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Mechanisms of coronary angiogenesis in response to stretch: role of VEGF and TGF-β

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Zheng, Wei, Elisabeth A. Seftor, Cynthia J. Meininger, Mary J. C. Hendrix, and Robert J. Tomanek. Mechanisms of coronary angiogenesis in response to stretch: role of VEGF and TGF-β. *Am J Physiol Heart Circ Physiol* 280: H909–H917, 2001.—To test the hypotheses that cyclic stretch of *1*) cardiac myocytes produces factors that trigger angiogenic events in coronary microvascular endothelial cells (CMEC) and *2*) CMEC enhances the expression of growth factors, cardiac myocytes and CMEC were subjected to cyclic stretch in a Flexercell Strain Unit. Vascular endothelial growth factor (VEGF) but not basic fibroblast growth factor mRNA and protein levels increased approximately twofold in myocytes after 1 h of stretch. CMEC DNA synthesis increased approximately twofold when conditioned medium from stretched myocytes or VEGF protein was added, and addition of VEGF neutralizing antibody blocked the increase. CMEC migration and tube formation increased with the addition of conditioned media but were markedly attenuated by VEGF neutralizing antibody. Myocyte tumor growth factor-β (TGF-β) increased 2.5-fold after 1 h of stretch, and the addition of TGF-β neutralizing antibodies inhibited the stretch-induced upregulation of VEGF. Stretch of CMEC increased VEGF mRNA in these cells (determined by Northern blot and RT-PCR) and increased the levels of VEGF protein (determined by ELISA analysis) in the conditioned media. Therefore, cyclic stretch of cardiac myocytes and CMEC appears to be an important primary stimulus for coronary angiogenesis through both paracrine and autocrine VEGF pathways. These data indicate that *1*) CMEC DNA synthesis, migration, and tube formation are increased in response to VEGF secreted from stretched cardiac myocytes; *2*) VEGF in CMEC subjected to stretch is upregulated and secreted; and *3*) TGF-β signaling may regulate VEGF expression in cardiac myocytes.

cardiac myocytes; endothelial cells; basic fibroblast growth factor; cell migration; cell proliferation; vascular endothelial growth factor; tumor growth factor-β

OUR KNOWLEDGE CONCERNING ANGIOGENESIS has been advanced by identifying growth factors and other molecules that initiate and regulate a cascade of events leading to neovascularization. Although the primary stimuli that activate angiogenic molecules have not been established for all models of angiogenesis, metabolic and mechanical factors are implicated as stimuli for both angiogenesis and vascular remodeling (reviewed in Ref. 8, 15, and 29). Previous work in our laboratory and reports by others suggest that mechanical factors play a major role in coronary angiogenesis (reviewed in Ref. 31). Taken together, data from these studies indicate that coronary angiogenesis is an adaptation to either increased coronary blood flow or increased diastolic filling. These findings provided the rationale for the hypothesis that stretch of the ventricular wall might trigger angiogenesis.

Evidence that vascular endothelial growth factor (VEGF), a key angiogenic factor, is upregulated by stretch of the ventricular wall was provided by Li and colleagues (18), who found a marked increase in VEGF mRNA after diastolic pressure had been increased to 35 mmHg for 30 min in an isolated Langendorff preparation. They also showed that this increase was mediated, at least in part, by tumor growth factor-β (TGF-β). To test the hypothesis that myocardial angiogenesis is a response to enhanced diastolic filling in a nonhypertrophic model, we administered the bradycardia drug alinidine to young rats for a period of 3 wk (37). Our data documented an increase in VEGF mRNA associated with the myocardial angiogenesis, i.e., a 23% increase in capillary length density. When VEGF neutralizing antibodies were administered to the bradycardia group, angiogenesis was completely prevented.

Evidence that stretch is a stimulus for growth factors has emerged from in vitro studies. Cyclic stretch triggers TGF-β secretion in mesangial (25) and smooth muscle (19) cells. The demonstration that VEGF secretion in rat cardiomyocytes occurs in response to cyclic stretch has provided direct evidence that stretch per se provides a trigger for VEGF secretion in this cell type (28). The current study was designed to test two hypotheses based on these findings. First, we tested the hypothesis that specific angiogenic events in coronary microvascular endothelial cells (CMEC) are triggered

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by stretch-activated growth factors in cardiomyocytes, which provide paracrine signals for CMEC. Second, we tested the hypothesis that stretch of endothelial cells serves to trigger autocrine signaling by growth factors.

MATERIALS AND METHODS

Cardiac myocyte cultures Primary cardiac myocyte cultures were prepared from ventricles of 2-day-old Sprague-Dawley rats, with modifications of the protocol described previously (1). Briefly, minced ventricular myocytes were placed into potassium glutamate solution [containing (in mM) 140 potassium glutamate, 16 $NaHCO_3$, 0.5 NaH_2PO_4, 25 HEPES, 16.5 dextrose, and 0.014 phenol red], which was obtained from the Hybridoma Facility at the University of Iowa. Cell dispersion was accomplished by digestion with 0.3% collagenase and then with 0.1% trypsin at 37°C for 10 min. The suspension from the first treatment was discarded, and the sequence was repeated until all tissue was dissociated. The dissociated cells were preplated into a 100-mm culture dish for 1 h in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) to reduce the number of contaminating nonmuscle cells. Cardiomyocyte-enriched suspensions were removed from the culture dish and plated on collagen I Bioflex plates (Flexcell International, Hillsborough, NC) at a density of 1×10^5 cells/ml. Primary cardiac myocytes were cultured for 48 h in 10% FBS-DMEM containing 1 mM sodium pyruvate and antibiotics. More than 90% of the cells were beating at the end of the experiment.

