Am J Physiol Heart Circ Physiol 291: H2057–H2066, 2006;
doi:10.1152/ajpheart.00494.2006.

Myometrial cells induce angiogenesis and salvage damaged myocardium

Ming-Li Huang,[1,2] Hai Tian,[1,3] Jun Wu,[1] Keiji Matsubayashi,[4] Richard D. Weisel,[1] and Ren-Ke Li[1]

[1]*Division of Cardiovascular Surgery and Toronto General Research Institute, Toronto General Hospital and University of Toronto, Toronto, Ontario, Canada;* [2]*Department of Gynecology and Obstetrics, First Clinical College of Harbin Medical University;* [3]*Division of Cardiovascular Surgery, Second Affiliated Hospital of Harbin Medical University, Harbin, China; and* [4]*Department of Surgery, Division of Cardiovascular Surgery, Shiga University of Medical Science, Shiga, Japan*

Submitted 15 May 2006; accepted in final form 13 June 2006

Huang, Ming-Li, Hai Tian, Jun Wu, Keiji Matsubayashi, Richard D. Weisel, and Ren-Ke Li. Myometrial cells induce angiogenesis and salvage damaged myocardium. *Am J Physiol Heart Circ Physiol* 291: H2057–H2066, 2006. First published June 16, 2006; doi:10.1152/ajpheart.00494.2006.—Characteristically, uterine myometrial cells (MCs) are proliferative, inducing angiogenesis within the female reproductive organ. We evaluated whether MCs implanted into myocardium could also induce angiogenesis and restore heart function after injury. MCs were isolated from the adult rat uterus and cultured for three studies: *1*) Intracellular VEGF levels were measured in MCs cultured with progesterone (10^{-11}, 10^{-9}, and 10^{-7} M) ($n = 6$ tests per group). *2*) Blood vessel density was evaluated 8 days after MCs (3×10^6 or 6×10^6), smooth muscle cells (SMCs), or endothelial cells ($n = 6$ rats per group) were injected with matrigel into the subcutaneous tissue of adult rats. *3*) MCs, SMCs (5×10^6/rat), or media were injected into a transmural scar 3 wk after cryoinjury in rat hearts ($n = 12$ rats per group), and heart function, blood vessel density, and myocardial scar size and thickness were evaluated 5 wk later. In *study 1*, cultured MCs expressed VEGF, with levels significantly ($P < 0.05$) upregulated by progesterone at an optimal dose of 10^{-11} M. In *study 2*, MCs injected into the subcutaneous tissue with matrigel induced significantly more blood vessels, especially large-diameter vessels, than did SMCs or endothelial cells ($P < 0.01$ for all groups). This angiogenic effect was greatest ($P < 0.01$) at higher doses of MCs and was enhanced by progesterone (10^{-11} M). In *study 3*, MCs implanted into the injured myocardium increased blood vessel density at the implant area, reduced scar size, and improved cardiac function relative to SMCs and media. Overall, MCs induced angiogenesis in vitro and in vivo, prevented cardiac remodeling, and improved heart functional recovery after cardiac injury.

cell transplantation; heart failure; ischemic cardiomyopathy; angiogenic effect; heart function

ANGIOGENESIS IS AN IMPORTANT physiological event that may be inadequate in the heart after coronary occlusion, resulting in progressive cardiac failure. In the female reproductive system, the myometrium undergoes extensive angiogenesis, and this capability might be employed to improve the recovery of the heart after injury.

In patients with ischemic cardiomyopathy, percutaneous transluminal coronary interventions or coronary bypass surgery will restore regional myocardial perfusion and improve heart function. However, ~10% of patients are not ideal candidates for these therapies because of diffuse coronary disease. Two treatments have become available for patients with diffuse atherosclerosis: transmyocardial laser revascularization (5, 8) and angiogenic protein or gene therapy (9, 16). Although preclinical results are encouraging, these techniques are complex and expensive, and the mechanisms of their beneficial effect remain obscure. Reliable therapies, which will establish a "biological bypass" without intraluminal or surgical interventions, would provide patients with new hope for full recovery.

Cell transplantation offers the promise of establishing a biological bypass. Recently, a number of researchers have transplanted a variety of cell types into the infarcted myocardium in an attempt to improve ventricular function (3, 11, 14, 15, 21). Implantation of endothelial cells and smooth muscle cells (SMCs) increased regional blood vessel density in the area of transplantation (12, 17). Bone marrow cells and angiogenic progenitor cells were also studied for their myocardial angiogenic potential, but the optimal cell type to increase perfusion in the ischemic myocardium has not yet been determined (1, 25).

Angiogenesis is a necessary process that, concomitant with the growth and regression of the human endometrium, provides an extensive blood supply to this rapidly changing tissue (23). The angiogenic mechanisms in the uterus are well established and of a significant magnitude, and this effect could be exploited to restore perfusion to the injured heart. Human uterine cells have previously been isolated and cultured (26). Uterine epithelial cells, myometrial cells (MCs), and endothelial cells have been identified with the use of immunohistochemistry, using antibodies against cytokeratin, α-smooth muscle actin, and factor VIII (26). In addition, VEGF and basic fibroblast growth factor have been identified in these cells (20, 22, 26). To date though, few in vitro or in vivo studies of the vasculogenic capacity of uterine cells have been published, and no attempt has been made to exploit this effect to increase perfusion to the injured myocardium.

The present study investigated the ex vivo angiogenic potential of MCs and the contribution of progesterone to blood vessel formation. In addition, the ability and efficacy of MCs to increase myocardial perfusion and improve cardiac structure and function after myocardial injury were also evaluated. We found that MCs have a significant capacity to induce angiogenesis, especially arteriolar vessels, and improve heart function after injury.

METHODS

Experimental animals. Female Lewis rats (200–250 g) were obtained from Charles River Canada (Saint-Constant, QC, Canada). Uterine tissue from adult rats was used for cell isolation, and inbred rats were used as the recipients of cell implants ($n = 6$ rats per group

Address for reprint requests and other correspondence: R.-K. Li, MaRS Centre, Toronto Medical Discovery Tower, 3rd Fl., Rm. 702, 101 College St., Toronto, ON, Canada, M5G 1L7 (e-mail: renkeli@uhnres.utoronto.ca).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

for matrigel study; $n = 12$ rats per group for myocardial injury study). All procedures were approved by the Animal Care Committee of the Toronto General Research Institute.

Cell isolation, culture, and identification. MCs were isolated from uterine tissue using enzymatic digestion and were cultured in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator (17). *Passage 4* cells were used for these studies. The purity of the cell preparation was identified immunohistochemically, using an antibody against α-smooth muscle actin.

SMCs and vascular endothelial cells were isolated from the aorta of adult rats using enzymatic digestion and cultured in IMDM culture media containing 10% FBS and antibiotics, as described previously (17). The SMCs and endothelial cells were identified using antibodies against α-smooth muscle actin and factor VIII, respectively. *Passage 4* cells were used for these studies.

Immunohistochemical staining was performed on cultured cells fixed with 100% methanol, as described previously (17). Endogenous peroxidase was blocked using 3% hydrogen peroxide, and the cells were incubated with antibodies against α-smooth muscle actin or factor VIII for 16 h at room temperature. Negative control samples were incubated in PBS, without the primary antibodies, under the same conditions. The cells were rinsed with PBS and incubated with secondary antibodies conjugated to peroxidase for 45 min at 37°C. The samples were washed three times with PBS, then immersed in diaminobenzidine-H_2O_2 (2 mg/ml diaminobenzidine and 0.03% H_2O_2 in 0.02 ml/l phosphate buffer) solution for 15 min. Finally, the samples were washed with PBS and photographed.

