A Novel Role for Vascular Endothelial Growth Factor as an Autocrine Survival Factor for Embryonic Stem Cells during Hypoxia*

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Vascular endothelial growth factor (VEGF) is best known for its angiogenic activity on endothelial cells, but it also affects neurons, pneumocytes, and other mature cell types as well as endothelial, neural, and hematopoietic progenitors. Here, we examined its effect on pluripotent embryonic stem (ES) cells under hypoxic stress. ES cells were found to produce VEGF and to express VEGF receptor-2 and neuropilin-1 (Nrp-1), a VEGF165 isoform-specific receptor. During hypoxia, expression levels of VEGF, Flk-1, and Nrp-1 were elevated. Inhibition or targeted gene inactivation of VEGF increased ES cell apoptosis during prolonged hypoxia (48 h) by about 10-fold. The survival activity of VEGF was specific since inhibition of other growth factors (including basic fibroblast growth factor, epidermal growth factor, insulin-like growth factor, platelet-derived growth factor, and placental growth factor) had no effect. Neuropilin-1 was involved in the VEGF-survival activity since overexpression of Nrp-1 decreased hypoxia-induced apoptosis about 3-fold. The hypoxia-response element, via which hypoxia-inducible transcription factors up-regulate VEGF expression under hypoxic conditions, was critical since targeted deletion of this element in the VEGF promoter enhanced hypoxia-induced ES cell apoptosis to the same extent as VEGF inhibition or gene inactivation. Thus, VEGF plays a critical role in survival of ES cells during prolonged hypoxia.

Blood vessels are critical for delivery of oxygen to growing mammalian tissues. Certain tissues such as the cornea and cartilage in the adult remain avascular and receive oxygen from diffusion of nearby blood vessels, as vessels would impede transmission of light or cause bleeding of joints, respectively. During development, the oocyte in the ovarian follicle receives oxygen through diffusion from perifollicular blood vessels through the follicular fluid, whereas the oxygen needed for cellular metabolism and division of the preimplantation embryo is supplied by diffusion from the fluids within the oviduct and uterus, respectively. Development of the early pre-implantation embryo therefore occurs in an environment with reduced oxygenation until the onset of vascularization after blastocyst implantation (1, 2). The mechanisms responsible for the adaptation of the blastocyst to hypoxic damage remain poorly characterized.

A particular growth factor that plays an essential role in the cellular adaptation to hypoxia is vascular endothelial growth factor (VEGF),1 one of the most important prototype angiogenic factors in health and disease (3–5). It is alternatively transcribed in different VEGF isoforms, which bind to VEGF receptor-2 (also named fetal liver kinase-1 (Flk-1) or kinase insert domain-containing receptor (KDR)) and VEGF receptor-1 (also named fms-like tyrosine kinase-1 (Flt-1)) (6, 7). The VEGF165 isoform, which is sufficient for normal vascular development (8–10), binds to neuropilin-1 (Nrp-1), a receptor for the collapsin/semaphorin family expressed on neuronal, endothelial, and hematopoietic cells (11–13). VEGF is rapidly and significantly up-regulated in response to hypoxia via activation of the hypoxia-inducible transcription factors HIF-1α and HIF-2α, which bind to the hypoxia-response element (HRE) in the VEGF promoter (14), leading to angiogenesis and restoration of tissue oxygenation.

Recent studies also indicated that VEGF may protect cells against hypoxic damage by directly stimulating their survival, independently of angiogenesis. For instance, VEGF appeared to be critical to prevent motor neuron degeneration, not only because it was essential to maintain sufficient perfusion but also because it exerted direct trophic effects on neuronal survival (15–17). Because VEGF not only affects differentiated cell types but also adult precursors of several lineages including endothelial and neural progenitor cells, which express Flk-1, and hematopoietic stem cells, which express Flt-1 (18–20), we examined in the present study whether VEGF also affects embryonic stem (ES) cells, the equivalent of the pluripotent inner cell mass of blastocysts. Because ES cells constitute a potential renewable source of progenitors for tissue regeneration via cellular transplantation (21, 22), a better understanding of the mechanisms protecting such stem cells against environmental stress is warranted. Our findings indicate that ES cells during prolonged hypoxia produce elevated levels of VEGF.