H9C2(2-1) rat embryo heart myoblasts were purchased from American Type Culture Collection and were grown under the same experimental conditions as the primary cardiac myocytes. For the serum-free condition, cells were washed with PBS and maintained in DMEM containing insulin-transferrin-selenium A (Life Technologies, Rockville, MD) for 16–20 h and then stretched.

Coronary microvascular endothelial cell cultures. CMEC were isolated by collagenase perfusion as previously described (34). Briefly, after the rat was anesthetized and heparinized, the hearts were removed and placed in ice-cold minimal essential medium (Joklik's modified buffer) and 5 mM HEPES and perfused using a static 40-mmHg hydrostatic pressure head. After a 10-min washout period, collagenase (0.7 mg/ml) was introduced to the perfusate and allowed to recirculate for 30–40 min. The ventricles were minced and placed in fresh collagenase-containing perfusate, and $CaCl_2$ (50 μM) was added. The cells were dispersed, filtered through a double layer of cheesecloth, and diluted 1:4 with buffer containing 0.1% of dialyzed bovine serum albumin. The resulting suspension was allowed to settle to separate the myocytes from CMEC. Further purification of CMEC was accomplished by sequential filtration through a series of 90-, 45-, 25-, and 15-μm nylon screens. We confirmed CMEC identity by the uptake of modified low-density lipoprotein and/or positive staining for factor VIII-related antigen.

CMEC from two to four hearts were pooled into one 60-mm gelatin-coated culture dish. Endothelial cells were cultured at 37°C under 10% CO_2 in 3 ml DMEM supplemented with 20% FBS, 2 mM L-glutamine, 20 mM D-glucose, 20 U/ml heparin, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. After the cells neared confluence, they were passaged by trypsinization in Dulbecco's PBS containing 0.25% trypsin and 0.02% EDTA and were used for experiments at *passages 7–9*. The cells were replated in various culture dishes depending on the experimental protocol.

Stimulation of cardiac myocytes and endothelial cells. To produce cyclic stretch in vitro, we employed a computerized Flexercell Strain Unit (Flexcell International). Cardiac myocytes or CMEC were seeded on a Bioflex culture plate with a type I collagen substrate. After serum starvation for 16 h, cultured cells were subjected to a 10% average surface elongation at 30 cycles/min (1-s stretch/1-s relaxation) for various time periods. This protocol was selected to provide a dynamic near-physiological strain stimulus. Cultured cells not subjected to cyclic stretch were used as controls.

To determine the role of TGF-β, this protein (1 or 10 ng/ml; R&D Systems, Minneapolis, MN) was added to the medium of cultured myocytes for a period of 4 h. To determine whether VEGF mRNA expression is dependent on TGF-β, we added TFG-β neutralizing antibodies (20 μg/ml; R&D Systems) to the medium before stretching the cells.

Northern blot assay. Northern blot analysis was used to quantify the abundance of VEGF, basic fibroblast growth factor (bFGF), and TGF-β mRNA in the cultured cells by modification of the method described previously (37). Briefly, total RNA was extracted from cardiocytes and CMEC using a STAT-60 (TEL-TEST "B", Friendswood, TX). Total RNA (10 μg) was separated in a 1.2% formaldehyde-agarose gel, transferred to nylon membrane by capillary blot, and fixed by ultraviolet cross-linking. Prehybridization of the filters was conducted for 1–2 h at 55°C in 50% formamide, 5× saline-sodium citrate (SSC), 4× Denhardt's solution, 1% SDS, 10% dextran sulfate, and 150 μg/μl heat-denatured, sheared salmon sperm DNA. Northern blot hybridization was performed with a random ^{32}P-labeled 630-bp cDNA fragment of rat VEGF (the kind gift of Dr. Kenneth Thomas, Merck Research Laboratories), a 400-bp fragment of bFGF cDNA, or 416-bp fragment of rat TGF-β_1 cDNA (36) at 42°C overnight. After incubation, the blots were washed in 1× SSC-1% SDS for 10 min at room temperature, followed by two 30-min washes in 0.2× SSC-1% SDS at 55°C, and then exposed to X-ray film (Fuji NIF film, Fisher Scientific, Pittsburgh, PA) at −70°C. Autoradiagrams were scanned using Adobe Photoshop 5.0, and a single band of either VEGF, bFGF, or TGF-β mRNA was normalized for each sample with respect to the density of the corresponding 18S mRNA signal to compensate for potential variation in RNA loading and transfer.

Western blot assay. For analysis of VEGF protein expression in cardiac myocytes, cells were lysed by addition of 0.5 ml radioimmunoprecipitation buffer [1% NP-40, 0.5% sodium deoxycholic acid, 0.1% SDS in PBS (pH 7.4), 1 μmol/l leupeptin, 5 μmol/l aprotinin, 1 mmol/l phenylmethylsulfonyl fluoride, and 1 μmol/l pepstatin] per each Flexcell plate well. Protein extracts (50 μg) were separated with 10% SDS-PAGE, transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) by electrotransfer, and blocked with 5% nonfat milk for 1 h at room temperature. The blots were incubated with VEGF rabbit polyclonal IgG (Santa Cruz Biotechnology, San Cruz, CA) diluted 1:500 in 1% milk and 0.05% Tris-buffered saline-Tween 20. The antigen-antibody complexes were visualized using anti-rabbit IgG-horseradish persoxidase (Santa Cruz Biotechnology) diluted 1:5,000 and an enhance chemiluminescence detection system (Santa Cruz Biotechnology).

