Vascular endothelial growth factor promotes cardiomyocyte differentiation of embryonic stem cells

Yu Chen,1,2,* Ivo Amende,1,* Thomas G. Hampton,1 Yinke Yang,1 Qingen Ke,1 Jiang-Yong Min,1 Yong-Fu Xiao,1 and James P. Morgan1,3

1Cardiovascular Division, Department of Medicine, the Charles A. Dana Research Institute and Harvard-Thorndike Laboratories, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts; 2Cell Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York; and 3Division of Cardiovascular Medicine, Cartas St. Elizabeth’s Medical Center, Boston, Massachusetts

Submitted 12 April 2005; accepted in final form 3 May 2006

We have previously reported that embryonic stem cells (ESCs) overexpressing VEGF cDNA significantly improved cardiac function in mice with myocardial infarction (25). However, the role of VEGF in cardiomyocyte differentiation of ESCs has not yet been clarified. Here, we studied the effects of VEGF on cardiomyocyte differentiation of mouse ESCs in vitro. To validate the cardiac-specific differentiation of the ESCs, we quantified the following specific cardiac proteins: α-myosin heavy chain (α-MHC), troponin I (cTn-I), and Nkx2.5 in differentiated ESCs. VEGF (20 ng/ml) significantly enhanced α-MHC, cTn-I, and Nkx2.5 expression in differentiated ESCs. Western blot analysis confirmed these findings. We found that VEGF receptor FMS-like tyrosine kinase-1 (Flk-1) and fetal liver kinase-1 (Flt-1) expression increased during ESC differentiation. Antibodies against Flk-1 totally blocked and against Flt-1 partially blocked VEGF-induced Nkx2.5-positive-stained cells. The ERK inhibitor PD-098059 abolished VEGF-induced cardiomyocyte differentiation of ESCs. Our results suggest that VEGF promotes cardiomyocyte differentiation predominantly by ERK-mediated Flk-1 activation and, to a lesser extent, by Flt-1 activation. These findings may be of significance for stem cell and growth factor therapies to regenerate failing cardiomyocytes.

MATERIALS AND METHODS

The mouse ESC line ES-D3 was obtained from American Type Culture Collection. Cells were maintained in knockout Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) with 15% fetal bovine serum (HyClone) at 37°C in a humidified atmosphere containing 5% CO2-95% O2. ESCs spontaneously differentiated into cardiomyocytes without leukemia inhibitory factor (LIF) (15, 25). LIF (1,000 U/ml) was added to the medium, therefore, to maintain self-renewal and pluripotency of ESCs.

Stem cell differentiation into cardiomyocytes. We used the “hanging drop” method to determine the effect of VEGF on ESC differentiation, as previously described (25). In brief, ESCs were dissociated by 0.25% trypsin and resuspended in knockout DMEM with 20% FBS in 0.25% trypsin and resuspended in knockout DMEM with 20% FBS. Cells were then placed on the underside of tissue culture dishes for 2 days to form cell aggregates. Cell aggregates were then transferred to bectrophotometric microscopes for 5 days to form embryoid bodies (EBs). The EBs were further cultured for 4 days on gelatin-coated dishes. Mouse recombinant VEGF(165) (Alpha Diagnostic International) at a concentration of 1 or 20 ng/ml was added to the culture medium on day 0 of ESC differentiation.

We also determined cardiomyocyte differentiation in ESCs overexpressing VEGF cDNA (25). VEGF cDNA (pHVEGF165) was a generous gift from Dr. Kenneth Walsh (St. Elizabeth’s Medical Center, Tufts University School of Medicine). Figure 1A depicts an ESC-derived cardiomyocyte, including multinucleation and striations.

* Y. Chen and I. Amende contributed equally to this study.

Address for reprint requests and other correspondence: J. P. Morgan, Harvard Medical School, Boston, MA 02215 (E-mail: james.morgan@caritaschristi.org).

http://www.ajpheart.org

0363-6135/06 $8.00 Copyright © 2006 the American Physiological Society

H1653
We determined the whole number of cells on day 0, day 3, day 6, and day 11 of differentiation in control EBs, EBs treated with 1 ng/ml VEGF, EBs treated with 20 ng/ml VEGF, and EBs treated with VEGF cDNA. EBs were dissociated into single cells by using PBS containing 15% FBS, 0.25% collagenase D (Boehringer Mannheim), and 0.25% collagenase XI (Sigma) (n = 6 for each group). The total cell number in each EB was then counted by using a hemocytometer.