1 The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; bFGF, basic fibroblast growth factor (FGF); EGF, epidermal growth factor; ES cell, embryonic stem cell; Flk-1, fetal liver kinase-1; Flt-1, fms-like tyrosine kinase-1; HIF, hypoxia-inducible factor; HRE, hypoxia-response element; IGF, insulin-like growth factor; LIF, leukemia inhibitory factor; NRP-1, neuropilin-1; PDGF, platelet-derived growth factor; PIGF, placenta growth factor; WT, wild type.
and its receptors Flk-1 and Nrp-1 via mechanisms involving the hypoxia-inducible transcription factors. Using genetic and pharmacological analyses, we uncover a novel role for VEGF as a survival factor for ES cells in hypoxia.

**EXPERIMENTAL PROCEDURES**

**Targeted ES Cell Clones and Materials**—ES cells deficient in VEGF (VEGF+/−), HIF-1α (HIF-1α−/−), or PIGF (PIGF+/−) were generated by homologous recombination and subsequent selection in high G418, as described previously (3, 23, 24). Mutation of the HIF-1α binding site in the VEGF promoter and generation of homozygous mutant (VEGF+/+) ES cells by selection in high G418 has been described in detail elsewhere (15). VEGF, basic fibroblast growth factor (bFGF), platelet-derived growth factor-BB (PDGF-B), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF) and PIGF, and monoclonal antibodies against VEGF, bFGF, PDGF-B, IGF-1, EGF, or PIGF were purchased from R&D Systems (Abingdon, UK). Leukemia inhibitory factor (LIF; EGFRO) was from Chemicon International (Temecula, CA). Culture media and reagents were from Invitrogen, HyClone (Logan, UT), and Roche Applied Science.

**Hypoxia Treatment of ES Cells**—Undifferentiated ES cells were cultured in 75-cm² flasks or 10-cm culture dishes on mitomycin C-treated embryonic fibroblasts in Dulbecco’s modified Eagle’s medium containing 15% heat-inactivated fetal calf serum, 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mm glutamine, and 20 ng/ml LIF. ES cells were detached with 0.05% trypsin and 0.02% EDTA and seeded at low cell density (10,000 cells/35-mm Petri dish, precoated for at least 24 h with 0.2% gelatin) in culture medium supplemented with 20 ng/ml LIF and 5% fetal calf serum. After 24 h ES cells were rinsed with phosphate-buffered saline, and fresh culture medium supplemented with 5% fetal calf serum but without LIF was added. Experiments were performed in an incubator at 37 °C under normoxia (N) by maintaining the cells in 95% air and 5% CO₂ but without LIF was added. Experiments were performed in an incubator at 37 °C under normoxia (N) by maintaining the cells in 95% air and 5% CO₂ or under hypoxia (H) to incubating cells in a humidified sealed chamber gassed with 5% O₂, 5% CO₂, and 94.5% N₂ (AGA, Lille, France) at a flow rate of 30 liters/h for 24 to 48 h. After incubation in normoxia or hypoxia for 24 or 48 h, ES cells were analyzed for expression of VEGF, Flk-1, and Nrp-1, or used for Northern blot analysis as described below. Growth factors and antibodies were added to the culture medium as indicated.

**Measurement of Apoptosis**—Apoptosis of ES cells was measured every 12 h during a 24- or 48-h (prolonged) incubation with a photometric enzyme immunoassay (cell death enzyme-linked immunosorbent assay, Roche Applied Science) for quantitation of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) (23). First, ES cells grown under normoxia or hypoxia as described above were rinsed with phosphate-buffered saline to remove the floating dead cells. Subsequently, ES cells were detached and assayed for apoptosis, which was expressed as the average number of oligonucleosomes per 10⁶ cells. Terminal deoxynucleotidyltransferase-mediated deoxyuridine nick ending-labeling of cultured ES cells was performed using a commercially available kit according to the manufacturer’s instructions (Trevigen, Gaithersburg, MD).