ELISA. VEGF levels in conditioned medium from unstretched and stretched CMEC were detected by ELISA (R&D Systems). Briefly, standards consisting of recombinant mouse VEGF were used at concentration of 0–1,000 pg/ml. VEGF standards and samples were pipetted into wells coated with antibody specific for mouse VEGF (cross-reaction with rat VEGF). After any unbound substances were washed

away, an enzyme-linked polyclonal antibody specific for VEGF was added to the wells. After a wash, a substrate solution was added. The absorbance of standards and samples was measured spectrophotometrically at 450 nm using a microplate reader. VEGF concentrations were calculated (in pg/ml) on the basis of the standard curve.

Preparation of conditioned media. Conditioned media were collected from cardiac myocytes that had been cultured under cyclic stretch for 1 h or nonstretch conditions, centrifuged at 1,000 *g* for 10 min, and then passed through a 0.22-μm filter. Some of conditioned medium was absorbed with VEGF neutralizing antibody (R&D Systems) for 30 min before use in CMEC. Conditioned medium was added to CMEC that had been washed in PBS.

Endothelial cell proliferation assay. DNA synthesis was estimated by quantifying the incorporation of [*methyl*-^{3}H]thymidine into trichloroactive acid-insoluble macromolecules. CMEC were seeded onto gelatin-coated membranes in wells and covered with DMEM supplemented with 20% FBS, and, after they approached confluence, they were arrested for 16 h in serum-free DMEM containing 0.1% BSA. They were then incubated for an additional 24 h with media from the following: *1*) stretched or unstretched myocytes, *2*) 20 ng/ml VEGF, or *3*) a mixture of medium from stretched myocytes plus 20 μg/ml VEGF neutralizing antibody. After 1 μCi [^{3}H]thymidine was added per well, the labeled cells were washed with ice-cold PBS, fixed in ice-cold 10% trichloroacetic acid, and then lysed in 0.3 N NaOH. The incorporated [^{3}H]thymidine was measured in 6 ml of counting cocktail 3a70B (Research Products, Mount Prospect, IL) in a liquid scintillation counter (Beckman Instruments).

In vitro migration of endothelial cells. Conditioned media were obtained from stretched or nonstretched myocytes as described for the proliferation studies. CMEC migration assays (4, 13) were performed in a 12-well analytic membrane invasion culture system (MICS) using a 10-μm gelatin-soaked polycarbonate membrane (Osmonics, Livermore, CA). Conditioned medium alone or conditioned medium containing 20 μg/ml of VEGF neutralizing antibody in DMEM supplemented with 0.1% BSA was placed in the lower wells of the MICS chamber. Subconfluent CMEC cultures were washed and trypsinized for the minimum time required to achieve cell detachment. After the filter was placed between the lower and upper chambers, 1×10^5 cells/ml of DMEM containing 0.1% BSA were seeded in the upper chamber and incubated for 6 h at 37°C in a humidified incubator with 5% CO_2. CMEC that had migrated to the lower chamber were trypsinized and collected onto a polylysine-treated 3-μm polycarbonate membrane by filtration in a Schleicher and Schuell minifold 1 apparatus. The cells were then fixed with methanol and stained using a Leukostat staining kit (Fisher Scientific, Pittsburgh, PA). The cells were examined microscopically for intactness and scored by counting 6–10 randomly selected high-power fields.

Endothelial cell tube formation. Three-dimentional cultures were established as described (27) with minor modifications. To observe tube formation in vitro, rat tail type collagen (1.5 mg/ml) was mixed with 2× medium 199 (M199) and neutralized with 1 N NaOH and 300 μl/well of collagen mixture, added to a four-well plate, and allowed to gel for 1 h in a humidified incubator at 37°C. After polymerization, the gels were soaked in DMEM containing 10% FBS for 30 min at 37°C. CMEC (1×10^5 cell/ml) were plated onto the gel in wells that contained conditioned medium from either stretched or unstretched myocytes, as described above. CMEC were allowed to form tubelike structures for 6–8 days in three-dimensional culture and were then photographed.

The collagen gels with tubelike structures were washed with 0.1 M sodium cacodylate buffer (pH 7.4), fixed in 2.5% gluteraldehyde for 1 h at room temperature, incubated in 1% osminum fixative for another 1 h, and washed again with sodium cacodylate buffer. The samples were dehydrated through a series of acetone and embedded in Spurrs. Ultrathin sections, stained with 5% uranyl acetate and saturated lead citrate, were observed with a Hitachi 7000 transmission electron microscope.

RT-PCR. Total RNA (2 μg) from CMEC was reverse transcribed in 20 μl of reaction volume containing 250 ng of random primers, 0.5 mmol/l each dNTP, 50 mmol/l Tris·HCl (pH 8.3), 75 mmol/l KCl, 3 mmol/l $MgCl_2$, 10 mmol/l dithiothreitol, and 200 units of moloney murine leukemia virus reverse transcriptase (Life Technologies). After 1 h of incubation at 37°C, samples were heated to 80°C for 10 min and then chilled on ice. Thermal cycling of 2 μl of cDNA from the reverse transcriptase mix was performed using the following specific primers for rat VEGF: sense, 5′CCAGCACATAGGAGAGATGAGCTTC-3′; and antisense, 5′-GGTGTGGTGGTGACATGGTTAATC-3′. PCR resulted in three bands (262, 394, and 466 bp) corresponding to the three principal VEGF isoforms 120, 164, and 188, respectively, expressed in the rat (5, 7, 9). Coamplification of the same DNA was performed for rat glyceraldehyde 3-phosphate dehydrogenase as an internal standard with the following primers: sense, 5′-AGGTCGGTGTCAACGGATTT-3′; and antisense, 5′-CAGCATCAAAGGTGGAGGAA-3′. Primers, obtained from the DNA facility at the University of Iowa, were added to the reaction mixture containing 50 mmol/l of Tris·HCl (pH 8.3), 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 0.2 mmol/l of each dNTP, and 2.5 units of Taq polymerase in a final volume of 50 μl. Amplication was performed in a thermocycler using the following parameters: 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min (30 cycles). The products were separated on a 1.2% agarose gel together with 100-bp DNA ladder, and the bands were visualized with ethidium bromide. The bands were excised, and their DNA sequences were determined.