Cell apoptosis was analyzed in ~10,000 cells on day 11 of differentiation by annexin V staining (BD Bioscience) (n = 3 for each group).

Flow cytometry to quantify cardiomyocyte differentiation. On day 11 of ESC differentiation, EBs were dissociated into single cells using PBS containing 15% FBS, 0.25% collagenase D (Boehringer Mannheim), and 0.25% collagenase XI (Sigma). The dissociated cells were then permeabilized with 70% ethanol for 30 min. Antibodies (2 μg/ml) against α-myosin heavy chain (α-MHC, goat, Berkeley Antibody), cTn-I, and Nkx2.5 (rabbit, Santa Cruz Biotechnology) were incubated with the permeabilized cells at 37°C for 1 h. The cells were then incubated with fluorescence-conjugated secondary antibodies for 1 h. The cells were analyzed by using flow cytometry (Becton Dickinson). Gates were established by nonspecific immunoglobulin binding in each experiment.

Western blot analysis to determine cardiac-specific proteins. We determined cardiac-specific protein expression in differentiated ESCs. The cells were harvested in RIPA buffer (Boston Bioproducts) on day 11 of differentiation. Sixty micrograms of protein in an equal volume of loading buffer (Boston Bioproducts) was boiled and separated by 4–15% SDS-PAGE ready gel and transferred to a polyvinylidene difluoride membrane (BioRad) for staining. The nonspecific binding sites of protein were blocked in PBS containing 1% BSA, 1.5% nonfat dry milk, 1% horse serum, and 0.1% Tween-20 for 1 h. The membrane was then incubated at 4°C for 16 h with primary antibodies against α-MHC, cTn-I, and Nkx2.5 (dilution 1:1,000). The antibody-positive proteins were conjugated with horseradish peroxidase (HRP)-linked secondary antibodies (dilution 1:2,000) and visualized with enhanced chemiluminescence luminol reagent (ECL) (Santa Cruz Biotechnology). To ensure a similar amount of protein in each sample, the polyvinylidene difluoride membranes were “stripped off,” re-probed with glyceraldehyde-3-phosphate dehydrogenase, developed with HRP-conjugated secondary antibodies, and visualized by ECL. Protein expression was quantified by densitometry.

Analyses of VEGF, VEGF receptors, and mitogen-activated protein kinases. Expression of VEGF receptors Flt-1 and Flk-1 in undifferentiated and differentiated ESCs was determined by using Western blot analysis. ESCs cultured in knockout DMEM with 15% FBS without LIF were harvested in RIPA buffer on day 0 (undifferentiated) and days 3, 6, and 11. Sixty micrograms of protein was used for Western blot analysis. Antibodies against Flt-1 (goat, polyclonal, Oncogene Research Products), Flk-1 (mouse, monoclonal, Calbiochem-Novabiochem), ERK (rabbit, polyclonal, Cell Signaling Technology), and JNK (rabbit, monoclonal, Cell Signaling Technology) were used as primary antibodies (dilution 1:500).

To determine if VEGF receptors Flk-1 and Flt-1 are required for VEGF-promoted ESC differentiation into cardiomyocytes, we added neutralizing antibodies against Flk-1 (0.3 μg/ml, anti-Flk-1) and against Flt-1 (8 μg/ml, anti-Flt-1) (R&D Systems) together with 20 ng/ml VEGF to the ESC culture medium at day 0 of ESC differentiation. The Nkx2.5-positive-stained cells were determined by flow cytometry on day 11 of differentiation. We examined expression of Flk-1 and CD31, early endothelial cell markers, in control and VEGF-treated EBs. Expression of Flk-1 and CD31 was determined by flow cytometry.

Immunoprecipitation of phosphorylated VEGF receptors and mitogen-activated protein kinases. ESCs were incubated with VEGF (20 ng/ml) for 15 min. Cells were harvested in a lysis buffer containing 1 mM benzamidine, 1 mM dithiothreitol, 10% glycerol, 80 mM glycerophosphate, 0.5 mM EDTA, 5 mM EGTA, 20 mM MOPS, 50 mM NaF, 5 mM Na3PO4, 1 mM Na2VO3, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100, at pH 7.0. The samples were vortexed and centrifuged at 12,000 g for 10 min at 4°C. The clarified supernatant was pre-cleared with protein A/G-Sepharose (Oncogene Research Products) for 1 h at 4°C. The pre-cleared samples were incubated with phosphotyrosine mAbs p-Thr-100 (Cell Signaling Technology) for 16 h at 4°C. The immune complex was analyzed by Western blot. Antibodies against phospho-Flk-1, phospho-Flt-1 (Santa Cruz Biotechnology), phospho-ERK, and phospho-JNK (Cell Signaling Technology) were used as primary antibodies.