**Expression of VEGF, PIGF, and Their Receptors**—VEGF in ES cell-conditioned medium was measured at the indicated times by enzyme-linked immunosorbent assay (R&D). For Northern blot analysis, total RNA isolated from 5 × 10⁶ cells cultured for 24 h was prepared and hybridized as described previously (3, 23). Hybridization probes were used for VEGF (a 655-bp BamHI-EcoRI fragment encompassing the entire open reading frame of the murine VEGF188 isoform cDNA), for PIGF (a 1391-bp NotI-XhoI fragment, encompassing the entire open reading frame), for VEGF receptor-1 (VEGFR-1 or Flt-1) (a PCR fragment encompassing nucleotides 421–1403 of the murine VEGFR-1 cDNA), for VEGF receptor-2 (VEGFR-2 or Flk-1) (a PCR fragment encompassing nucleotides 3361–4440 of the murine VEGFR-2 cDNA), and for neuropilin-1 (Nrp-1) (a PCR fragment encompassing nucleotides 358–761 of the murine Nrp-1 cDNA).

**Neuropilin-1 Overexpression in WT ES Cells**—WT ES cells were electroporated with a plasmid bearing the human neuropilin-1 (NRP-1) gene under control of a cytomegalovirus promoter and the hygromycin phosphotransferase gene as a selection marker. WT ES cells transfected with a control plasmid only bearing the selection marker were used as controls. Overexpression of NRP-1 was quantified by reverse transcription-PCR as the number of mRNA copies per 100 mRNA copies of hygaxanthine guanine phosphoribosyltransferase. Total RNA was extracted from ES cells using TRIZOL reagent (Invitrogen) and reverse- transcribed with Superscript II (Invitrogen). Quantitative real time reverse transcription-PCR was performed according to the manufacturer’s protocol (PerkinElmer Life Sciences) using the following forward and reverse primer and probe (P), labeled with fluorescent dye (FAM or JOE) and quencher (TAMRA) as described previously (8): for NRP-1 forward, 5′-TGGAAAGGGCCCTACATA-3′; reverse, 5′-GGCTTGCTGCTCATCAGCT-3′; probe, 5′-FAM-CCGACCACTCACAACGGGAGATCGT-TAMRA-3′; for hygaxanthine guanine phosphoribosyltransferase forward, 5′-TTTATGACTGGAAGCTGACTGTAAATGCT-3′; reverse, 5′-TACCAGCTGAAATTATATATCCAAATCT-3′; probe, 5′-JOE-TGAAGATCATC-TCCACAAATTTTGTGCCTC-3′.

**Statistics**—All data are expressed as the mean ± S.D. Comparisons of values were made using a non-parametric Mann-Whitney U test. Significance was defined as two-sided p < 0.05.

**RESULTS**

**Expression of VEGF and Its Receptors by ES Cells and Up-regulation by Hypoxia**—Undifferentiated wild type (WT) ES cells were found to produce detectable amounts of VEGF in normoxia, which were up-regulated when the cells were cultured in hypoxia (Table I). ES cells also produced the VEGF homologue PlGF, but its expression was not inducible by hypoxia (Fig. 1A). Hypoxic induction of VEGF was mediated via binding of HIF-1α to the HRE as both HIF-1α−/− ES cells and ES cells with a deletion of the HRE in the VEGF promoter (VEGF−/− ES cells) were unable to up-regulate VEGF in response to hypoxia (Table I; Fig. 1B and C).

The survival function of VEGF for endothelial cells is known to be mediated via VEGF receptor type-2 (VEGFR-2; also termed Flk-1 or kinase insert domain-containing receptor) (25, 26). Northern blot analysis revealed that ES cells expressed VEGFR-2 and that expression of VEGFR-2 in WT ES cells was increased in response to hypoxia (Fig. 1A). Hypoxic induction of VEGFR-2 in WT ES cells was HIF-1α-dependent, since similar expression levels were detected in HIF-1α−/− ES cells in normoxia and hypoxia (Fig. 1A). In contrast, hypoxic induction of VEGFR-1 (also termed Flt-1), was retained in HIF-1α−/− ES cells, whereas expression of PIGF, a Flt-1-specific ligand, was only slightly induced by hypoxia (Fig. 1A). These data may reflect the differential roles of Flk-1 and Flt-1.

