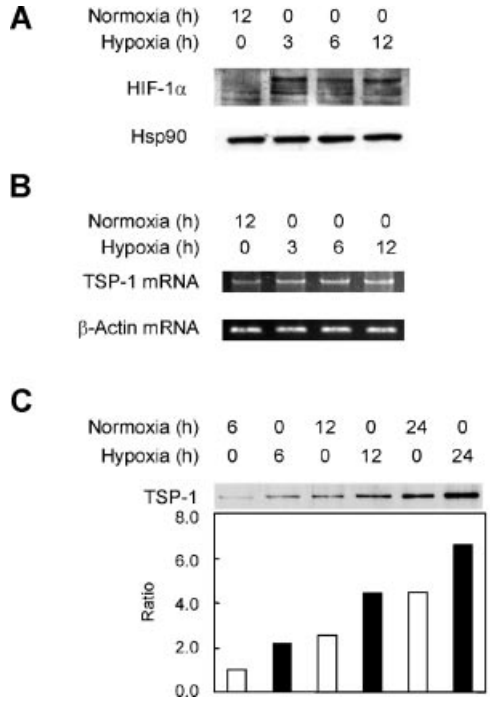

Fig. 1. The stabilization of HIF-1α protein and expression of the mRNA and protein of TSP-1 in a time-dependent manner. CASMCs were exposed to 1% O_2, 5% CO_2 for 3, 6, 12, or 24 h. **A**: Whole cell lysates were analyzed by SDS–PAGE with a 7.5% polyacrylamide gel and immunoblotted with anti-HIF-1 and anti-Hsp90 (as loading control) antibodies. **B**: The mRNA levels of TSP-1 and β-actin (as a loading control) were analyzed by RT-PCR. **C**: Culture medium was analyzed by SDS–PAGE with a 10% polyacrylamide gel and immunoblotted with anti-TSP-1 IgG. The bar in the graph represents the intensity of TSP-1 protein levels. Data are expressed as a ratio of the control (normoxia at 6 h).

mRNA Expression of TSP-1-Receptors

We next examined the mRNA levels of TSP-1-receptors: integrin β_1, integrin β_3, and CD36. Under hypoxic conditions, the mRNA expression of integrin β_3 was significantly induced for 3–12 h, but the levels of integrin β_1 and CD36 mRNA were only slightly increased for 12 h (Fig. 2).

Hypoxia-Induced Cell Proliferation and Effect of TSP-1

To test the influences of hypoxia and TSP-1 on the proliferation of human CASMCs, the cells were incubated for 12 h under hypoxic conditions, and DNA synthesis was evaluated based on the incorporation of [^{3}H]thymidine into the cells. As shown in Figure 3, hypoxia significantly enhanced the cell proliferation compared to normoxia. We next examined whether or not a neutralizing antibody against TSP-1 (anti-TSP-1 IgG) inhibits hypoxia-induced cell proliferation. However, anti-TSP-1 IgG did not affect cell proliferation under hypoxic or normoxic conditions.

Hypoxia-Induced Cell Migration and Effect of TSP-1

To examine whether or not hypoxia induces cell migration, human CASMCs were incubated in a Boyden chamber under normoxic or hypoxic conditions. As seen in Figure 4A,B, hypoxia stimulated the migration of more cells than did normoxia. To examine the effect of TSP-1 on cell migration, we assessed the migration in the presence of anti-TSP-1 IgG. Anti-TSP-1 IgG significantly inhibited cell migration stimulated for 12 h under hypoxic conditions (Fig. 4C,D), but non-immune IgG did not (data not shown). We next examined the effect of RGD peptide, which binds to the receptor for TSP-1, on the migration of CASMCs under hypoxia. The results in Figure 5 showed that the peptide significantly inhibited hypoxia-induced migration.

