Age-dependent Defect in Vascular Endothelial Growth Factor Expression Is Associated with Reduced Hypoxia-inducible Factor 1 Activity*

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Previous studies have indicated that advanced age is associated with impaired angiogenesis in part because of reduced levels of vascular endothelial growth factor (VEGF) expression. To investigate potential mechanisms responsible for this age-dependent defect in VEGF expression, aortic smooth muscle cells isolated from young rabbits (ages 6–8 months) or old rabbits (ages 4–5 years) were exposed to normoxic (21% oxygen) or hypoxic (0.1% oxygen) conditions. Hypoxia-induced VEGF expression was significantly lower in old versus young cells. VEGF mRNA stability in hypoxic conditions was similar in both young and old cells. However, transient transfection with a luciferase reporter gene that was transcriptionally regulated by the VEGF promoter revealed a significant defect in VEGF up-regulation following hypoxia in old versus young cells (a 43% increase in luciferase activity, \( p < 0.05 \)); this difference was not seen when a deletion construct lacking the hypoxia-inducible 1 (HIF-1) binding site was used. Moreover, although HIF-1α-mRNA expression was shown to be similar in young and old smooth muscle cells, HIF-1α protein and DNA binding activity were significantly reduced in old versus young smooth muscle cells that were exposed to hypoxia. We propose that age-dependent reduction in hypoxia-induced VEGF expression results from reduced HIF-1 activity and may explain the previously described age-dependent impairment of angiogenesis in response to ischemia.

One of the characteristics of aging is the decline in the ability of the organism to respond to different types of stress. For instance, we have recently demonstrated that advanced age is associated with a defect in compensatory neovascularization in response to tissue ischemia (1). Such impaired angiogenesis in ischemic tissues of old animals was found to be associated with reduced expression of vascular endothelial growth factor (VEGF), an endothelial-specific growth factor that is essential for embryonic (2, 3) and postnatal (4) neovascularization. The molecular alterations responsible for this age-dependent decline in VEGF expression, however, have not been elucidated.

Among those factors that have been implicated in the regulation of VEGF expression, hypoxia appears to play a major role, both in vitro (5) and in vivo (6). The transcriptional and post-transcriptional mechanisms involved in the hypoxic regulation of VEGF are similar to those factors responsible for erythropoietin (Epo) expression. Transcriptional regulation of VEGF expression at the 5'-promoter of VEGF is conferred by a sequence that is homologous to the hypoxia-inducible factor 1 (HIF-1) binding site within the Epo gene (7). HIF-1 is a heterodimeric (HIF-1α and HIF-1β) basic helix-loop-helix protein that activates the transcription of the Epo gene in hypoxic cells (8). When reporter genes containing VEGF sequences are co-transfected with expression vectors that encode HIF-1α and HIF-1β, the reporter gene transcription is much higher in both hypoxic and normoxic cells than in cells transfected with the reporter gene alone (9). Moreover, the results of gene-targeting experiments have shown that HIF-1α deficiency is associated with a complete loss of VEGF gene induction under hypoxic conditions, marked reduction in vascularization of embryos, and early lethality by embryonic day 10.5 (10).

In this study, we used an in vitro system to investigate potential transcriptional and post-transcriptional mechanisms involved in the age-dependent defect of VEGF induction by hypoxia. Our results indicate that the reduction in VEGF expression with aging is primarily attributed to a lower transcriptional activity under hypoxic conditions. Thus, this lower transcriptional activity is related to an age-dependent defect in HIF-1 activity.

MATERIALS AND METHODS

Cell Culture— Cultures of primary vascular smooth muscle cells (VSMCs) were isolated from the aortas of young New Zealand White rabbits (ages 6–8 months) or old New Zealand White rabbits (ages 4–5 years) as described by Mader (11). The maximum age for New Zealand White rabbits was previously reported to be 7 years (12). Cells were incubated in Dulbecco’s modified Eagle’s medium (Sigma), supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 200 units/ml penicillin, and 0.25 mg/ml streptomycin. Cells were cultured at 37 °C in a humidified atmosphere of normoxic conditions (5% CO2, 21% O2, and 74% N2), or placed in a modular incubator chamber (Billups-Rothenberg, New York), flushed with hypoxic gas (95% N2, and 5% CO2), and incubated at 37 °C in a humidified atmosphere. All experiments were performed with early passage (P1-P3) rabbit VSMCs at 70% confluence. VEGF ELISA—VSMCs from young and old rabbits were incubated under normoxic or hypoxic conditions for a 24-h period. The supernatant was collected, and VEGF concentrations were determined using a VEGF ELISA kit (R&D Systems). The results were expressed as ng/mg protein.