Nrp-1, a selective receptor for the VEGF_165 isoform is known to assist Flk-1 as a co-receptor (13). Therefore, the expression and potential role of Nrp-1 in ES cell survival was studied. WT ES cells expressed Nrp-1 in normoxia, and expression of Nrp-1 was increased in response to hypoxia (Fig. 1A). Hypoxic induction of Nrp-1 was blocked in HIF-1α−/− ES cells, indicating that HIF-1α was involved (Fig. 1A).
ES Cell Apoptosis during Prolonged Hypoxia—To study whether VEGF affects survival of ES cells, we first optimized the experimental conditions to induce ES cell apoptosis. To quantify apoptosis the number of oligonucleosomes per 10^5 adherent cells was determined or the number of terminal deoxynucleotidyl transferase-mediated deoxyuridine nick end-labeling-positive cells was counted, yielding overall comparable results. ES cell apoptosis was found to be dependent on the cellular differentiation and on the duration and degree of hypoxia. 

When cultured in the presence of LIF, ES cells remained undifferentiated, as determined by their high nucleus-to-cytosol ratio and typical growth in small cell clumps. In contrast, when cultured for three subsequent passages without LIF, ES cells grew as monolayers of differentiated cells with flattened fibroblast-like appearance and a high cytosol-to-nucleus ratio. When undifferentiated WT ES cells were cultured for 24 h in progressively lower O_2 concentrations, an increasing fraction of WT ES cells became apoptotic (Fig. 2A). In contrast, differentiated WT ES cells without LIF became resistant to apoptosis when cultured under low (0.5%) oxygen conditions for 24 h (not shown). After normoxia for 24 h, the number of oligonucleosomes per 10^5 cells was low (41 ± 5 with LIF and 31 ± 2 without LIF). When ES cells were cultured for 24 h at 0.5% O_2, the number of oligonucleosomes per 10^5 cells was 340 ± 23 with LIF versus 52 ± 5 without LIF (p < 0.05 versus with LIF).

Interestingly, ES cell apoptosis was also dependent on the duration of the hypoxic stress. Based on the finding that a fraction of undifferentiated WT ES cells became apoptotic when exposed to 0.5% O_2 for 24 h, we had anticipated that longer periods of hypoxia would even further aggravate ES cell apoptosis. Surprisingly, however, the residual ES cells, which were still viable after 24 h of hypoxia, became resistant to apoptosis when stressed for another 24 h of hypoxia (i.e. from 24 to 48 h; phase B; Fig. 3A, solid line). This was not due to ES cell differentiation, as the colony morphology typical for undifferentiated cultures was retained. These data, therefore, indicate that a survival mechanism rescued the residual ES cells during prolonged hypoxia.

Role of VEGF in ES Cell Survival during Prolonged Hypoxia; Pharmacological Evidence—Because VEGF functions as a survival factor for various cell types and is strongly induced by hypoxia in ES cells, we examined whether survival of WT ES cells under prolonged hypoxia was related to the hypoxic induction of VEGF. Supplementation of various neutralizing antibodies to the culture medium of WT ES cells, which were grown under hypoxia for 48 h, revealed that only anti-VEGF antibodies prevented survival of ES cells and maintained apoptosis during prolonged hypoxia (i.e. between 24 and 48 h) (Fig. 2B and Fig. 3A, dotted line). In contrast, antibodies against bFGF, PDGF-BB, EGF, IGF-1, or PlGF did not affect apoptosis (Fig. 2B), indicating that the survival activity was mediated by VEGF. Furthermore, apoptosis of WT ES cells cultured under hypoxia for 24 h was prevented by supplementation of culture medium, conditioned by WT ES cells under hypoxia for 24 h or for 48 h (Fig. 2, C and D), indicating that the survival factor VEGF was secreted in the medium at significant amounts already after 24 h of hypoxia. Accordingly, only anti-VEGF antibodies inhibited the survival activity present in culture medium, conditioned by WT ES cells under hypoxia for 24 or 48 h (Fig. 2, C–D).