Involvement of HIF-1α in the Increase of TSP-1 and Cell Migration

To test then involvement of HIF-1α in the hypoxia-induced expression of TSP-1, HIF-1α-antisense plasmid or sense plasmid was transiently introduced into human CASMCs to generate HIF-1α-knockdown (HIF-1α^-) or HIF-1α-overexpressing cells (HIF-1α^+). Results in Figure 6 showed that HIF-1α was less stable in HIF-1α^- cells even under hypoxic conditions, and little or no expression of the mRNA and protein of TSP-1 was observed, although both were apparently induced in the cells transfected with empty vector (control). On the other hand, the mRNA and protein levels of TSP-1 and the HIF-1α protein level were higher in HIF-1α^+ cells than in the control cells under hypoxia. We next examined the role of HIF-1α in the hypoxia-induced proliferation and migration of CASMCs. As shown in Figure 7A, under hypoxic conditions, the incorporation of thymidine was reduced in HIF-1α^- cells, whereas it was enhanced in HIF-1α^+ cells, compared with the control cells (empty vector), although similar results were obtained in each cell type under

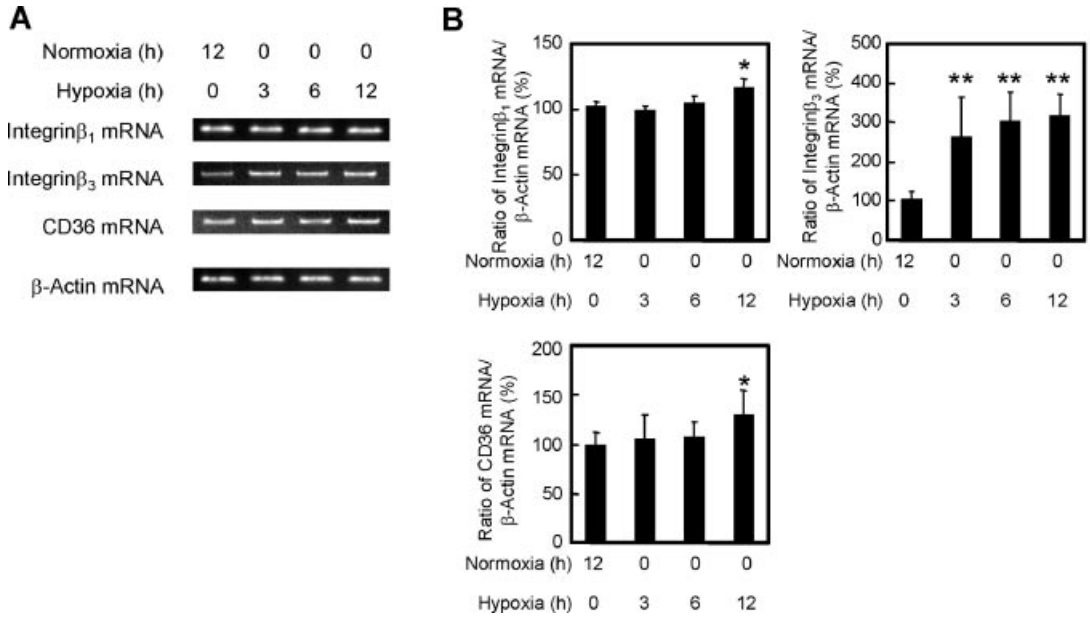

Fig. 2. The mRNA expression of some receptors for TSP-1. CASMCs were exposed to 1% O_2, 5% CO_2 for 3, 6, 12, or 24 h. **A**: Integrin β_1, Integrin β_3, and CD36 mRNA as receptor of TSP-1 were analyzed by RT-PCR. **B**: Data are the mean ± SD of four different cell preparations and expressed as a percentage of the control (normoxia). $^*P < 0.05$ or $^{**}P < 0.005$ versus normoxia for 12 h.

normoxic conditions. Figure 7B shows that cell migration was reduced in HIF-1α^- and increased in HIF-1α^+ cells, compared with control cells under hypoxia.