Progesterone stimulation study. MCs were cultured in media containing 10% charcoal-stripped FBS for 7 days for stabilization. The cells were then cultured in media containing progesterone at concentrations of 10^{-11} M, 10^{-9} M, or 10^{-7} M for 2 or 5 days. SMCs and endothelial cells cultured with progesterone were also examined, and MCs cultured in regular media containing the reagent for the progesterone solution were used as controls ($n = 6$ tests per group per time point). The collected cells were sonicated, and intracellular protein levels were measured with a Bio-Rad Protein Assay Kit for which bovine serum albumin (Sigma) was used as the standard. VEGF levels in the cultured cells were measured with a commercial ELISA kit, according to the suggested protocol. To identify progesterone receptors, each group of cultured cells was also stained with an anti-progesterone receptor antibody.

Preparation of cells for implantation. Before implantation, the cultured MCs, SMCs, and endothelial cells were detached with 0.05% trypsin. After centrifugation, the cell pellet was resuspended in growth factor-reduced matrigel matrix (BD Biosciences) at 4°C for implantation.

To evaluate the contribution of MCs to vessel formation, the MCs, SMCs, and endothelial cells were necrosed by boiling at 100°C for 10 min. They were then cultured for 24 h to assess the survival rate. The living or necrosed cells were mixed with matrigel as described above for implantation.

In vivo matrigel study. Adult female rats were anesthetized with an intramuscular injection of ketamine (20 mg/kg), followed by an intraperitoneal injection of pentobarbital sodium (30 mg/kg). MCs (3×10^6 or 6×10^6), SMCs (6×10^6), endothelial cells (6×10^6), or culture media mixed with 0.5 ml of matrigel at 4°C were injected into the abdominal subcutaneous tissue. To assess the effect of progesterone on the angiogenic potential of MCs, cells (6×10^6 per group) mixed with matrigel containing 10^{-11} M progesterone were injected into the subcutaneous tissue. In another three groups, necrosed cells (6×10^6 per group: MCs, SMCs, and endothelial cells) were mixed with matrigel and injected into the subcutaneous tissue ($n = 6$ rats per group). The animals were given Penlong XL (150,000 U/ml benzathine penicillin G and 150,000 U/ml procaine penicillin G; 0.3 ml im) after operation.

Histology and immunohistochemistry. Eight days after implantation, the matrigel nodules from all groups were carefully excised under anesthesia. Each nodule was examined, and the vessel network was photographed. The nodules were then fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 10-μm sections. The sections were stained with hematoxylin and eosin as described in the manufacturer's specifications (Sigma, St. Louis, MO). To quantify blood vessel density, arteriolar vessels and capillaries were counted in five random, high-power, 0.2-mm^2 fields from each of three 10-μm sections per nodule, per group; counts were averaged to estimate the number of vessels per 0.2 mm^2 in the matrigel nodules. Immunohistochemical staining with a monoclonal antibody against α-smooth muscle actin was used to identify SMCs in the blood vessels.

Myocardial injury and cell implantation. Under isoflurane anesthesia, rats were intubated and ventilated with a mixture of room air, oxygen, and 2–3% isoflurane using a Harvard ventilator. The heart was exposed through a 2-cm left lateral thoracotomy. With the use of a 0.8×1.2 cm liquid nitrogen probe, the left ventricular free wall was cryoinjured 14 times, for a duration of 1 min each time, as previously described (17, 18). The thoracotomy was closed in layers with 3-0 silk continuous sutures, and postoperative care was as described in *In vivo matrigel study*. Three weeks later, the animals were randomly assigned to one of three groups: MC implant, SMC implant, or media (control) implant ($n = 12$ rats per group). In each animal, the heart was exposed through a medial sternotomy under general anesthesia, and a single injection (5×10^6 MCs or aortic SMCs in 0.07 ml of culture media or 0.07 ml of culture media alone) was given directly into the center of the scarred region using a tuberculin syringe. Scar sizes were comparable among animals at the time of cell transplant (because each myocardial scar was created by using the same 0.8×1.2 cm probe), so cells injected directly into the center of the scar were distributed similarly in all animals. The chest was closed with 3-0 silk sutures.

Functional measurement, ventricular morphology, and histology. Five weeks after cell implantation, heart function was evaluated in each rat by using a Langendorff preparation (17, 18). The systolic and diastolic pressures were measured at an intraventricular balloon volume of 0.04 ml, and the balloon volume was then increased by 0.02-ml increments until the left ventricular end diastolic pressure reached 30 mmHg. Developed pressure was calculated as the difference between the peak systolic and end-diastolic pressures. The maximal positive and negative first derivatives of the time course of pressure generation ($+dP/dt$, $-dP/dt$) were also recorded at each balloon volume. At the end of the study, the hearts were arrested with a KCl solution and then fixed at a ventricular pressure of 30 mmHg with 10% phosphate-buffered formalin solution.

After the atria and great arteries were excised, the fixed hearts were cut into 2-mm-thick sections. Both the apical and basal aspects of each section were digitally photographed and quantified by using computerized planimetry. The epicardial surface areas of the left ventricular free wall and scar tissue were measured as previously described (11). The left ventricular total scar area and the scar thickness were calculated.

To quantify blood vessel density, 2-mm sections (from morphological studies) from each heart were sectioned into 10-μm sections and then stained with hematoxylin and eosin. The total number of vessels per 0.2 mm^2 in the myocardial scar area of each group was estimated as described for matrigel nodules in *Histology and immunohistochemistry*.

Statistical analyses. Between-group comparisons were made using a repeated-measures (or two-way) ANOVA. The Langendorff hemodynamic data were evaluated by an analysis of covariance (ANCOVA) with balloon volume as the covariate and either systolic, diastolic, or developed pressure as the independent variable to compare the differences between groups or their interaction. When the *F*-ratio was significant ($P < 0.05$), differences were specified by Tukey's multiple range test. VEGF levels, vascular density, and cardiac infarct size and thickness were analyzed by using unpaired Student's *t*-tests. Differences were deemed significant when *P* values were <0.05. All data are presented as means ± SE.

RESULTS

Cell culture and identification. MCs were isolated from the muscle layer of uterine tissue, whereas SMCs and endothelial cells were isolated from aortic tissue and used as controls for the angiogenesis studies (Fig. 1, *A–C*). Immunohistochemical staining of cultured MCs and SMCs showed that 93% and 96% (respectively) of the cells were positive for α-smooth muscle actin,