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tand was collected and VEGF levels were determined by using an immunoassay according to the manufacturer's instructions (Quantikine R&D Systems). Results were compared with a standard curve of human VEGF with a lower detection limit of 5 pg/ml. Samples were checked by serial dilution and were performed at least in duplicate. VEGF levels were normalized relative to the protein concentration of VSMC cellular extracts (Bio-Rad protein assay).

**Western Blot—**VSMCs (P1) from young and old rabbits were incubated under normoxic or hypoxic conditions for a 24-h period, washed with phosphate-buffered saline, and lysed in lysis buffer. Total protein extracts were quantified with the Bio-Rad protein assay. The protein extract (100 µg per sample) was separated on a 4–12% polyacrylamide gel (Bio-Rad) and electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked with 10% nonfat dry milk in 0.2% Tween phosphate-buffered saline and then probed with VEGF rabbit polyclonal antibody (1:200 dilution, Santa Cruz Biotechnology, Inc.), an HIF-1α mouse monoclonal antibody (1:400 dilution, Novus Biologicals), or an actin goat polyclonal antibody (1:2000 dilution, Santa Cruz Biotechnology). Filters were hybridized overnight with 32P-cDNA of VEGF (1.0 µg/ml, Amersham Pharmacia Biotech) at room temperature, followed by exposure to hyperfilms (Amersham Pharmacia Biotech). Each experiment was repeated at least twice with different cellular extracts. Representative results are shown below.

**Northern Blot—**Total cellular RNA was isolated from VSMCs by acid guanidinium phenol/chloroform extraction (13). The total denatured RNA (15 µg) was subjected to electrophoresis in a 1.0% agarose/formaldehyde gel and then transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech). A 675-bp EcoRI/BglII fragment of pSV-VEGF.21 (14), or a 345-bp fragment of human HIF-1α cDNA (pBlue-script/HIF-1α 3.2–3.7T) (8) were used as specific probes. All probes were radio-labeled by random-primed labeling (Rediprime kit, Amersham Pharmacia Biotech). After 24 h, luciferase and alkaline phosphatase activities were measured in the VSMCs in old and young rabbits. Results are expressed as the ratio of luciferase to alkaline phosphatase activities.

**HIF-1α Immunohistochemistry—**Cells were plated on four-well chamber slides before being exposed to either normoxic or hypoxic culture conditions for 6 h. Cells were fixed in 10% neutral buffered formalin for 10 min before staining. They were then incubated in 3% hydrogen peroxide to block endogenous peroxidase and 10% fetal bovine serum before exposure to normoxic or hypoxic conditions. After 24 h, luciferase and alkaline phosphatase activities were measured in the VSMCs in old and young rabbits. Results are expressed as the ratio of luciferase to alkaline phosphatase activities.

**Electromobility Shift Assay (EMSA)—**EMSA was performed in a reporter construct and with 30 µg of LipofectAMINE reagent (Life Technologies, Inc.). After transfection, VSMCs were allowed to recover for 3 h in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum before exposure to normoxic or hypoxic conditions. For transfection efficiency, luciferase activity was normalized relative to the level of alkaline phosphatase activity that was produced from co-transfected pSVAPAP plasmid (0.5 µg), which contained the reporter gene under the control of the simian virus 40 enhancer-promoter. The cells were incubated with the transfection mixture for 3 h and then exposed to normoxic or hypoxic conditions. After 24 h, luciferase and alkaline phosphatase activities were measured in the VSMCs and old and young rabbits. Results are expressed as the ratio of luciferase to alkaline phosphatase activities.

**Fig. 1.** VEGF expression in young and old VSMCs exposed to normoxic or hypoxic conditions. A, Northern blot analysis showing a lower degree of VEGF mRNA induction by hypoxia in old VSMCs compared with young VSMCs. B, VEGF protein secretion in culture medium of VSMCs as measured by ELISA. The magnitude of VEGF induction by hypoxia is significantly reduced in old versus young VSMCs. C, Western blot experiments on cellular extracts from young and old VSMCs exposed to hypoxic conditions. Actin protein level is used as a loading control.