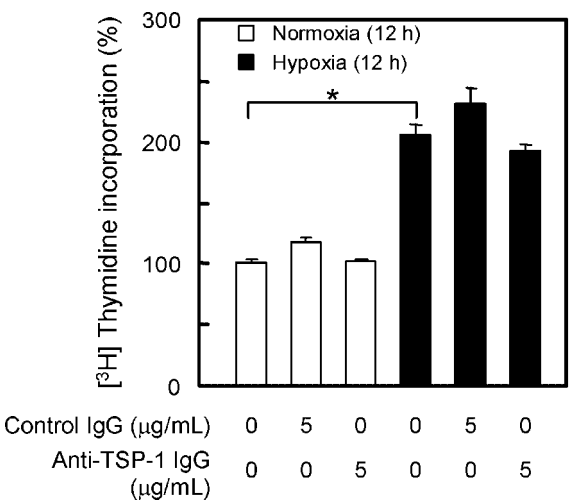

Fig. 3. Cell proliferation estimated by [^{3}H]thymidine incorporation and effect of anti-TSP-1 antibody. CASMCs were incubated under normoxic or hypoxic conditions for12 h in the presence or absence of a neutralizing antibody against TSP-1 (anti-TSP-1 IgG) and non-immune IgG (Control IgG). The incorporation of [^{3}H]thymidine was analyzed with a liquid scintillation counter. Data are the mean ± SD of three different cell preparations and expressed as a percentage of the control in the absence of antibody during normoxia. $^*P < 0.001$.

DISCUSSION

The migration of VSMCs from the media into the neointimal and their subsequent proliferation are important in the pathogenesis of atherosclerosis. This process is regulated by multiple factors, including growth factors, and involves changes in the interaction of VSMCs with the extracellular matrix. TSP-1 expression has been shown to be up-regulated in a number of animal models of vascular diseases, including carotid artery angioplasty and diet-induced atherosclerosis [Roth et al., 1998; Chen et al., 1999]. Therefore, TSP-1 is also implicated in the pathogenesis of vascular diseases.

We examined in the present study the involvement of TSP-1 in the hypoxia-induced migration of human CASMCs. Our results showed that hypoxia enhances the expression of TSP-1 and integrin β_3, with the stimulation of cell migration. The hypoxia-induced migration was markedly inhibited by a neutralizing antibody against TSP-1 and by RGD peptide that binds to integrin β_3 to block the interaction with TSP-1. Similar results have been reported, although they were from an experiment in which TSP-1 was added. It has been reported by Patel et al. [1997] that TSP-1 elicits chemotaxis and DNA synthesis in human VSMCs, and