We further determined if activation of ERK or JNK pathways were essential for VEGF-induced cardiomyocyte differentiation. ESCs were pre-incubated with PD-098059, the inhibitor for ERK (10 μM, Sigma), or SP-600125, the inhibitor for JNK (10 μM, Cell Signaling Technology) for 10 min at 4°C. Flow cytometry was performed as described above.
VEGF enhanced cardiomyocyte differentiation of ESCs. VEGF (1 ng/ml), VEGF (20 ng/ml) and VEGF cDNA significantly increased α-MHC, cTn-I, and Nkx2.5-positive-stained cells: 35 ± 2%, 46 ± 3%, and 39 ± 3% for α-MHC compared with 26 ± 1% (controls); 27 ± 2%, 33 ± 1%, and 34 ± 3% for cTn-I compared with 21 ± 2% (controls); and 34 ± 3%, 46 ± 5%, and 45 ± 4% for Nkx2.5 compared with 26 ± 1% (controls) (P < 0.05, n = 6) (Fig. 1).

We determined if VEGF altered the whole number of cells during differentiation. The number of cells increased from day 0 to day 11 of differentiation in control EBs, EBs treated with 1 ng/ml VEGF, EBs treated with 20 ng/ml VEGF, and EBs treated with VEGF cDNA. The number of cells did not signifi-

analyzed ~10,000 cells using flow cytometry and determined the percentage of apoptotic cells. Cell proliferation was determined in PD-098059-pretreated cells on day 11 in differentiated EBs by trypsinizing 20 EBs and counting the number of cells (n = 3 for apoptosis and n = 6 for proliferation).

Immunohistochemistry. We used immunohistochemistry staining to determine NKx2.5, one of the cardiac-specific transcription factors, in ESC-derived cardiomyocytes on day 11 of differentiation. Briefly, the cells were fixed in 4% paraformaldehyde for 10 min on day 11 of differentiation. The cells were then boiled in 10 mM sodium citrate buffer (pH 6.0) for 1 min and cooled down to unmask the antigen. The cells were further incubated in 1% H2O2 for 10 min at room temperature to block endogenous peroxidase. An antibody against NKx2.5 (rabbit, diluted 1:200, Santa Cruz Biotechnology) was incubated with the cells at 4°C for 16 h. The immunological reaction was obtained by using Vectastain Elite ABC reagent (Vector Laboratories) and visualized in a solution containing diaminobenzidine, which produced a yellow-brown color of antibody-positive cells. Nuclei were stained using hematoxylin. Wide-field images were obtained with either a ×5 or ×40 objective microscope lens (model E-400, Nikon) equipped with a back-illuminated CCD camera (model Y-FL, Nikon) and processed with Spot 4.0 software (Diagnostic Instruments).

Data analysis. Values are presented as means ± SD. Results between two individual groups were compared by the unpaired Student’s t-test. Data from more than two experimental groups were statistically compared by one-way ANOVA. Differences were considered significant with P < 0.05.
protein phosphorylation of Flk-1 and Flt-1 in ESCs by using immunoprecipitation. VEGF activated Flk-1 and Flt-1 in undifferentiated and differentiated ESCs (n = 3) (Fig. 3).

We used antibodies against Flk-1 and Flt-1. Anti-Flk-1 antibody significantly blocked VEGF-induced NKx2.5-positive-stained cells from 47 ± 3% to 7 ± 2% determined by flow cytometry to VEGF (P < 0.05, n = 3). Anti-Flt-1 antibody partially blocked VEGF-induced NKx2.5-positive-stained cells (42 ± 3%) on day 11 of differentiation (n = 3; NS) (Fig. 4).