Statistical analysis. Values are expressed as means ± SE. ANOVA and the *t*-test followed by a Bonferroni adjustment for multiple comparisons were used for comparisons of more than two groups. Significance of mean differences was noted when $P \leq 0.05$.

RESULTS

Stretch induces VEGF gene expression and protein upregulation in cardiac myocytes. Primary cardiac myocytes and H9C2(2-1) myoblasts were serum deprived for 16–20 h before the experiment and then exposed to cyclic stretch (10% elongation and 30 cycles/min) for various time periods. VEGF mRNA expression was determined over an 18-h time period. As illustrated in Fig. 1*A*, VEGF mRNA levels in both cell types were upregulated by stretch with a similar time course, as evidenced by a 1.6-fold increase in both cell types. A significant increase ($P < 0.01$) was noted after only 30 min of stretch and was maintained for 6 h, with maximal levels (2.3- to 2.5-fold) occurring at 1 h. VEGF protein levels, determined by immunobloting, were higher in stretched than nonstretched myocytes (Fig. 1*B*). The increased levels in response to stretch occurred in a similar time-dependent manner in both types of myocytes, with the increase apparent as early as 30 min after initiation of stretch. Maximal levels

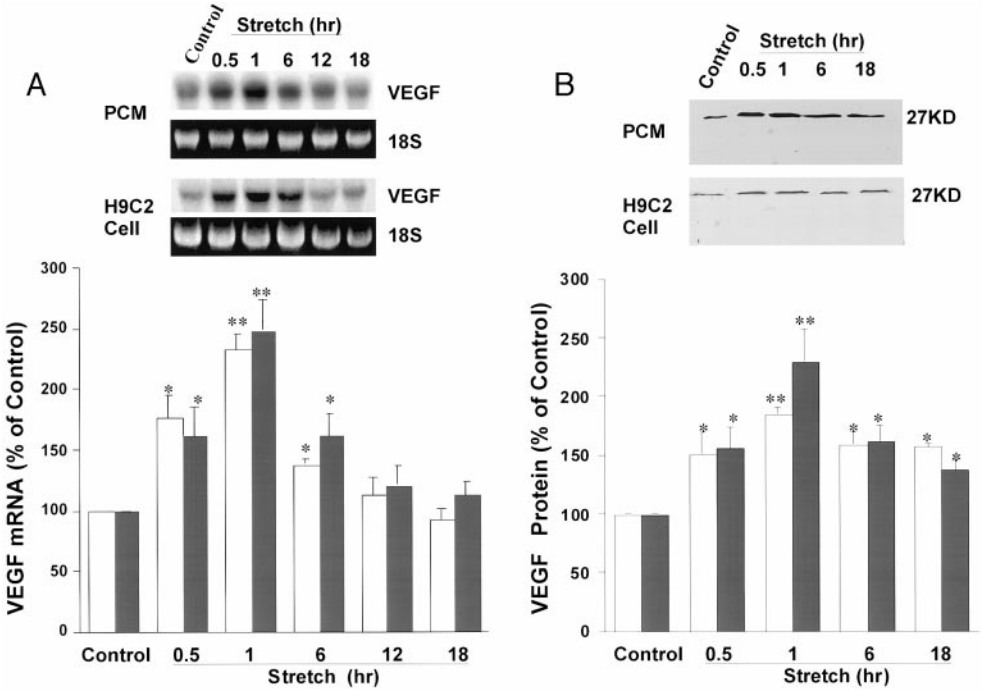

Fig. 1. Time course of stretch-induced expression of vascular endothelial growth factor (VEGF) in primary cardiac myocytes (PCM; open bars) and H9C2(2-1) (H9C2; closed bars) cells. Confluent monolayers of cardiac myocytes were stretched (30 cycles/min, 10% elongation) for a various periods of time, and VEGF mRNA (*A*; *bottom*) and protein (*B*; *bottom*) were determined by Northern blot (*A*; *top*) and Western blot assays (*B*; *top*). The VEGF mRNA and protein levels were compared in stretched and nonstretched cardiac myocytes. Similar results were obtained in 3 experiments. The values are expressed as means ± SE. Densitometric scanning of blots was used to determine the level of VEGF mRNA or protein in cells treated with stretch relative to nonstretched (control) cells. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

occurred at 1 h [1.9-fold, $P < 0.01$; or 2.3-fold, $P < 0.01$ in primary cardiac myocytes and a H9C2(2-1) cell, respectively] and then remained elevated up to 18 h.

TGF-β plays a role in stretch-induced upregulation of VEGF. With the use of Northern blot analysis, we found that mRNA levels of TGF-β increased 2.5-fold in cardiac myocytes after 1 h of stretch (Fig. 2*A*). We then showed that both stretch and exogenous TGF-β (1 or 10 ng/ml) induced the upregulation of VEGF mRNA (Fig. 2*B*). When anti-TGF-β neutralizing antibodies were added to the culture medium and the myocytes were then stretched, the stretch-induced VEGF expression was totally inhibited, indicating that VEGF upregulation in response to stretch is dependent on TGF-β. Addition of anti-TBF-β neutralizing antibodies to nonstretched myocytes had no effect on VEGF mRNA. In contrast, as seen in Fig. 3, bFGF mRNA was not significantly altered in myocytes subjected to stretch, although slightly higher values were noted 1–18 h after initiation of stretch compared with the controls.