Moreover, recombinant VEGF165 inhibited apoptosis of WT ES cells both during short term (<24 h) and prolonged (>24 h) hypoxia, indicating that the VEGF-mediated survival signals overruled the death pathway in response to acute hypoxia (Figs. 3A, gray line and 2E). In contrast, the addition of recombinant PDGF-BB, EGF, IGF-1, or PlGF (Fig. 2E) was ineffective. Recombinant bFGF also rendered ES cells refractory to hypoxia-induced apoptosis, similarly as observed for VEGF (Fig. 2E). However, antibodies against bFGF did not affect survival of ES cells cultured under hypoxic conditions for 24 h with culture medium conditioned by ES cells under hypoxia for 24 h (Fig. 2C), whereas these antibodies also did not inhibit survival of ES cells cultured under hypoxia with medium conditioned by ES cells under hypoxia for 48 h (Fig. 2D). Therefore, endogenous bFGF is unlikely to contribute to the survival of ES cells during short term (<24 h) or during prolonged hypoxia (>24 h). Conversely, an immunoneutralizing antibody to VEGF maintained susceptibility of WT ES cells to apoptosis beyond 24 h of hypoxia (Fig. 3A; dotted line). These findings together with the observation that WT ES cells became refractory to apoptosis after 24 h of hypoxia suggested that VEGF was only produced at significant levels beyond 24 h. VEGF was indeed secreted at high levels by WT ES cells beyond 24 h and remained highly expressed during 48 h (Table I). Consistent with its inability to affect ES cell apoptosis during hypoxia, PlGF expression was not induced by hypoxia (Fig. 1A).

Role of VEGF in ES Cell Survival during Prolonged Hypoxia; Genetic Evidence—To extend the VEGF immunoneutralization data, apoptosis was studied in ES cells lacking VEGF (VEGF−/−). VEGF−/− ES cells became apoptotic in response to short term hypoxia (<24 h), but in contrast to WT ES cells VEGF−/− ES cells remained susceptible to apoptosis up to 48 h of hypoxia, most likely because they did not produce the survival factor VEGF (Fig. 3B). The survival activity of VEGF was specific, as PlGF-deficient (PlGF−/−) ES cells became apoptotic in response to 24 h of hypoxia, but like WT ES cells PlGF−/− ES cells became refractory to hypoxia-induced apoptosis upon prolonged hypoxia (48 h) (Fig. 3C). The lack of an effect of PlGF on apoptosis in WT cells was not due to absent PlGF expression (Fig. 1A).

Genetic Evidence for a HIF-1α → HRE → VEGF Survival Pathway during Prolonged Hypoxia—HIF-1α−/− ES cells were unable to up-regulate VEGF in response to hypoxia, consistent
with the observation that hypoxic induction of VEGF expression is mediated by HIF-1α, more particularly via binding of HIF-1α to a specific HRE in the VEGF promoter (27). ES cells in which the HRE in the VEGF promoter was mutated by gene targeting (VEGF−/−) could indeed not up-regulate VEGF expression in response to hypoxia (15), similarly to the lack of hypoxic up-regulation of VEGF in HIF-1α−/− ES cells (Table I; Fig. 1B). VEGF+/+ ES cells became apoptotic during the first 24 h of hypoxia, and like VEGF−/− ES cells, remained sensitive to apoptosis during prolonged hypoxia (up to 48 h; Fig. 3D).

Role of Neuropilin-1 in Survival of ES Cells during Hypoxic Stress—To study whether Nrp-1 plays a role in cell survival, WT ES cells were stably transfected with a plasmid containing the human NRP-1 gene (NRP-1+ ES cells), resulting in 30-fold higher NRP-1 expression levels than in WT ES cells transfected with a control plasmid (mRNA copies per 100 mRNA copies of hypoxanthine guanine phosphoribosyltransferase: 0.86 ± 0.03 for WT ES cells versus 27 ± 6 for NRP-1+ ES cells, p < 0.05 by Mann-Whitney U test, n = 6). Because we anticipated that NRP-1 would enhance the VEGF-dependent survival effect, apoptosis of WT and NRP-1+ ES cells was studied at 24 h of hypoxia, i.e. at a time when VEGF levels were half-maximally elevated (Table I). NRP-1 expression levels determined the survival response of WT ES cells in hypoxia, since ES cells overexpressing human NRP-1 were more resistant to hypoxic stress than mock-transfected clones. The oligonucleosomes per 10^5 ES cells after 24 h were 35 ± 4 for WT cells versus 40 ± 2 for NRP-1+ ES cells in normoxia (p = not significant) and 340 ± 21 for WT cells versus 110 ± 9 for NRP-1+ ES cells in hypoxia (p < 0.05; n = 6).