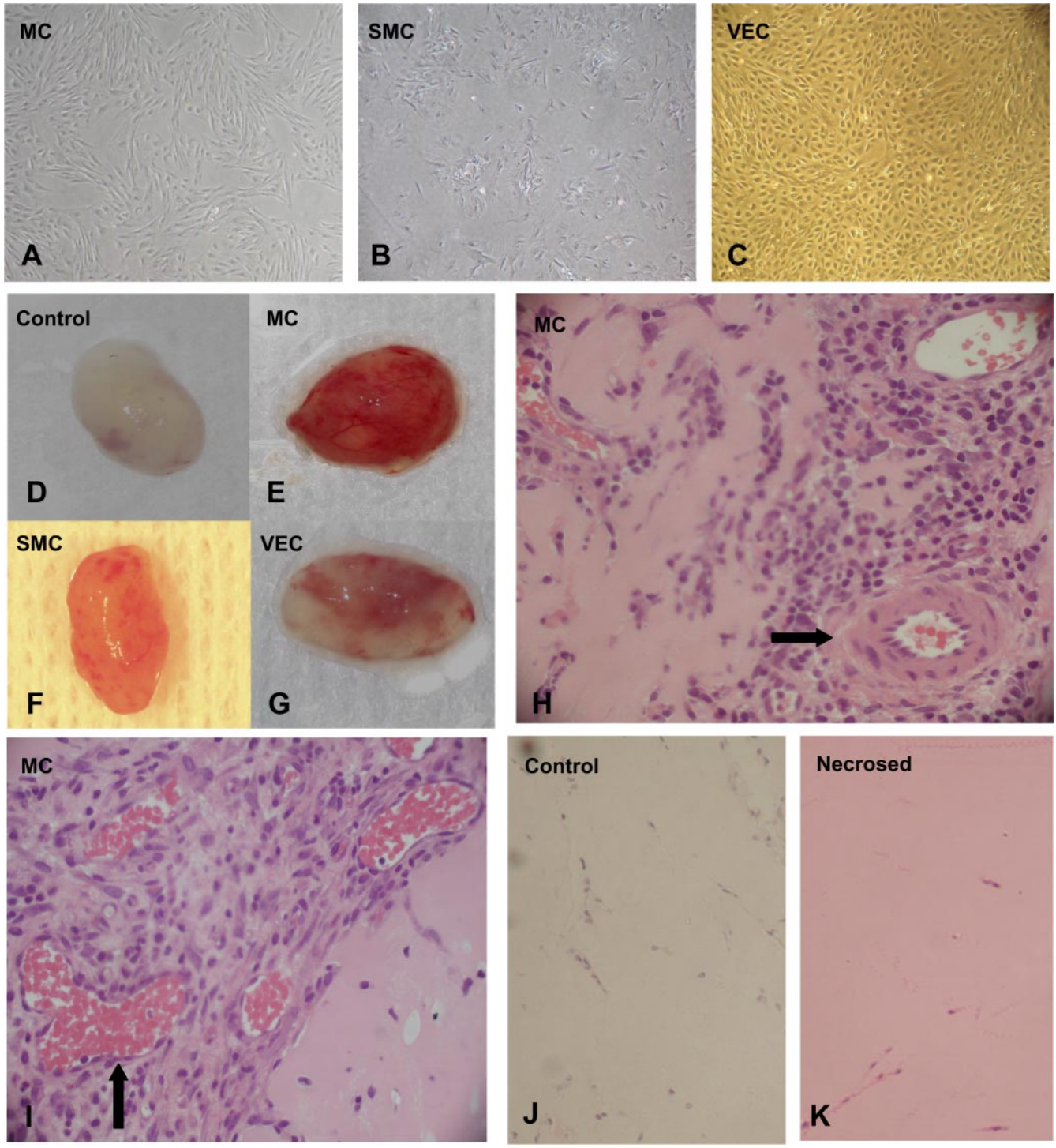

Fig. 1. *A–C*: cells in culture. Appearance of cultured myometrial cells (MC; *A*), smooth muscle cells (SMC; *B*), and endothelial cells (VEC; *C*) under a light microscope. *D–G*: vessel formation in matrigel nodules. Eight days after cell implantation with matrigel, control matrigel nodules were pale and semitransparent (*D*), whereas blood vessels were clearly visible in nodules of MC (*E*), SMC (*F*), and VEC (*G*) cell groups. *H–K*: histological studies. Light microphotographs (hematoxylin and eosin staining) confirm that arteriolar structures (arrow in *H*; MC group) and capillaries (arrow in *I*; MC group) were present in all cell groups at 8 days after cell implantation. Few blood vessels were observed in control nodules (*J*) or in nodules resulting from implantation of necrosed cells (*K*; MC group). Magnification is ×100 in *A–C*, *J*, and *K* and ×400 in *H* and *I*. $n = 6$ Rats per group in matrigel study.

whereas 92% of cultured endothelial cells were positive for the antibody against factor VIII.

Angiogenesis in matrigel nodules. MCs, SMCs, endothelial cells, or media (no cells) were mixed with matrigel and implanted into abdominal subcutaneous tissue. Additional groups had necrosed cells or progesterone-treated cells implanted with the matrigel. Eight days after implantation, matrigel nodules were observed in all groups. Control matrigel nodules (without cells) and necrosed cell nodules were pale and semitransparent (Fig. 1*D*), whereas blood vessels were visible in the matrigel nodules of all cell groups (Fig. 1, *E–G*). Histological studies using hematoxylin and eosin staining and light microscopy confirmed the presence of arteriolar structures (vessels with smooth muscle rings; Fig. 1*H*) and capillaries (Fig. 1*I*) in the nodules of all cell groups. However, few such vessels were evident in the matrigel control nodules (Fig. 1*J*) or in the nodules resulting after implantation with necrosed cells (Fig. 1*K*).

Dose response of MCs to induce angiogenesis. Implantation of a higher dose (6×10^6) of MCs induced significantly more total blood vessels ($P < 0.01$), capillaries ($P < 0.01$), and arteriolar vessels ($P < 0.01$) within the matrigel nodules than did implantation of a lower dose (3×10^6) after 8 days (Fig. 2). There were no significant differences between the control group and the low-dose MC group; therefore, only results from the group injected with 6×10^6 MCs are presented in the results for the matrigel study.

Relative angiogenic potential of implanted cells. As shown in Fig. 3, the numbers of blood vessels (capillaries and arteriolar vessels) found in the nodules of the MC group 8 days after injection with matrigel were significantly greater ($P < 0.01$ for all groups) than those in the SMC, endothelial cell, and control groups, although the implanted SMCs and endothelial cells stimulated significantly greater capillary formation than was seen in the control group ($P < 0.05$). Overall, the total number of blood vessels in the MC group was significantly greater ($P < 0.01$) than in the other groups.

Contribution of implanted cells to neovessel formation. To evaluate the angiogenic potential of the implanted cells, necrotic MCs, SMCs, and endothelial cells were generated by heating cells from each group at 100°C for 10 min. In vitro culture examination showed that 100% of these necrosed cells did not survive, and subcutaneous matrigel implantation of the cells produced very few blood vessels within the resultant nodule (Fig. 1*K*) after 8 days. Because the total numbers of blood vessels induced by the normal MCs, SMCs, and endothelial cells were significantly greater ($P < 0.01$ for all groups) than those induced by the heat-damaged cells (Fig. 4), the angiogenic effect was therefore produced by the biological activity of the implanted cells.