VEGF from stretched myocytes induces proliferation of endothelial cells. Because VEGF has been previously shown to stimulate proliferation, migration, and tube formation of endothelial cells, we hypothesized that

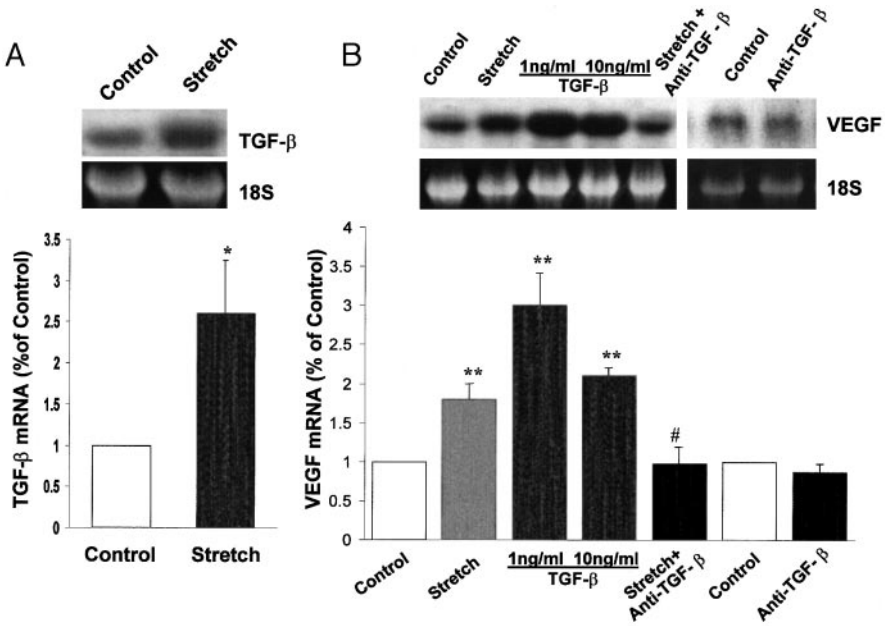

Fig. 2. Northern blot analysis of tumor growth factor-β (TGF-β) mRNA (*A*; *top*) and VEGF mRNA (*B*; *top*). *A*; *bottom*: TGF-β mRNA was significantly increased (more than 2.5-fold) in cardiac myocytes subjected to cyclic stretch for 1 h and compared with nonstretched (control) cells. *B*; *bottom*: stretch (for 1 h) and TGF-β induced the upregulation of VEGF mRNA, whereas stretch-induced VEGF expression was inhibited by addition of anti-TGF-β (20 μg/ml) before stretch. Results are expressed as means ± SE of 3–4 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control; $\#P < 0.05$ compared with stretch.

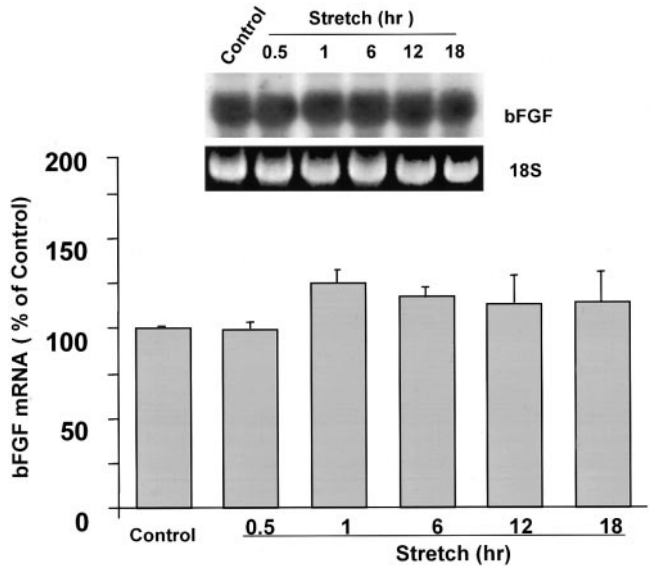

Fig. 3. Basic fibroblast growth factor (bFGF) mRNA in stretched and nonstretched primary cardiac myocytes. No significant difference is indicated between stretched and nonstretched (control) cells. *Top*: Northern blot; *bottom*: time course. Data represent means ± SE of 3 experiments.

VEGF, released from stretched myocytes, might stimulate these angiogenic characteristics in our model. As shown in Fig. 4, conditioned medium in which myocytes were stretched for 1 h increased CMEC DNA synthesis more than twofold over the control group. Addition of recombinant $VEGF_{165}$ (20 ng/ml) to the CMEC medium had a similar effect. VEGF neutralizing antibody (20 μg/ml) resulted in an almost total block of conditioned medium-stimulated proliferation, suggesting that soluble VEGF produced by the stretched myocyte mediates proliferation in CMEC via a paracrine mechanism.

Conditioned medium from stretched myocytes increases endothelial cell migration and tube formation. The impact of conditioned medium on endothelial cell migration, shown in Fig. 5, indicates that the conditioned medium from myocytes markedly increases CMEC migration ($P = 0.004$). Addition of VEGF neutralizing antibodies to the medium inhibited the more than twofold increase by 60% ($P < 0.05$). We then tested the hypothesis that tube formation by CMEC is enhanced by the conditioned media from stretched myocytes. Accordingly, CMEC were seeded on the three-dimensional type I collagen gels and exposed to basal media (Fig. 6*A*), conditioned medium from nonstretched myocytes (Fig. 6*B*), or conditioned medium from stretched myocytes (Fig. 6*C*). Media were changed daily. After 6–8 days of treatment, endothelial cells cultured in either basal medium or medium from unstretched cells were minimally spread and did not form networks of cell cords. In contrast, CMEC stimulated with media from stretched myocytes appeared in networks of cords. That these cell cords formed tubular structures was verified by transmission electron microscopy (Fig. 6*E*). The organization of CMEC into networks and tube structures in the conditioned medium from stretched myocytes was inhibited by addition of VEGF neutralizing antibodies to the conditioned medium (Fig. 6*D*). VEGF antibodies not only partially inhibited tube formation but also decreased the number of cells. Thus the latter observation suggests that VEGF may stimulate CMEC proliferation and/or facilitate survival of these cells.