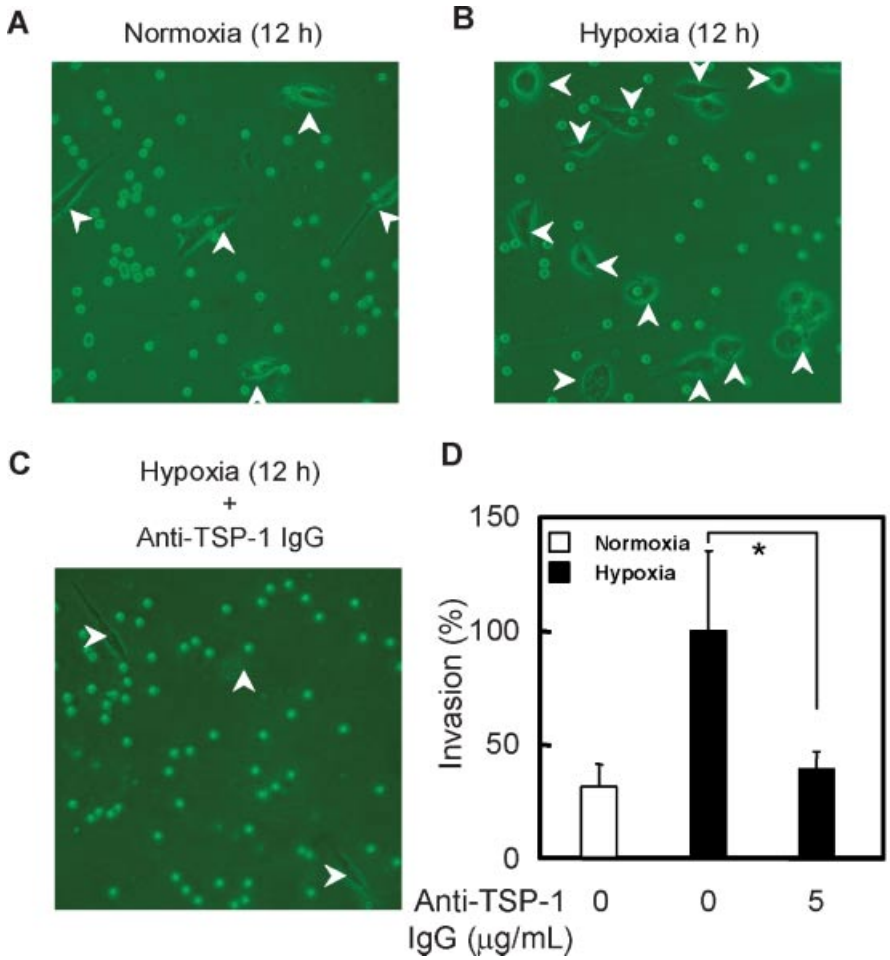

Fig. 4. Enhancement of cell migration and the inhibition by anti-TSP-1 antibody. The migration of CASMCs was evaluated using the matrigel invasion system. After incubation at 37°C for 12 h in the presence or absence of a neutralizing antibody against TSP-1 (anti-TSP-1 IgG), photos of cells that had migrated from the upper to lower side of the membranes were taken with the use of a microscope. Data are the mean ± SD of six different cell preparations and expressed as a percentage of the number of cells migrated in the absence of antibody during hypoxia. $*P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the chemotactic response, but not DNA synthesis, is inhibited by RGD peptide. Lymn et al. [2002] reported that the TSP-1-induced chemotaxis of human VSMCs is suppressed by RGD peptide and anti-$\alpha_v\beta_3$ antibody, while TSP-1-induced DNA synthesis is independent of RDG and suppressed by anti-$\alpha_3\beta_1$ antibody. Thus, considering these findings together with the present results, we suggest that hypoxia induces expression of TSP-1 and integrin β_3 and that hypoxia-induced migration of human CASMCs is elicited via their association with each other. Our data show, for the first time to our knowledge, a mechanism underlying hypoxia-mediated cell migration.

We also showed that a neutralizing anti-TSP-1 antibody does not affect hypoxia-induced DNA synthesis. This raises the possibility that TSP-1 is not involved in hypoxia-induced cell proliferation. However, evidence has accumulated indicating that some growth factors, such as PDGF and fibroblast growth factor, in addition to TSP-1 are involved in the hypoxia-induced proliferation of VSMCs [Parenti et al., 2002; Schultz et al., 2006]. We also obtained evidence that VEGF has a critical role in the proliferation of human CASMCs under hypoxic conditions [Osada-Oka et al., 2008]. Therefore, in the present study, we could not establish a substantial role for TSP-1 in the proliferation of VSMCs.

PDGF markedly stimulates the migration of VSMCs [Yabkowitz et al., 1993; Ikeda et al., 1997; Kohno et al., 2002]. PDGF-BB increased

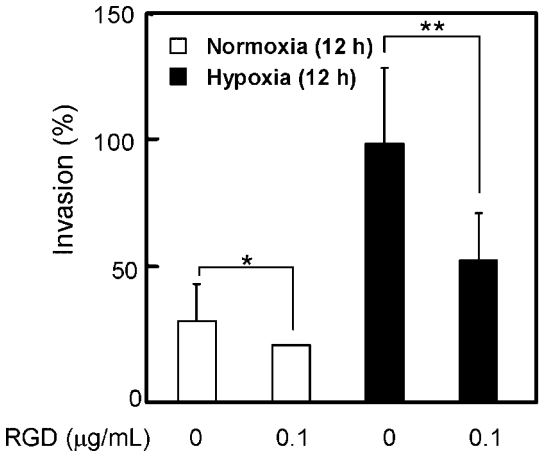

Fig. 5. Inhibition of cell migration by RGD peptide. The migration of CASMCs was evaluated using the matrigel invasion system. After incubation at 37°C for 12 h in the presence or absence of RGD peptide, photos of cells that had migrated from the upper to lower side of the membranes were taken with the use of a microscope. The ratio of the number of cells that migrated was the mean ± SD of six different cell preparations and expressed as a percentage of the control in the absence of RGD peptide during hypoxia. $*P < 0.05$ and $**P < 0.005$.