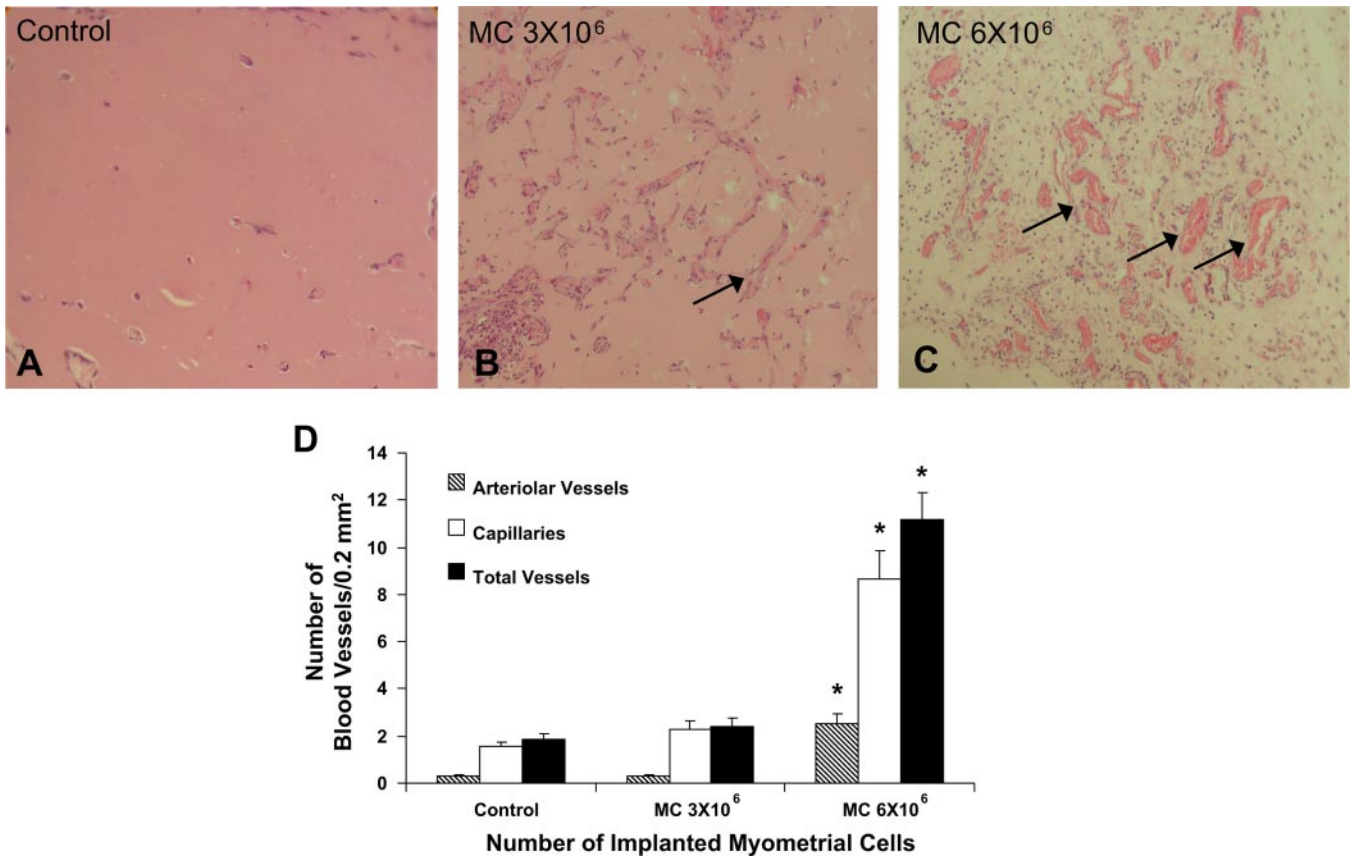

Fig. 2. Dose response of MC-induced vessel formation. *A–C*: light microphotographs (hematoxylin and eosin staining) taken 8 days after cell implantation with matrigel, showing blood vessels forming in matrigel nodules of control group (*A*) and after injection of MCs at concentrations of 3 million (MC 3×10^6, arrow in *B*) or 6 million (MC 6×10^6, arrows in *C*). *D*: after 8 days, implantation of MC 6×10^6 produced significantly more arteriolar vessels, capillaries, and total blood vessels than implantation of MC 3×10^6. There were no significant differences between MC 3×10^6 group and control group. Magnification is ×100 in *A–C*. Data represent means ± SE ($n = 6$ rats per group). *$P < 0.01$ for each vessel type compared with MC 3×10^6 and control groups.

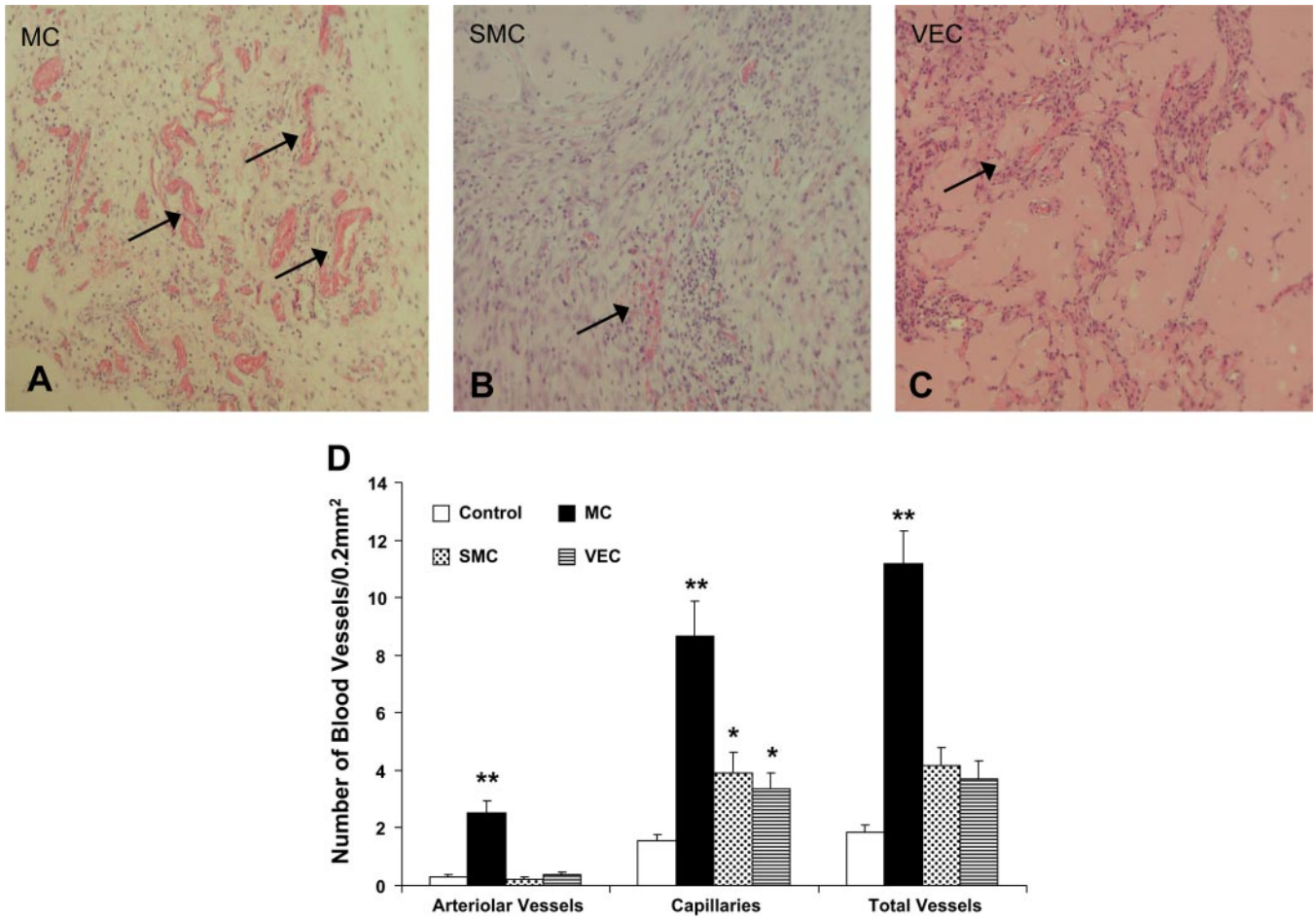

Fig. 3. Quantification of vessel formation. *A–C*: light microphotographs (hematoxylin and eosin staining) showing blood vessels forming in matrigel nodules of MC (arrows in *A*), SMC (arrow in *B*), and VEC (arrow in *C*) groups 8 days after cell implantation with matrigel. *D*: numbers of blood vessels (arteriolar vessels, capillaries, and total vessels) formed in MC group 8 days after cell and matrigel injection were significantly greater than those in SMC, VEC, or control groups, although implanted SMCs and VECs stimulated significantly greater capillary formation than was seen in the control group. Magnification is ×100 in *A–C*. Data represent means ± SE ($n = 6$ rats per group). *$P < 0.05$ and **$P < 0.01$.