**DISCUSSION**

Cells deprived of oxygen are at risk for irreversible damage and even death. In many cases hypoxic cells will up-regulate angiogenic factors that stimulate the formation of new blood vessels to restore oxygenation, but alternative mechanisms may secure the viability of the ischemic cells before onset of angiogenesis, a process that requires at least several days. In this study we provide evidence that the angiogenic factor VEGF contributes to the survival of ES cells independently of angiogenesis, likely by directly activating VEGFR-2 on ES cells. Such a dual survival activity of VEGF, via a direct trophic effect on the target cell and via an indirect effect on blood vessels, has been documented for motor neurons (15–17). For ES cells, which have to survive the hypoxic environment in the oviduct and uterus, VEGF may not only enable the implanting...
Fig. 3. ES cell apoptosis and survival during short (24 h) and long term (48 h) hypoxia. Apoptosis is expressed as mean numbers of oligonucleosomes/10° cells ± S.D. (n = 6). A, WT ES cells exhibit apoptosis up to 24 h of hypoxia (0.5% O₂) but subsequently become refractory and survive prolonged hypoxia (up to 48 h; solid line). Supplementation of VEGF (50 nM) suppresses apoptosis (gray line), whereas ES cells remain sensitive to apoptosis during prolonged hypoxia in the presence of a monoclonal anti-VEGF antibody (100 μg/ml; dotted line). B-D, apoptosis of VEGF−/− cells (B), PIGF−/− cells (C), and VEGF+ cells (D) (dotted lines) with their respective WT control cells (solid lines) in response to normoxia (N) or hypoxia (H, 0.5% O₂). Loss of VEGF expression (VEGF−/− cells; B) or of hypoxic induction of VEGF expression (VEGF+ cells; D) causes ES cells to remain sensitive to apoptosis during prolonged hypoxia.

Embryo to immediately induce angiogenesis at the implantation site (28) but may also contribute to securing proper development before onset of vascularization after implantation of the embryo in the uterine wall. Blastocyst-stage embryos are particularly vulnerable to hypoxic damage before they implant in the vascularized uterine tissue, and early hypoxia was shown to have a devastating impact on implantation and embryonic development in mice and rats (1, 29, 30). This is indeed a critical stage of development as the pluripotent undifferentiated embryonic stem cells, from which all lineages subsequently differentiate, develop in the inner cell mass in the blastocyst just before implantation. Survival of ES cells not only is of utmost importance for normal development in vivo but also for safe manipulation and proper differentiation in vitro. Examples of applications involving the handling of ES cells and preimplantation embryos in stressful conditions include in vitro fertilization procedures, morula and blastocyst manipulation for transgenesis in experimental animal models, and in vitro embryo culture. ES cells also constitute a potential renewable source of more differentiated progenitors for tissue regeneration via cellular transplantation (21, 22). Surprisingly, however, little is known about the mechanisms regulating survival of ES cells and preimplantation embryos in stressful conditions such as hypoxia.

We previously demonstrated that ES cells undergo apoptosis in response to short term hypoxia for less than 24 h (23). Here, we provide novel findings that this cell death pathway is overruled by a survival pathway during prolonged hypoxia (48 h) and that this involves HIF-1α-dependent up-regulation of VEGF which, in an autocrine action involving VEGFR-2 and Nrp-1, protects against apoptosis.

VEGF, the prototypic angiogenic factor, is a survival factor for ES cells, as supplementation of exogenous VEGF₆₅ prevents apoptosis in response to both short (<24 h) and long term hypoxia (>24 h). In addition, apoptosis was sustained during prolonged hypoxia if (i) when ES cells were treated with anti-VEGF antibodies, (ii) when ES cells lacked VEGF (VEGF−/−), or (iii) when ES cells were unable to up-regulate VEGF during hypoxia as a result of targeted mutation of the HIF-1α binding site (VEGF−/−). The survival effect was specific for VEGF as ES cells lacking PIGF (PIGF−/−) or WT ES cells treated with antibodies against bFGF, PDGF-BB, EGF, IGF-1, or PIGF survived prolonged hypoxia. Interestingly, supplementation of recombinant bFGF also rescued WT ES cells from hypoxia-induced apoptosis. However, several studies have demonstrated that expression of VEGF but not of bFGF is enhanced during hypoxia in various cell types and tissues (31–34). Furthermore, we observed that the addition of antibodies against bFGF did not increase apoptosis of ES cells during short term hypoxia or during prolonged hypoxia. Therefore, it may be concluded that endogenous bFGF most likely does not contribute to the survival response of ES cells during hypoxic stress. Taken together our data clearly demonstrate an important role for VEGF as a survival factor for ES cells during hypoxic stress.