**Fig. 2.** VEGF mRNA half-life in young and old VSMCs under hypoxic conditions. Cells were exposed to hypoxia for 24 h and then treated with actinomycin D (ActD) 1, 2, or 3 h before total RNA extraction. Northern blotting was used to evaluate VEGF mRNA expression. No significant difference in VEGF mRNA half-life was found between young and old VSMCs.
buffer containing 10 mM Tris-HCl (pH 7.5), 4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, and 0.05 mg/ml poly(dI-dC)/poly(dI-dC). After 10 min of preincubation in ice, 20 µg of whole cell extracts were incubated for 30 min with 1 pmol (4 × 10⁴ cpm) of ³²P-labeled double-stranded oligonucleotide probe spanning the VEGF HIF-1 binding site (5'-TGCA(T/A)CTGGGCTCCAACAG-3'; HIF-1 site is underlined) (9). Unlabeled oligonucleotide was added to the preincubation mixture for competition assays (50-fold molar excess). Binding reactions were performed with a final volume of 15 µl (20 µg of cell extract), separated at 4 °C in non-denaturing 4% acrylamide gels in TBE-running buffer. Each condition was performed in duplicate. The gel was dried and exposed to Kodak XAR-5 films.

RESULTS

Age-dependent Deficit of VEGF Induction by Hypoxia—To study the effect of aging on VEGF expression in vitro, VSMCs that were isolated from young or old rabbits were exposed to normoxic or hypoxic conditions for 24 h, and the VEGF expression was evaluated at mRNA and protein levels. As shown in Fig. 1A, VEGF mRNA levels were low and similar among young and old VSMCs under normoxic conditions. VEGF levels were significantly increased by hypoxia in both groups. However, the magnitude of VEGF induction by hypoxia in old VSMCs (157% increase versus normoxia) was significantly lower than that of young VSMCs (273% increase versus normoxia). Similar findings were observed at the protein level. VEGF levels in the supernatant ELISA were low and similar among young and old VSMCs in normoxic conditions (Fig. 1B). Although VEGF values were significantly higher in both young and old VSMCs under hypoxic conditions, the magnitude of VEGF induction was significantly reduced in old versus young VSMCs (100 versus 213% induction by hypoxia). This finding was confirmed by Western blot experiments on cellular extracts from VSMCs, which were exposed to hypoxic conditions. As seen in Fig. 1C, VEGF levels were significantly reduced in old versus young hypoxic VSMCs. These results indicate that aging impairs VEGF induction under hypoxic conditions.

No Effect of Aging on VEGF mRNA Half-life under Hypoxic Conditions—Because VEGF expression is regulated not only at the transcriptional level but post-translationally as well (15), we investigated whether VEGF mRNA half-life under hypoxic conditions was reduced by aging. VSMCs were exposed to hypoxic conditions for 24 h before the addition of 5 µg/ml actinomycin D to block gene transcription. RNA isolation was performed at different time-points, and the VEGF mRNA half-life was determined after Northern blotting. As shown in Fig. 2, no significant difference in mRNA half-life was found between young and old VSMCs (2.4 versus 2.8 h, p = not significant), indicating that the age-dependent impairment of VEGF induction under hypoxic conditions is not because of a decrease in mRNA stability.

Reduction of VEGF Gene Transcription in Old VSMCs—To investigate whether the transcriptional regulation of VEGF is affected by aging, we transfected VSMCs that were isolated from young or old rabbits with a reporter construct containing the luciferase gene under the transcriptional control of the VEGF promoter. The cells were then exposed to hypoxic conditions for a 24-h period, and promoter activities were measured in young and old VSMCs (Fig. 3). VEGF promoter activity achieved under hypoxia in old VSMCs was almost half of that in young VSMCs (2.1 ± 0.3 versus 3.9 ± 0.1, p < 0.01). However, when a promoter construct lacking the HIF-1 binding site (Pst, Fig. 3), was transfected instead of the full-length reporter gene, VEGF promoter activity was not increased from hypoxia and no significant difference was found between the young and old VSMCs. This finding implicates HIF-1α in the age-dependent defect of VEGF induction by hypoxia.

Effect of Aging on HIF-1 Expression and Activity—Next, we evaluated HIF-1α expression at the mRNA and protein levels in young and old VSMCs (Fig. 4). HIF-1α mRNA was not up-regulated by hypoxia, and similar levels were found in young and old VSMCs under normoxic or hypoxic conditions (Fig. 4A). At the protein level, HIF-1α expression was barely detectable under normoxic conditions. Under hypoxic condi-
conditions at both the mRNA and protein levels. This indicates that aging is associated with a defect in VEGF up-regulation under hypoxic conditions.