Effects of progesterone on angiogenic potential of MCs. At 2 and 5 days after incubation with various concentrations of progesterone, cellular VEGF levels rose significantly ($P < 0.01$) in MCs cultured with 10^{-11} M progesterone compared with those cultured at higher concentrations, or controls (Fig. 5*A*). MCs cultured with progesterone also had more progesterone receptors (arrows in Fig. 5*B*) than those cultured without progesterone (arrow in Fig. 5*C*) and more receptors than endothelial cells or SMCs cultured with progesterone (Fig. 5, *D–E*).

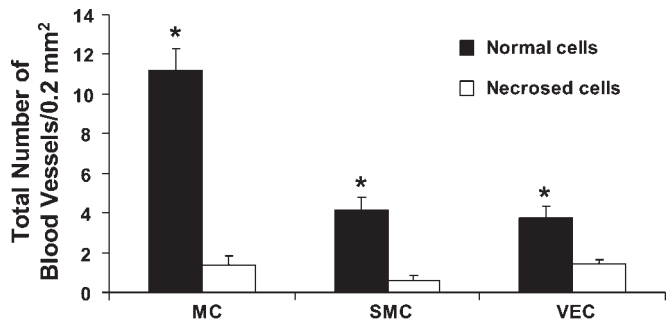

Fig. 4. Vessel formation in normal vs. necrosed cells. Total numbers of blood vessels induced 8 days after matrigel injection with normal MCs, SMCs, or VECs were significantly greater than those induced after injection of matrigel with heat-damaged (necrosed) cells. Data represent means ± SE ($n = 6$ rats per group). *$P < 0.01$.

Eight days after implantation with matrigel into the subcutaneous tissue, MCs treated with 10^{-11} M progesterone stimulated more arteriolar vessels and more total blood vessels ($P < 0.05$) than did untreated cells (Fig. 5*F*).

Effects of cell implantation on damaged hearts. Five weeks after cell injection, the group implanted with MCs exhibited significantly higher ($P < 0.05$) systolic and developed pressures by ANCOVA than those implanted with SMCs or media (at similar left ventricular end-diastolic pressures, between −5 and 35 mmHg). The maximal +d*P*/d*t* and minimum −d*P*/d*t* values were also improved ($P < 0.05$) in the MC implant group compared with the other two groups (Fig. 6).

The ratio of scar area to left ventricle free wall area in the MC implant group was significantly smaller ($P < 0.05$) than in the SMC implant or control (media injected) groups (Fig. 7*A*).

Implanted MCs also increased ($P < 0.01$) scar thickness compared with SMCs and controls (Fig. 7*B*).

Five weeks after cell injection into damaged heart tissue, the total number of arterioles and capillaries in the myocardial scar area was significantly greater ($P < 0.01$) in the group implanted with MCs than in the groups implanted with SMCs or media (Fig. 8), though more ($P < 0.01$) vessels were observed in the SMC group than in the controls.

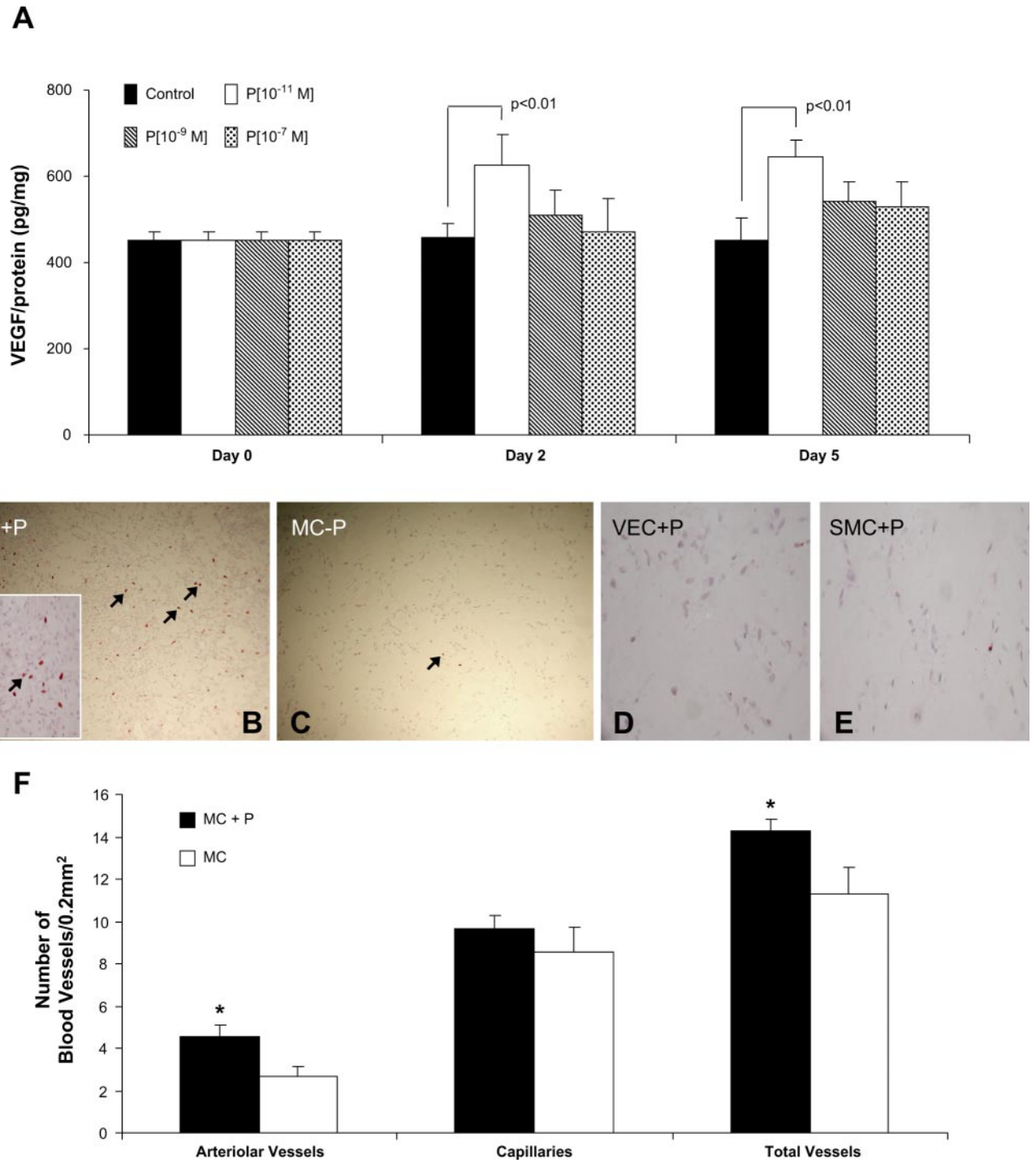

Fig. 5. Effects of progesterone on MCs. *A*: at 2 and 5 days after incubation with progesterone, VEGF levels rose significantly ($P < 0.01$) in MCs cultured with progesterone at a concentration of 10^{-11} M (P[10^{-11} M]) relative to those cultured at higher concentrations (P[10^{-9} M], P[10^{-7} M]) or without progesterone (Control) ($n = 6$ tests per group per time point). *B–E*: light microphotographs (staining with an anti-progesterone receptor antibody) revealed that MCs cultured with progesterone (MC + P, arrows in *B*, large panel and *inset*) had more progesterone receptors than MCs cultured without progesterone (MC − P, arrow in *C*), and VECs (VEC + P; *D*) or SMCs (SMC + P; *E*) cultured with progesterone. *F*: MCs treated with 10^{-11} M progesterone (MC + P) stimulated significantly more arteriolar vessels and total blood vessels than untreated MCs (MC) 8 days after subcutaneous tissue implantation of cells with matrigel ($n = 6$ rats per goup). Magnification is ×100 in *B* and *C*; ×400 in *inset* of *B*; and ×400 in *D* and *E*. All data represent means ± SE. *$P < 0.05$.