We determined expression of the endothelial cell markers, Flk-1 and CD31 (26). We found that VEGF (20 ng/ml) significantly increased expression of Flk-1 in differentiated ESCs on days 3 and 6 and slightly decreased expression of Flk-1 in day 11: 8 ± 1% on day 3 compared with 4 ± 1% (controls); 30 ± 3% on day 6 compared with 16 ± 1% (controls); and 23 ± 2% on day 11 compared with 12 ± 2% (controls) (P < 0.05 for days 3, 6, and 11, n = 3). VEGF (20 ng/ml) only slightly increased expression of CD31 in differentiated ESCs: 24 ± 2% on day 3 compared with 21 ± 3% (controls); 5 ± 1% on day 6 compared with 3 ± 1% (controls); and below 1% on day 11 in VEGF-treated and control cells (P < 0.05 for day 6, n = 3), suggesting that the greater production of Flk-1 and Flt-1 was not associated with substantial differentiation of endothelial cells.

VEGF increases cardiomyocyte differentiation of ESCs by ERK and JNK activation. Binding of VEGF to its receptors activates mitogen-activated protein kinases (8, 19, 21). We determined if ERK and JNK were involved in VEGF-induced cardiomyocyte differentiation. ERK and JNK protein expression did not change in VEGF-treated and untreated ESCs. ERK2 (42 kDa) but not ERK1 (44 kDa) was phosphorylated in VEGF-untreated differentiated ESCs. VEGF, however, stimulated phosphorylation of ERK1 and ERK2 in VEGF-treated differentiated ESCs. Phosphorylated JNK was present in VEGF-untreated and VEGF-treated ESCs (Fig. 5).

We further determined if activation of ERK or JNK pathways was essential for VEGF-induced cardiomyocyte differ-

![Image](http://ajpheart.physiology.org/Downloadedfrom/10.2203.357)
entiation. PD-098059, the inhibitor for ERK, significantly decreased VEGF-induced α-MHC-positive-stained cells, from 47 ± 3% to 31 ± 3% (P < 0.05, n = 3). SP-600125, the inhibitor for JNK, however, did not significantly decrease VEGF-induced α-MHC-positive-stained cells (from 47 ± 3% to 41 ± 4%; n = 3, NS) (Fig. 5).

We determined the percentage of apoptotic and the number of proliferating cells in VEGF-treated cells and PD-098059-pretreated cells. PD-098059 pretreatment did not significantly change the percentage of apoptotic cells (28 ± 4%) or the number of proliferating cells (24,500 ± 1,258) compared with VEGF-treated cells (22 ± 3% and 22,167 ± 2,347) on day 11 of differentiation (n = 3 for apoptosis, NS; n = 6 for proliferation, NS).

**DISCUSSION**

The main findings of the present study are the following: 1) VEGF significantly increased cardiac-specific protein α-MHC, cTn-I, and Nkx2.5 expression in differentiated ESCs; 2) VEGF receptors Flt-1 and Flk-1 were significantly increased in differentiated ESCs compared with undifferentiated ESCs; 3) VEGF activated Flt-1 and Flk-1 in undifferentiated and differentiated ESCs; 4) VEGF significantly increased cardiomyocyte differentiation of ESCs by activating ERK signaling pathways; 5) antibodies against Flk-1 completely blocked and Flt-1 partially blocked VEGF-induced Nkx2.5 expression. Our results indicate that VEGF promotes cardiomyocyte differentiation primarily by ERK-mediated Flk-1 activation and, to a lesser extent, by Flt-1 activation. We had previously shown that expression of VEGF in transplanted ESCs improved cardiac function in mice with myocardial infarction (25). Our present findings suggest some mechanistic pathways by which VEGF promotes stem cell efficacy for restoration of cardiac function.

VEGF increased α-MHC, cTn-I, and Nkx2.5 positive-stained cells determined by flow cytometry (Fig. 1). We further confirmed, by using Western blot analysis, upregulation of α-MHC, cTn-I, and Nkx2.5 by VEGF (Fig. 2). VEGF has been shown to strongly increase endocardial cell proliferation and impact the growth rate of the myocardium (6). Others have reported the capability of VEGF to upregulate cardiac-specific proteins. Pimentel et al. (17), for example, demonstrated that VEGF enhanced the expression of the cardiac-specific protein connexin43 in the mouse myocardium. We now show that VEGF can enhance the expression of cardiac-specific proteins in ESCs. Regenerated cardiomyocytes derived from stem cells could possibly replace injured cardiomyocytes and improve cardiac function (2). The present findings suggest that improvement of cardiac function by ESCs overexpressing VEGF in mice with myocardial infarction may have resulted from VEGF-induced cardiomyocyte regeneration.