Stretch-induced VEGF expression in CMEC. To test the hypothesis that VEGF is upregulated by stretch of

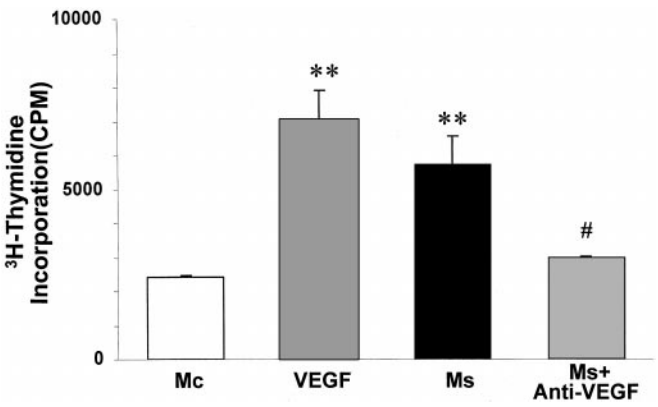

Fig. 4. Effects of VEGF and conditioned medium in the presence or absence of VEGF neutralizing antibodies on coronary microvascular endothelial cell (CMEC) DNA synthesis. Subconfluent CMECs were growth arrested for 16 h in serum-free medium. Conditioned media from nonstretched (control) cells (Mc), VEGF (20 ng/ml) in serum-free medium, and conditioned media from myocytes (Ms) stretched for 1 h in absence or presence of anti-VEGF antibody (20 μg/ml) were added to CMEC. After 24 h, 1 μCi [^{3}H]thymidine was added to the media for 6 h. The means ± SE represent radioactivity. Experiments were conducted in triplicate. cpm, Counts per minute. $^{**}P < 0.01$ significantly different from Mc. #$P < 0.01$ significantly different from Ms.

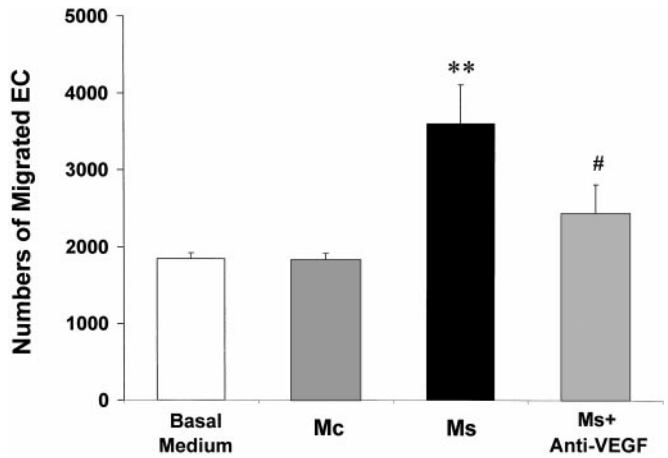

Fig. 5. Chemotactic response of CMEC to conditioned medium from myocytes stretched (Ms) for 1 h and nonstretched (Mc) myocytes. CMEC were suspended in DMEM in the upper compartment, and media were placed in the lower compartment of the migration chamber, as described in MATERIALS AND METHODS. The chamber was incubated at 37°C for 6 h. The migration of cells was examined microscopically. Data are presented as the means ± SE from four experiments. $^{**}P < 0.01$, significant only different from basal control; #$P < 0.05$, significantly different from conditioned medium from stretched myocyte alone.