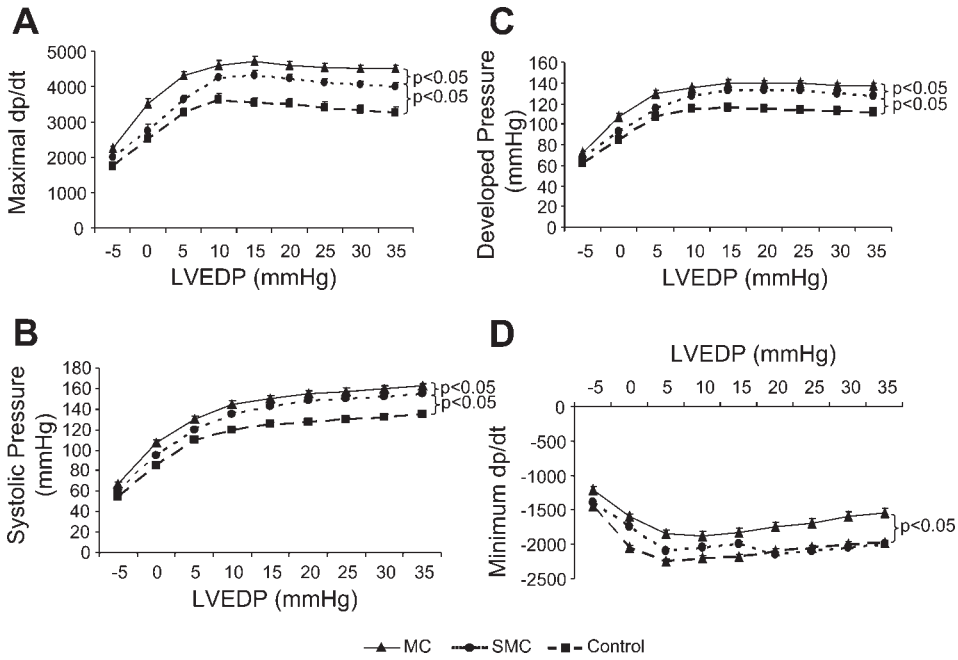

Fig. 6. Cardiac function. *A–D*: 5 wk after cell injection, at left ventricular (LV) end-diastolic pressure (LVEDP) within the −5- to 35-mmHg range, the group implanted with MCs displayed significantly higher ($P < 0.05$) values for maximal positive first derivative of time course of LV pressure generation (maximal +dP/d*t*; *A*), systolic pressure (*B*), and developed pressure (*C*) than groups implanted with SMCs or media (Control). Minimum −dP/d*t* values were also improved ($P < 0.05$) in MC implant group compared with the other two groups at LVEDP within the −5- to 15-mmHg range (*D*). Data represent means ± SE ($n = 12$ rats per group).

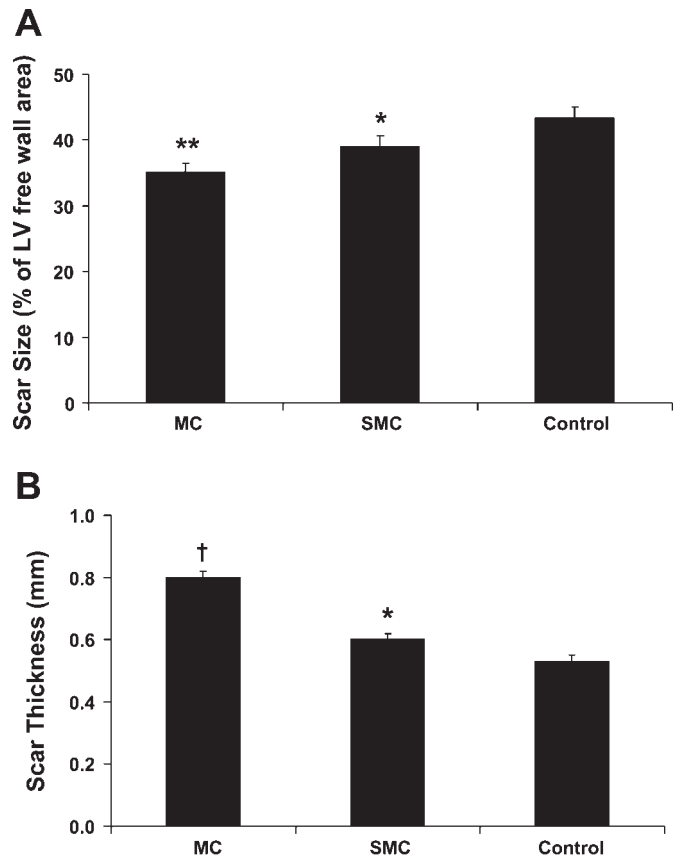

Fig. 7. Scar size and thickness. *A*: 5 wk after cell injection, myocardial scar size (expressed as percentage of LV free wall area) was significantly reduced in the group implanted with MCs compared with groups implanted with SMCs or media (Control). *B*: implanted MCs also increased scar thickness relative to SMCs and controls. Data represent means ± SE ($n = 12$ rats per group). *$P <$ 0.05 vs. Control; **$P < 0.05$ vs. SMC; †$P < 0.01$ vs. SMC.

DISCUSSION

Cell transplantation into the myocardium has been under extensive investigation because of its potential to restore heart function after myocardial infarction (4). Although several mechanisms have been proposed to explain the functional improvements associated with cell transplantation, it is likely that angiogenesis derived from the implanted cells plays a particularly important role. Significant angiogenesis has previously been demonstrated after implantation of skeletal muscle cells, bone marrow cells, SMCs, and endothelial cells.

This study describes the angiogenic potential of MCs, and the dose-sensitive augmentation of this effect by progesterone. We hypothesized that the extent and type of angiogenesis induced are determined by the type of cells implanted, and we demonstrated that MCs induced greater angiogenesis than either aortic SMCs or aortic endothelial cells. Of particular note, MCs induced more arteriolar structures (larger vessels with smooth muscle contributions to the media) than other cell types. This effect was dose dependent, with an optimal dose of 6×10^6 cells in matrigel for subcutaneous tissue injection. These findings suggest that uterine cells have a significant angiogenic potential that might be exploited to induce angiogenesis in the heart. To that point, an in vivo myocardial implantation study using the experimentally determined optimal cell dose demonstrated that MCs implanted into damaged myocardium increased blood vessel density, prevented cardiac thinning and dilatation (increased scar thickness and decreased infarct size), and improved heart function. These effects were greater than those produced by implanting SMCs or media without cells.