Several growth factors and cytokines previously have been demonstrated to exhibit similar protective effects against hypoxia-induced apoptosis for other cell types but not yet for ES cells. Indeed, a survival activity has been documented for bFGF, PDGF-B, VEGF, nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3 and erythropoietin for hypoxic neurons (15, 35–41), acidic fibroblast growth factor, IGF-I and erythropoietin for hypoxic myocardial cells (42–46), VEGF for hypoxic chondrocytes (47), or hepatocyte growth factor and erythropoietin for hypoxic renal cells (48, 49). Growth factors may also protect cells against apoptosis in response to other stimuli, as exemplified by the ability of erythropoietin, c-kit ligand, or interleukin-3 to block p53-mediated apoptosis of hematopoietic cell lines (50–52) by the protective effect of IGF-I or EGF on epithelial cells or lymphocytes against Fas-induced apoptosis (53–55) or by the inhibition by IGF-1 of glutamate-induced apoptosis of oligodendrocyte progenitors (56). In addition, IGF-1 and PDGF inhibit apoptosis of fibroblasts during serum starvation (57), whereas VEGF protects HepG2 and endothelial cells against apoptosis induced by TNF-α or serum deprivation (25, 58). Furthermore, chronic hypoxia in the lung
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not only protects smooth muscle cells from apoptosis but can even stimulate these cells to replicate possibly via induction by PDGF-B and endothelin-1, which are secreted by endothelial cells (59, 60).

VEGFR-2 has been documented on hemangioblasts, smooth muscle cells, placental trophoblasts, pulmonary epithelial cells, Schwann cells, non-endothelial embryonic cells, and motor neurons (61, 62). Its expression was also documented in undifferentiated ES cells (63–66). Our present findings indicate that VEGFR-2 is expressed in ES cells and that its expression is induced by hypoxia via a HIF-1α-dependent pathway. Because VEGF induces survival signals in endothelial cells via activation of VEGF-2 (25), VEGFR-2 is a likely candidate to mediate the survival function of VEGF in ES cells. VEGF-1 is less likely to be implicated in survival of ES cells since its expression is only minimally regulated by HIF-1α and its ligand PIGF failed to protect ES cells against apoptosis.

Nrp-1 has been implicated in endothelial migration as a co-receptor for VEGFR-2, and accumulating data suggest its possible role in VEGF-dependent survival in adult cells including endothelial, neuronal, and tumor cells (11, 15, 67–69). However, involvement of Nrp-1 in survival of ES cells had not been documented yet. Our findings revealed that overexpression of human Nrp-1 in WT ES cells decreased apoptosis in response to hypoxia, which up-regulates VEGF (see Fig. 1A and Table I). Because survival during prolonged hypoxia depended on VEGF, NRP-1 likely enhanced the VEGF-survival effect as a co-receptor of VEGFR-2. Overexpression of NRP-1 may have enabled ES cells to respond to lower levels of VEGF, which were insufficient to protect WT ES cells against apoptosis during initial hypoxia. Because neuropilin-1 only has a short intracellular domain and cannot transduce intracellular signals by itself, it has been suggested that complex formation of NRP-1 with a co-receptor is necessary for signal transduction (12, 70). The VEGF-dependent survival signaling is assumed to be mediated via the VEGFR-2; however, NRP-1 may also mediate a VEGF survival response independent of VEGFR-2, since VEGF was able to induce survival of NRP-1-expressing tumor cells and podocytes that did not express VEGFR-2 (71, 72).

In conclusion, our results show that hypoxic stress in ES cells is characterized by HIF-1α-dependent up-regulation of VEGF and its receptors VEGFR-2 and Nrp-1 and reveal a beneficial effect of Nrp-1 on the survival response of ES cells in hypoxia and a novel role for VEGF as an autocrine survival factor for ES cells during prolonged hypoxic conditions.

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