The biological activities of VEGF are mainly mediated by the two receptors Flt-1 and Flk-1 (5). In our study, we found that Flt-1 and Flk-1 expression were significantly increased in differentiated ESCs compared with undifferentiated ESCs (Fig. 3). Flt-1 is known to be expressed in rat cardiomyocytes (20). Flk-1 is one of the lateral mesoderm early markers, where cardiogenesis occurs. It has been shown, moreover, that Flk1+CD31−VE-cadherin− cells could act as cardiohemangioblasts to form cardiac cells (12). These findings are consistent with our data suggesting the VEGF-induced cardiomyocyte differentiation is through activation of Flk-1 and Flt-1 receptors.

VEGF significantly increased phosphorylation of ERK but not JNK. The specific ERK inhibitor PD-098059, but not the specific JNK inhibitor SP-600125, abolished VEGF-induced cardiomyocyte differentiation of ESCs (Fig. 4). These data suggest that the effects of VEGF on cardiomyocyte differentiation of ESCs are mediated by ERK pathways. This is consistent with previous findings demonstrating that activation of ERK is essential for cell differentiation induced by growth factors, including VEGF (3, 14, 17).

**Limitations.** The time points of cardiomyocyte differentiation we studied include the early stage of cardiomyocyte differentiation, rather than mid- and late stages of maturation (22).

The ESCs we used predominantly differentiate into cardiomyocytes; differentiation into endothelial cells and their progenitors is possible but unlikely. Iida et al. (12), for example, showed that FLK1+ cells from EBs formed spontaneously contracting colonies, indicating that Flk1+ cells could serve as

---

Fig. 5. A: Western blot showing the expression and phosphorylation (P) of ERK and JNK. ESCs were cultured for 3, 6, and 11 days by using the “hanging drop” method and then incubated with VEGF (20 ng/ml) for 15 min (n = 3). ERK and JNK protein expression did not change in VEGF-treated and untreated ESCs (control). ERK2 (42 kDa) but not ERK1 (44 kDa) was phosphorylated in VEGF-untreated differentiated ESCs. VEGF, however, stimulated phosphorylation of ERK1 and ERK2. Phosphorylated JNK was present in VEGF-untreated ESCs but did not change in VEGF-treated ESCs. B: extent of VEGF-induced cardiomyocyte differentiation determined by flow cytometry. ESCs were preincubated with either the ERK inhibitor PD-098059 or the JNK inhibitor SP-600125 for 30 min before VEGF (20 ng/ml) was added to the culture medium. PD-098059 but not SP-600125 significantly attenuated VEGF-induced α-MHC-positive-stained cells (⁎P < 0.05 vs. VEGF-untreated control, ⁎⁎P < 0.05 vs. VEGF, n = 3).
cardiac cells. Their findings support our findings that VEGF promoted cardiomyocyte differentiation through activation of its receptors, Flk-1 and Flt-1. Given the ESCs studied, any changes caused by endothelial cells and their progenitors are likely a secondary effect.

Future experiments remain to be done that include double staining to definitely demonstrate that differentiated cardiomyocytes of EBs can be simultaneously labeled with anti-α-MHC and anti-Flk-1 or anti-Flt-1 antibodies.

We did not determine if VEGF promoted a preferred type of cardiomyocyte. The ESCs that differentiated into cardiomyocytes, however, were not quiescent but contracted rhythmically in the culture medium, suggesting that these cells had ventricular cardiomyocyte capabilities. We have also shown, moreover, that the ESCs that differentiated into cardiomyocytes had action potentials and response to Ca2+ stimulation typical of normal adult ESC-derived cardiomyocytes (25). Identification of the type of cardiomyocyte that differentiates will be important for the therapeutic application of VEGF for promoting ESCs for the failing heart.

In conclusion, VEGF significantly increased cardiac-specific protein expression in differentiated ESCs. VEGF receptors Flk-1 and Flk-1 were significantly upregulated during ESC differentiation. ERK-mediated Flk-1 upregulation is required for VEGF to enhance cardiomyocyte differentiation of ESCs. Our findings indicate that VEGF stimulates ESCs to differentiate into cardiomyocytes. The present study provides a basis for additional studies to explore the potential for VEGF to promote cardiomyocyte differentiation of ESCs to regenerate failing myocardium.

REFERENCES