Although the mechanism by which implanted cells stimulate angiogenesis has not been fully elucidated, we believe that the cells contribute significantly to the angiogenic effect. Our matrigel study showed that, similar to the control (media without cells) group, myometrial, aortic smooth muscle, and aortic endothelial cells necrosed by heating did not increase

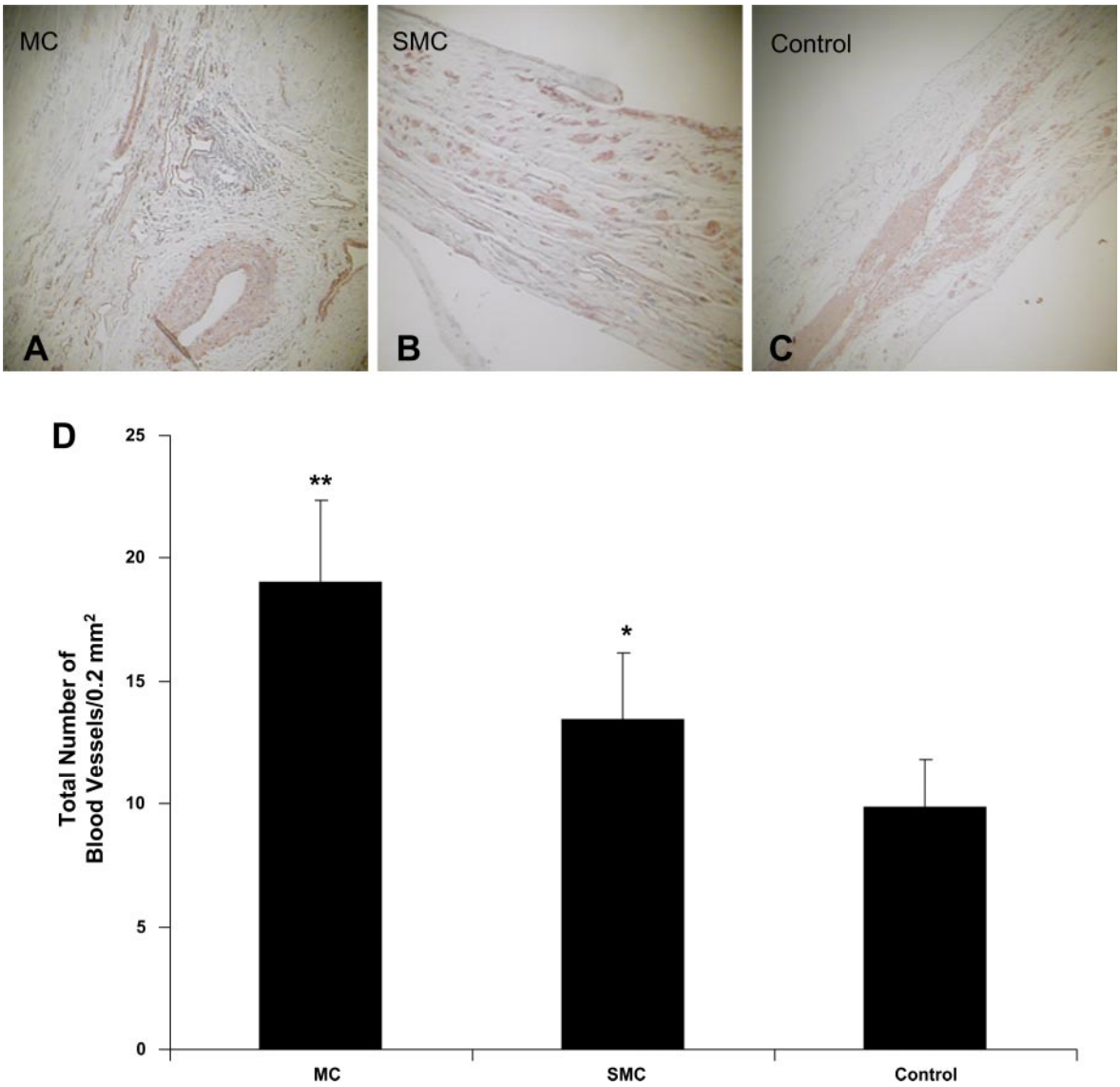

Fig. 8. Vessel formation in damaged heart. *A–C*: light microphotographs (hematoxylin and eosin staining) showing blood vessels forming 5 wk after cell injection in myocardial scar area of hearts implanted with MCs (*A*), SMCs (*B*), or media (Control; *C*) 3 wk after cryoinjury. *D*: at 5 wk after cell injection, total number of blood vessels in myocardial scar area was significantly greater in MC group than in either of the other two groups. Magnification is ×100 in *A–C*. Data represent means ± SE ($n = 12$ rats per group). *$P < 0.01$ vs. Control; **$P < 0.01$ vs. SMC.

vascular structures significantly after subcutaneous implantation with matrigel. These data suggest that the angiogenic effect resulted mainly from the biological activity of the implanted cells. These cells may contribute to angiogenesis in at least two ways: *1*) After they are implanted into the myocardium, the cells likely secrete angiogenic factors that stimulate regional angiogenesis. However, different cell types produce different types and amounts of such factors, some of which may favor arteriolar formation, while others favor capillary formation. Before implantation, we identified high levels of angiogenic factors (VEGF) in the MCs compared with the SMCs and endothelial cells; this level was twice as high as in the media control group (data not shown). *2*) Angiogenic factors secreted by the implanted cells may attract angiogenic cells necessary for blood vessel formation—-a process in which cells such as SMCs and endothelial cells may participate. We have previously shown that transplanted cells were incorporated into newly formed blood vessels (12).

Endometrial angiogenesis proceeds under the control of the ovarian steroids estrogen and progesterone, via their receptors, which function as hormone-activated transcription factors and modulators of gene expression in the targeted cells (2, 7). To date, most research has focused on the effects of estrogen on endometrial angiogenesis, and, in general, estrogen is thought to stimulate new vessel growth. In contrast, few studies have been performed on the effects of progesterone on endometrial angiogenesis (6, 19). Previous studies have produced conflicting results, either supporting a proangiogenic role (10) or an antiangiogenic role (24) for progesterone. There is, therefore, a need for further research to elucidate the role of progesterone in the process of blood vessel formation.

Our data indicate that cultured MCs express VEGF, and that VEGF levels are upregulated significantly by progesterone, at

an optimal dose of 10^{-11} M. Cells treated with progesterone stimulated more blood vessel formation than did untreated cells after they were implanted into subcutaneous tissue with matrigel. These results are in contrast to those produced in previous studies (24), which showed that progesterone inhibits endothelial cell proliferation through a nuclear receptor-mediated mechanism. However, the discrepancy may be partially explained by the fact that the previous studies were performed in a different species, with different cell types and under different experimental conditions than ours. Notably, Vazquez et al. (24) used a higher dose of progesterone (10^{-6} M) than the much lower concentration (10^{-11} M) at which we observed angiogenic effects.

Our data agree with that of Kayisli et al. (10), who reported that progesterone (at a relatively low concentration of 10^{-10} M) had proliferative and angiogenic effects on human endometrial endothelial cells in culture, indicated by an enhanced capacity to form angiogenic patterns such as tubelike structures and sprouts 5–8 days after treatment. In that study, the authors suggested that the progesterone-induced proliferation was most likely caused by a direct, but nongenomic, effect of progesterone on human endometrial endothelial cells.