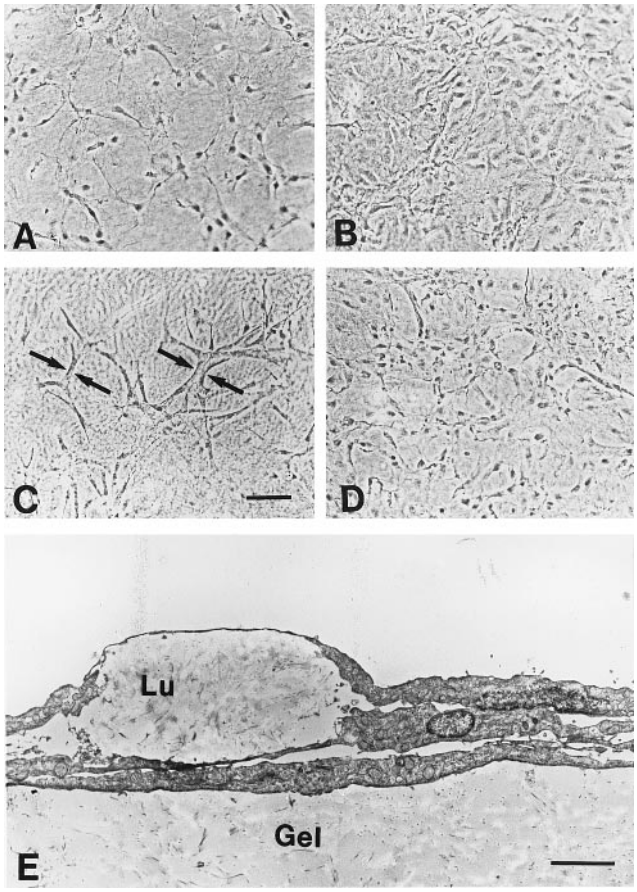

Fig. 6. Tube formation in CMEC in 3-dimensional (3D) cultures. CMEC were seeded in 3D cultures for 6–8 days in 4 distinct media. Phase-contrast micrographs (*A–D*) illustrate cell associations. When cells were incubated in basal medium (*A*) or conditioned medium from nonstretched myocytes (*B*), they were minimally spread out. In contrast, cells exposed to conditioned medium from myocytes stretched for 1 h (*C*) assemble into tubular structures. When VEGF neutralizing antibodies were added to the conditioned medium (*D*), tube formation was inhibited. *E*: representative electron micrograph of cross-sectioned tubelike structure from a 3D culture treated with conditioned medium from stretched myocytes illustrates that CMEC migrated into the gel and formed lumens (Lu). Bar (*A–D*) = 100 μm; bar (*E*) = 2 μm.

CMEC, we determined VEGF mRNA over time during cyclic stretch. The protocol was identical to that used for myocyte cyclic stretch. As shown in Fig. 7*A*, VEGF mRNA was increased 1.4-fold 1 h after stretch and then declined. RT-PCR analysis (Fig. 7*B*) confirmed induction of all three major VEGF isoforms in CMEC. To document increased VEGF protein secretion by stretched CMEC, we measured the protein in the conditioned media of stretched and nonstretched CMEC (Fig. 8). The data illustrate that VEGF protein significantly increased after 1 h of stretch and increased two- and fivefold by 6 and 18 h of stimulation, respectively. Thus stretch of CMEC provides a potential autocrine mechanism for VEGF.

DISCUSSION

Previous work (18) indicated that distention of the ventricle upregulates VEGF in the heart and that TGF-β plays a role in this upregulation. Subsequently, cyclic stretch was found to stimulate VEGF secretion in isolated cardiac myocytes. On the basis of this background, we tested hypotheses regarding paracrine angiogenic signals from stretched cardiac myocytes and autocrine signaling from stretched endothelial cells. Our data provide new evidence that angiogenic factors from cardiac myocytes subjected to stretch enhance the following critical angiogenic events in CMEC: DNA synthesis, migration, and tube formation. VEGF upregulation by stretch is mediated by TGF-β, because upregulation of the former was prevented by blocking the later with neutralizing antibodies. A second major finding is that stretch of CMEC upregulates VEGF.

Endothelial cells are subjected to mechanical forces, which alter their functional and structural properties, i.e., mechanotransduction (reviewed in Ref. 8). Such forces include shear stress, pressure, and stretch. Our experiments were based on the hypothesis that stretch within the ventricular wall constitutes a mechanical force that triggers the synthesis and release of growth factors as well as the cascade of angiogenic events leading to microvessel formation. This hypothesis was supported by our recent work that documented a VEGF-dependent angiogenesis in an in vivo model of enhanced diastolic filling associated with bradycardia (37) and by the finding that distension of the ventricle in an ex vivo system stimulates VEGF upregulation (18). Seko et al. (28) recently showed that cyclic stretch of cardiac myocytes can induce VEGF secretion, which is, at least in part, mediated by TGF-β. Our data are in agreement with these findings.

To our knowledge, our experiments are the first demonstration that cardiac myocytes subjected to stretch release factors that directly evoke *1*) DNA synthesis, *2*) migration, and *3*) tube formation by CMEC. We also documented that the conditioned media from myocytes subjected to cyclic stretch, which increased DNA synthesis in the CMEC by about the same magnitude as addition of VEGF, could be blocked by the addition of anti-VEGF neutralizing antibodies to the conditioned media. Thus proliferation of CMEC is dependent on VEGF signaling. In contrast, addition of the neutralizing antibodies to the conditioned media from stretched myocytes in the migration assay markedly limited, but did not abolish, the increase in CMEC migration, a finding suggesting that another promigratory factor(s) is released by the stretched myocytes. VEGF upregulation in myocytes is dependent on TGF-β, because the addition of TGF-β neutralizing antibodies completely negates the VEGF increase in myocytes subjected to stretch, a finding that is in agreement with Seko et al. (28). However, we noted that addition of 10 ng of TGF-β protein elicited a smaller increase in VEGF mRNA than did 1 ng of the protein. This finding is consistent with previous work on endothelial cells in vitro, which showed that low doses of TGF-β stimulate angiogenic events, whereas high doses are inhibitory (24). Although the increase in VEGF mRNA associated with TGF-β is probably due to increased transcription, we cannot rule out the possi-