Hypoxia-stimulated VEGF expression has been attributed to increases in both transcriptional and post-transcriptional mechanisms (15–22). Previous investigators have demonstrated the central role of hypoxia in transcriptional up-regulation of VEGF expression (14, 23). In this study, transfection experiments with a reporter gene under the control of the VEGF promoter clearly demonstrate that aging is associated with a reduction in hypoxia-induced VEGF transcriptional activity. Importantly, the age-dependent reduction in transcriptional activity was not seen when VSMCs were transfected with a reporter construct lacking the HIF-1 binding site.

HIF-1 is a transcription factor that is known to be involved in several physiological responses to hypoxia. These responses are mediated by specific genes that are transcriptionally induced by HIF-1, such as the genes for glycolytic enzymes (24), erythropoietin (25, 26), and VEGF (9, 25). HIF-1 has been shown to be essential for VEGF transcriptional activation, both in vitro (9) and in vivo (10). Using 5′-flanking sequences mediating transcriptional activation of reporter gene expression, for example, Forsythe et al. (9) showed that a 47-bp sequence located at 985 to 939 bp 5′ to the VEGF transcription initiation site was necessary for VEGF induction in hypoxic Hep3B cells. A HIF-1 binding site was demonstrated in the 47-bp response element, and a 5-bp substitution eliminated the ability of the element to bind HIF-1 and activate transcription in response to hypoxia. Moreover, the results of gene-targeting experiments have shown that HIF-1 null mice fail to up-regulate VEGF expression under hypoxic conditions, leading to a marked reduction in vascularization of the developing embryo and early lethality by embryonic day 10.5 (10). Collectively, these data confirm the essential role of HIF-1 in VEGF induction under hypoxic conditions.

In this study, we found that HIF-1 mRNA levels were not induced by hypoxia and were similar among young and old VSMCs. This finding is consistent with a previous study in which HIF-1α and HIF-1β mRNAs are shown to be constitutively expressed in HeLa and Hep3B cells with no significant induction by hypoxia. In fact, the activation of HIF-1 by hypoxia is shown to be primarily determined by the stabilization of HIF-1α protein (27). Our analysis of HIF-1α protein expression under hypoxic conditions suggested that the stabilization of HIF-1α was impaired with aging. The exact mechanisms involved in this age-dependent reduction of HIF-1α protein expression are unknown. HIF-1α protein has been shown to be rapidly degraded by the ubiquitin-protease system under normoxic conditions and stabilized by hypoxia through redox-in-
duced changes (28–30). Whether these mechanisms are affected by aging remains to be determined.

Increase in mRNA stability constitutes a second important control point for the hypoxic induction of VEGF in different cell lines (15, 19). Stabilization of VEGF mRNA by hypoxia is thought to be mediated by the binding of sequence-specific RNA-binding proteins (21, 22) to the sequences in both the 3′- and 5′-untranslated region of VEGF mRNA (18). Our findings indicated that VEGF mRNA half-life under hypoxic conditions was similar in VSMCs that were isolated from young and old animals. This finding suggests that the mechanisms involved in VEGF mRNA stabilization by hypoxia are not compromised with aging.

The results of EMSA in the current experiments indicated that DNA binding activity of HIF-1 under hypoxic conditions was significantly reduced in VSMCs that were isolated from old animals. Whether this was solely because of a reduction of HIF-1α protein expression is unclear. It is also possible that aging leads to a reduction in the ability of HIF-1 to bind to the hypoxia response element within the VEGF promoter, or that the ability of HIF-1α to form active heterodimers is reduced. Such post-translational loss of function has previously been described with aging for other proteins (31, 32) and transcription factors (33, 34).

The current findings implicate age-dependent defects in HIF-1 activity as being primarily responsible for reduced expression of VEGF and impaired angiogenesis associated with aging (1). HIF-1 is also involved in the induction of other genes, including Epo and glycolytic enzymes. Reduced expression of HIF-1 activity as being primarily responsible for reduced expression of VEGF and impaired angiogenesis associated with aging (1). HIF-1 is also involved in the induction of other genes, including Epo and glycolytic enzymes. Reduced expression of Epo has been observed in old animals (36) and in elderly patients (37, 38). The findings of this study thus suggest that an age-dependent defect in HIF-1 action could result in a reduction in the expression of genes that are directly involved in the physiological responses to hypoxia.

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