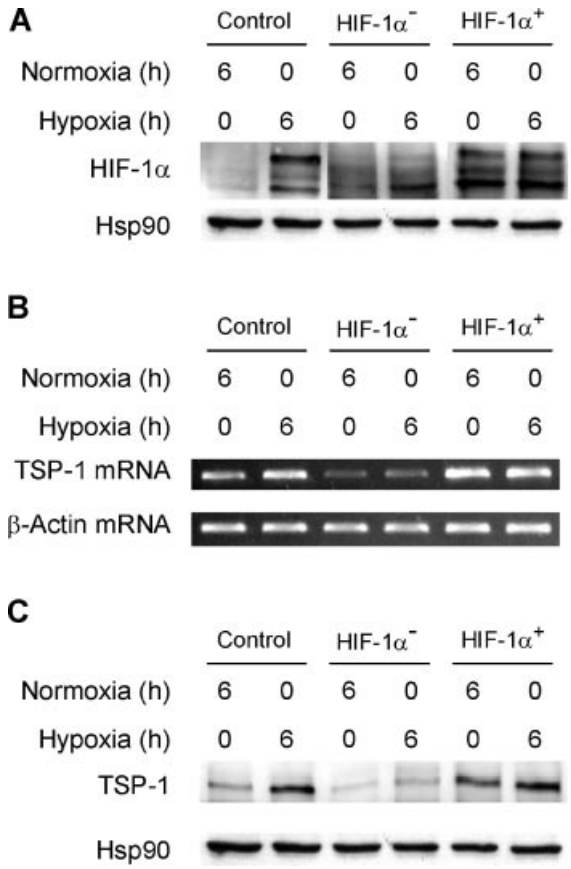

Fig. 6. Expression of TSP-1 in HIF-1α-knockdown and HIF-1α-overexpressing CASMCs. HIF-1α-knockdown and HIF-1α-overexpressing cells derived from CASMCs (HIF-1α^- and HIF-1α^+ cells) were exposed to 1% O_2, 5% CO_2 for 6 h. **A**: Whole cell lysates were analyzed by SDS–PAGE with a 7.5% polyacrylamide gel and immunoblotted with anti-HIF-1 and anti-Hsp90 (as loading control) antibodies. **B**: The mRNA levels of TSP-1 and β-actin (as loading control) were analyzed by RT-PCR. **C**: Culture medium was analyzed by SDS–PAGE with a 10% polyacrylamide gel and immunoblotted with anti-TSP-1 IgG.

the intimal thickening and the migration of VSMCs from the media to intima in a rat model of balloon angioplasty [Jawien et al., 1992]. In addition, hypoxia is known to potentiate PDGF expression [Zhang et al., 2003]. It seems likely, therefore, that under the conditions used in the present study, PDGF is implicated in the migration of human CASMCs exposed to hypoxia. However, little expression of PDGF protein was elicited by 12 h of hypoxia, at which time significant levels of TSP-1 were expressed (data not shown). Thus, we suggest the role of PDGF in the hypoxia-induced migration of the cells to be a minor one.

A number of cellular responses to hypoxia are dependent on the stabilization of a transcription factor, HIF-1α. We confirmed that hypoxic responses are HIF-1α-dependent through the knockdown or over-expression of HIF-1α. Our results indicated that HIF-1α-knockdown leads to a decrease in the hypoxia-induced expression of TSP-1 mRNA and protein in human CASMCs. Concurrently, DNA synthesis and migration were reduced. In contrast, HIF-1α-overexpression led to an enhancement of HIF-1α's stabilization and the expression of TSP-1 to potentiate migration of the cells under hypoxic conditions. Our results showed, therefore that hypoxia-induced HIF-1α's stabilization plays an important role in the induction of TSP-1 and in the migration of human CASMCs. It has been reported that hypoxia accelerates the expression of integrin $\alpha_v\beta_3$, but not integrin $\alpha_v\beta_5$, in human endothelial cells to potentiate their attachment to fibrinogen [Walton et al., 2000]. Furthermore, in HIF-1α-deficient trophoblast stem cells, hypoxia-induced migration was reduced compared with that in control cells, in parallel with a decrease in the expression of integrin $\alpha_v\beta_3$ [Cowden Dahl et al., 2005]. These results also indicate that hypoxia-induced cell migration is dependent upon an increase in the cell surface expression of integrin β_3.