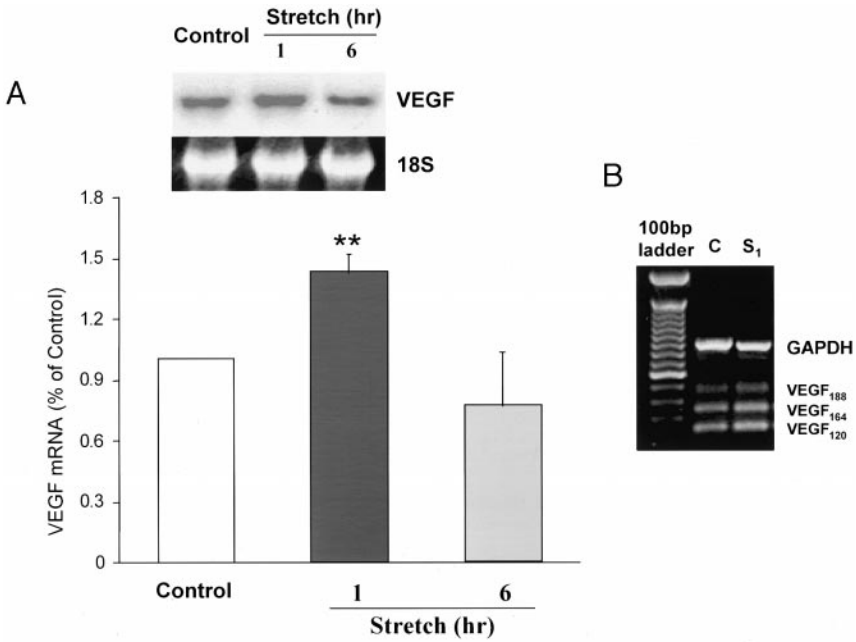

Fig. 7. Northern blot (*A*; *top*) and RT-PCR analysis (*B*) of VEGF mRNA in CMEC. VEGF mRNA was upregulated after 1 h of stretch and then declined after 6 h of stretch (*A*; *bottom*). Similar results were obtained in 4 experiments. Results are expressed as means ± SE. $^{**}P < 0.05$ compared with control. Densitometric scanning of blots was used to determine the level of VEGF mRNA in stretched and nonstretched CMEC. RT-PCR analysis showed simultaneous induction of mRNA for 3 principal VEGF isoforms (120, 164, and 188) by alternative splicing after stretch. C, control; S_1, after 1 h of stretch; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

blity that the increase is due to a longer half-life of the message. Both latent and active forms are elevated when smooth muscle cells are stretched (19).

Mechanical stretch has previously been shown to stimulate growth of neonatal cardiac myocytes (2) and to increase the activities of mitogen-activated protein (MAP) kinase, S6 peptide kinase, c-fos mRNA, and protein synthesis (17, 35). Stretch of cardiac myocytes was shown to rapidly activate several second messenger pathways in addition to Ras/MAP kinase, e.g., tyrosine kinases, protein kinase C, and phospholipase C and D, but not cAMP (26). Moreover, stretch has been shown to activate all three MAP kinase family members, extracellular signal-regulated protein kinase, and the focal adhesion kinase p125 (28). That study also demonstrated that VEGF and TGF-β are, at least partially, responsible for extracellular signal-regulated protein kinase activation by stretch. Thus the recruitment of VEGF and TGF-β by stretch is documented by these investigators as well as our own data. Stretch also has been shown to increase TGF-β (25) and VEGF production (12) in mesangial cells. In vascular smooth muscle cells, stretch increases TGF-β secretion (19) and phosphorylates the platelet-derived growth factor-α receptor (14). These data indicate that growth factors in several cell types are triggered by stretch.

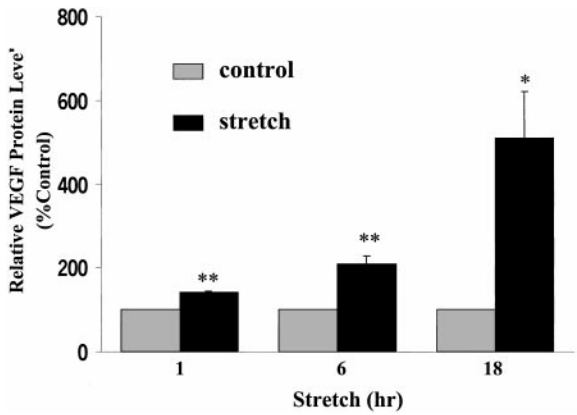

Fig. 8. VEGF protein in the conditioned media of nonstretched (control) and stretched CMEC (as a percentage of control) after 1, 6, and 18 h. Significant differences between the mean of the 2 groups are indicated: $^{*}P < 0.05$ and $^{**}P < 0.01$.

Our finding that stretch of CMEC upregulates VEGF is not only novel but perhaps unexpected, because endothelial cells do not express VEGF under normal conditions. However, under hypoxic conditions, endothelial cells have been shown to express (20) and secrete (23) VEGF. Stretch affects changes in endothelial cells, which may promote their growth, e.g., via prostacyclin accumulation (21) and alignment of stress fibers along the long axis of the cell (38). It has also been postulated that stretch inhibits protein phosphatase 2A, a growth suppressor (22).

The role of blood flow in angiogenesis during development was demonstrated during the early 1900's (6, 30). Capillary growth has been found to occur when blood flow is increased in the heart via chronic administration of a vasodilator (33) and in skeletal muscle via electrical stimulation (15). Although increased blood flow may enhance capillary growth by shear stress, wall tension, or stretch (16), our in vitro data show that stretch in the absence of the other flow-related forces acts as a primary stimulus for activation of angiogenesis. This evidence is consistent with in vivo experiments that demonstrated chronic stretch of skeletal muscle in the absence of enhanced blood flow stimulated marked capillary proliferation (10, 23).

Although the role of hypoxia in upregulation of VEGF is well established (reviewed in Ref. 11), concrete evidence that stretch also plays a similar role is

relatively new (18, 28). Accordingly, both metabolic and mechanical factors serve to activate VEGF and the angiogenic cascade. During heart development, both can be considered as potential activators of vascularization because rapid increases in cardiac mass could cause focal hypoxia and could also stretch developing vascular structures. Studies in adult hearts have induced angiogenesis under conditions of exercise training, dipyridamole treatment, arteriovenous shunts, thyroid hormone treatment, and chronic bradycardia (reviewed in Ref. 32). These models are associated with either increased coronary flow or enhanced diastolic filling, both of which provide mechanical stimuli for angiogenesis. With the use of the arteriovenous shunt model, we demonstrated growth of both capillaries and arterioles (3). On the basis of the findings of these studies and those of the current study, there is ample evidence to suggest that interventions that encompass mechanical factors, e.g., stretch, may be clinically useful in promoting myocardial vascularization.

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