In the current study, we did not directly compare the effects of estrogens versus progesterones on MC-induced angiogenesis. In native uterine tissue, both hormones participate in blood vessel formation and maintain the uterine lining. Future studies will require a more complete evaluation of the effects of estrogen, progesterone, and a combination of the two on vessel formation in vitro and in an animal model of ischemic cardiomyopathy. To determine whether ovarian function is required for the angiogenic response to MC implantation, additional studies using ovariectomized rats will be required. Furthermore, as this initial, proof of concept study was performed in female rats, future studies will also need to establish whether the effects observed here in females would also benefit males. Finally, a complete elucidation of the factors involved in the angiogenic response to progesterone therapy would be valuable. Altogether, such studies will help determine whether hormonal stimulation of angiogenesis could augment the beneficial effects associated with MC transplantation, and clarify the clinical relevance of this new approach to biological bypass.

Because the angiogenic effect of progesterone therapy was found to be dose sensitive in our rat studies, the clinical application of such an approach will require extensive safety and efficacy studies. For example, recent studies indicate that long-term administration of progesterone may increase the incidence of hormone-sensitive cancers or precipitate fatal cardiovascular events in humans. Still, cell transplantation augmented by short-term hormone treatment given after a myocardial infarction may offer the promise to restore ventricular function and improve quality of life in postmenopausal women.

In conclusion, the present study demonstrated that MCs exhibit a greater angiogenic potential than do SMCs or endothelial cells. Progesterone had a positive effect on blood vessel formation, as evidenced by increased levels of VEGF in cells cultured with progesterone, and higher blood vessel density within matrigel nodules containing the hormone. This study further showed that MC implantation into myocardial scar tissue limits infarct size and improves cardiac function, possibly mediated by enhanced angiogenesis.

ACKNOWLEDGMENTS

R.-K. Li is a Career Investigator of the Heart and Stroke Foundation of Canada and a Canada Research Chair in Cardiac Regeneration.

GRANTS

This study was funded by grants from Canadian Institutes of Health Research (MOP62698 and MOP14795) and Heart and Stroke Foundation of Ontario (T5206 and NA5294) (to R.-K. Li).

REFERENCES

1. **Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, Grunwald F, Aicher A, Urbich C, Martin H, Hoelzer D, Dimmeler S, and Zeiher AM.** Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation* 106: 3009–3017, 2002.
2. **Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J, and Nilsson S.** Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. *Mol Pharmacol* 54: 105–112, 1998.
3. **Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, and Anversa P.** Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* 114: 763–776, 2003.
4. **Brehm M and Strauer BE.** Stem cell therapy in postinfarction chronic coronary heart disease. *Nat Clin Pract Cardiovasc Med* 3, *Suppl* 1: S101–S104, 2006.
5. **Burkhoff D, Schmidt S, Schulman SP, Myers J, Resar J, Becker LC, Weiss J, and Jones JW.** Transmyocardial laser revascularisation compared with continued medical therapy for treatment of refractory angina pectoris: a prospective randomised trial. ATLANTIC Investigators Angina Treatments-Lasers and Normal Therapies in Comparison. *Lancet* 354: 885–890, 1999.
6. **Goodger AM and Rogers PA.** Uterine endothelial cell proliferation before and after embryo implantation in rats. *J Reprod Fertil* 99: 451–457, 1993.
7. **Hewitt SC and Korach KS.** Progesterone action and responses in the alphaERKO mouse. *Steroids* 65: 551–557, 2000.
8. **Horvath KA.** Results of prospective randomized controlled trials of transmyocardial laser revascularization. *Heart Surg Forum* 5: 33–39, 2002.
9. **Isner JM.** Myocardial gene therapy. *Nature* 415: 234–239, 2002.
10. **Kayisli UA, Luk J, Guzeloglu-Kayisli O, Seval Y, Demir R, and Arici A.** Regulation of angiogenic activity of human endometrial endothelial cells in culture by ovarian steroids. *J Clin Endocrinol Metab* 89: 5794–5802, 2004.
11. **Kim BO, Tian H, Prasongsukarn K, Wu J, Angoulvant D, Wnendt S, Muhs A, Spitkovsky D, and Li RK.** Cell transplantation improves ventricular function after a myocardial infarction: a preclinical study of human unrestricted somatic stem cells in a porcine model. *Circulation* 112: I96–104, 2005.
12. **Kim EJ, Li RK, Weisel RD, Mickle DA, Jia ZQ, Tomita S, Sakai T, and Yau TM.** Angiogenesis by endothelial cell transplantation. *J Thorac Cardiovasc Surg* 122: 963–971, 2001.
14. **Kofidis T, de Bruin JL, Hoyt G, Ho Y, Tanaka M, Yamane T, Lebl DR, Swijnenburg RJ, Chang CP, Quertermous T, and Robbins RC.** Myocardial restoration with embryonic stem cell bioartificial tissue transplantation. *J Heart Lung Transplant* 24: 737–744, 2005.
15. **Laflamme MA and Murry CE.** Regenerating the heart. *Nat Biotechnol* 23: 845–856, 2005.
16. **Leotta E, Patejunas G, Murphy G, Szokol J, McGregor L, Carbray J, Hamawy A, Winchester D, Hackett N, Crystal R, and Rosengart T.** Gene therapy with adenovirus-mediated myocardial transfer of vascular endothelial growth factor 121 improves cardiac performance in a pacing model of congestive heart failure. *J Thorac Cardiovasc Surg* 123: 1101–1113, 2002.
17. **Li RK, Jia ZQ, Weisel RD, Merante F, and Mickle DA.** Smooth muscle cell transplantation into myocardial scar tissue improves heart function. *J Mol Cell Cardiol* 31: 513–522, 1999.

18. **Li RK, Jia ZQ, Weisel RD, Mickle DA, Zhang J, Mohabeer MK, Rao V, and Ivanov J.** Cardiomyocyte transplantation improves heart function. *Ann Thorac Surg* 62: 654–660, 1996.
19. **Ma W, Tan J, Matsumoto H, Robert B, Abrahamson DR, Das SK, and Dey SK.** Adult tissue angiogenesis: evidence for negative regulation by estrogen in the uterus. *Mol Endocrinol* 15: 1983–1992, 2001.
20. **Popovici RM, Irwin JC, Giaccia AJ, and Giudice LC.** Hypoxia and cAMP stimulate vascular endothelial growth factor (VEGF) in human endometrial stromal cells: potential relevance to menstruation and endometrial regeneration. *J Clin Endocrinol Metab* 84: 2245–2248, 1999.
21. **Reinecke H, Minami E, Poppa V, and Murry CE.** Evidence for fusion between cardiac and skeletal muscle cells. *Circ Res* 94: e56-e60, 2004.
22. **Shifren JL, Tseng JF, Zaloudek CJ, Ryan IP, Meng YG, Ferrara N, Jaffe RB, and Taylor RN.** Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. *J Clin Endocrinol Metab* 81: 3112–3118, 1996.
23. **Smith SK.** Angiogenesis, vascular endothelial growth factor and the endometrium. *Hum Reprod Update* 4: 509–519, 1998.
24. **Vazquez F, Rodriguez-Manzaneque JC, Lydon JP, Edwards DP, O'Malley BW, and Iruela-Arispe ML.** Progesterone regulates proliferation of endothelial cells. *J Biol Chem* 274: 2185–2192, 1999.
25. **Yoon YS, Wecker A, Heyd L, Park JS, Tkebuchava T, Kusano K, Hanley A, Scadova H, Qin G, Cha DH, Johnson KL, Aikawa R, Asahara T, and Losordo DW.** Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. *J Clin Invest* 115: 326–338, 2005.
26. **Zhang L, Rees MC, and Bicknell R.** The isolation and long-term culture of normal human endometrial epithelium and stroma. Expression of mRNAs for angiogenic polypeptides basally and on oestrogen and progesterone challenges. *J Cell Sci* 108: 323–331, 1995.