Taken together, the present results show that hypoxia elicits RGD-sensitive migration of human CASMCs with increases in levels of

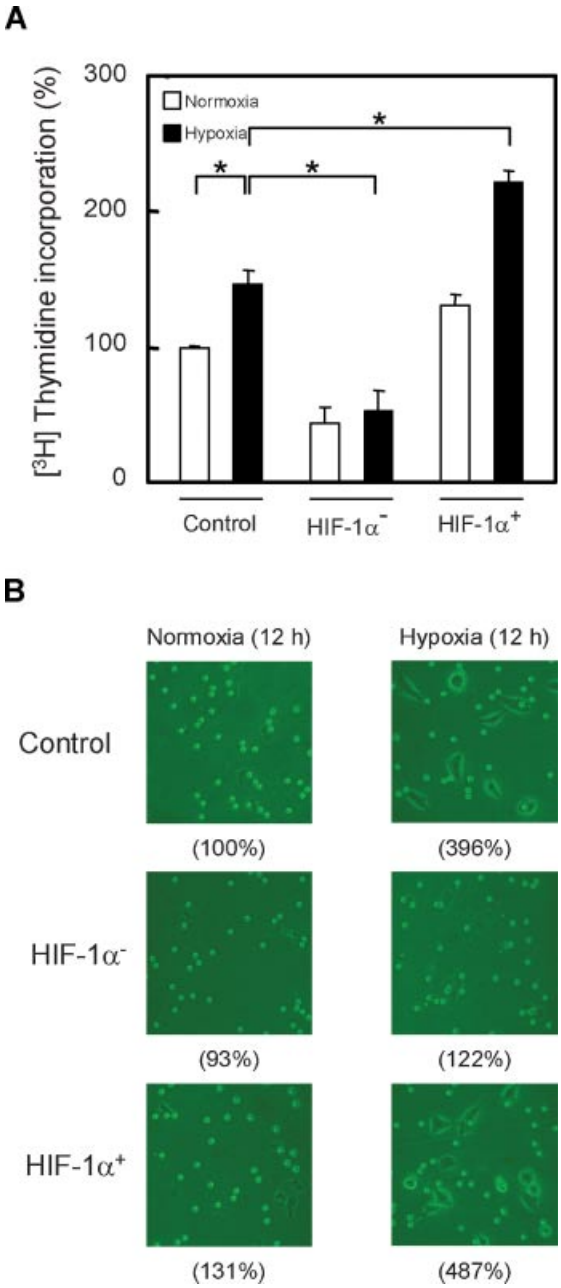

Fig. 7. Proliferation and migration in HIF-1α knockdown and HIF-1α-overexpressing CASMCs. HIF-1α$^-$ and HIF-1α$^+$ cells were incubated under normoxic or hypoxic conditions for 12 h. **A**: The incorporation of [^{3}H]thymidine was analyzed with a liquid scintillation counter. Data are the mean ± SD of three different cell preparations and expressed as a percentage of control cells during normoxia. *$P < 0.001$. **B**: The migration of CASMCs was evaluated using the matrigel invasion system, and photos of cells that had migrated from the upper to lower side of the membranes were taken with the use of a microscope. The number under each picture is the mean of two different cell preparations and expressed as a percentage of control cells during normoxia. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TSP-1 and integrin β_3, which contribute to the migration. These hypoxia-induced responses are fully dependent upon the stabilization of HIF-1α. Thus we suggest that HIF-1α plays an important role in the pathogenesis of atherosclerosis.

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