Skin-derived microorgan autotransplantation as a novel approach for therapeutic angiogenesis

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1Divisions of Cardiology and Cardiac Surgery, Laval Hospital, Quebec City, Quebec, Canada; 2Beth Israel Deaconess Medical Center-Harvard Medical School, Boston, Massachusetts; 3Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem, Israel; and 4First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China

Submitted 28 January 2007; accepted in final form 10 September 2007

Voisine P, Rosinberg A, Wykrzykowska JJ, Shamis Y, Wu GF, Appelbaum E, Li J, Sellke FW, Pinto D, Gibson CM, Mitrani E, Laham RJ. Skin-derived microorgan autotransplantation as a novel approach for therapeutic angiogenesis. Am J Physiol Heart Circ Physiol 294: H213–H219, 2008. First published October 26, 2007; doi:10.1152/ajpheart.00112.2007.—Despite promising preclinical results, transient single-factor-based therapeutic angiogenesis has shown no definitive benefits in clinical trials. The use of skin-derived microorgans (SMOs), capable of sustained expression of angiogenic factors and sustained viability with their cellular and extracellular elements, constitutes an alternative strategy. We sought to evaluate the efficacy of SMO implantation in a porcine model of chronic myocardial ischemia. Eighteen pigs underwent placement of an anorectal constrictor on the proximal circumflex artery. Three weeks later, split-thickness skin biopsies were harvested and pigs were randomized to lateral wall implantation of either 8 or 16 SMOs or blank injections. The procedure was safe and resulted in no adverse events. Three weeks after treatment, SMO implantation resulted in significant improvement of lateral wall perfusion during pacing, assessed by isotope-labeled microspheres [post- vs. pretreatment ratios of lateral anterior wall blood flow were 1.31 ± 0.09 (SMOs) and 1.04 ± 0.06 (controls); P = 0.03]. No significant difference in angiographic scores was observed. Microvascular relaxation in response to VEGF was impaired in the ischemic territory of the control group but returned to normal after SMO implantation, indicating restoration of endothelial function. Molecular studies showed significant increases in VEGF and CD31 expression in the ischemic area of treated animals. Morphometric analysis showed increased neovascularization with SMO treatment. Autotransplantation of SMOs constitutes a novel approach for safe and effective therapeutic angiogenesis with improvement in perfusion, normalization of microvascular reactivity, and increased expression of VEGF and CD31.

growth factors; myocardial ischemia

AS MANY AS TWELVE PERCENT of patients undergoing coronary angiography have end-stage coronary artery disease (CAD) that is not amenable to treatment with conventional revascularization (22). Among patients who are candidates for surgical revascularization, a significant proportion have at least one ungraftable myocardial territory due to poor target vessels and distal runoff (25). Many of these patients have residual angina despite maximal medical therapy. Therapeutic angiogenesis has been proposed as an alternative treatment strategy for these patients (9, 15). However, clinical trials testing the efficacy of single growth factors have thus far not met the expectations of preliminary studies (12, 19, 28). It is possible that inadequate sample size or lack of sensitive end points may have limited the ability to detect an effect in these trials. Alternatively, the high incidence of risk factors for endothelial dysfunction (such as smoking or elevated cholesterol) in patients may have impaired the angiogenic potential of key growth factors (29). In addition, the inherent complexity of the angiogenic process in patients with end-stage CAD may warrant more complex strategies with delivery of multiple growth factors and angiogenic modulators for a prolonged duration (4).

Therapeutic angiogenesis could be improved on several levels. The development of new vessels within the myocardium in response to ischemia is the result of a cascade of events involving a wide variety of molecules (4, 5), and single growth factor approaches may be too simplistic. In this regard, strategies designed at overexpressing “master switch” regulatory genes such as related transcriptional enhancer factor-1 (RTEF-1) and hypoxia-inducible factor (HIF)-1α are being explored (6, 27, 30). The combined use of multiple growth factors that play key roles in the angiogenic process could be another suitable option. The route and duration of growth factor delivery represent two other aspects that could be optimized. The ultimate goal would be tissue-specific administration and sustained release of growth factors (17, 18). A third approach involving cell-based therapy with stem cells or endothelial progenitor cells is being investigated but may be limited by the viability of administered cells (7, 15, 23).

In view of these important limitations to successful therapeutic angiogenesis, we studied the concept of using small skin fragments (termed skin microorgans, SMOs) to promote collateral vessel development (10, 11). SMOs are skin organ fragments that preserve the epidermal mesenchymal interactions and the basic organ microstructure. SMOs have been shown to function as self-sustained units that can be cultured without serum or exogenous factors for several weeks in vitro and transcribe tissue-specific genes including a wide array of angiogenic factors (10, 11, 21). SMOs display a number of features that could overcome the problems associated with single growth factor approaches in vivo. They are prepared from autologous primary cells and have the potential to function locally, by paracrine, coordinated, sustained secretion of a whole spectrum of angiogenic factors. Accordingly, the pursual of this approach offers promise for this clinical need.

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pose of this study was to investigate the viability of SMOs cultured in both normoxic and hypoxic conditions in vitro and to evaluate the angiogenic potential of SMO autotransplantation in a porcine model of chronic myocardial ischemia.

METHODS

SMO preparation. Split thickness biopsies of ~1-mm depth were taken from the back region of Yorkshire pigs and cut into 10-mm-long, 0.3-mm-wide sections. The prepared SMOs were washed with DMEM (Cellgro, Herndon, VA) containing 1% PSN Antibiotic Mixture (GIBCO-BRL, Carlsbad, CA) and divided into three portions. The first portion was used for intramyocardial implantation in the porcine model with chronic myocardial ischemia, the second portion was used for viability testing, and the third portion was used for sterility testing.

SMO culture in vitro. SMOs were cultured in normoxic conditions (at 37°C in humidified 95% air-5% CO2) submerged in serum-free and SMOs [8 in (lanthanum) for perfusion analysis. After these baseline studies the left atrium (with a catheter positioned retrogradely in the left midventricle level, and then sectioned into systematically identified spheres (lutetium at rest and ytterbium under pacing) were injected as blood sampling. Coronary angiography was performed, and an arterial sheath was inserted for catheterization and distribution. Three weeks later, the animals were brought back for sterility testing.

In vivo experimental design. Eighteen Yorkshire pigs of either sex weighing 15–20 kg (5–6 wk of age) were used. All animal protocols were reviewed and approved by the Harvard Medical Area Institutional Animal Care and Use Committee. All animals received humane care in compliance with the National Research Council’s Guide for the Care and Use of Laboratory Animals (1996). The animals were anesthetized, prepped, and draped in the lateral decubitus position. Under sterile conditions, a left thoracotomy was performed through the fourth intercostal space during mechanical ventilation. The pericardium was opened, and an amiodar constrictor of 2.25- to 2.5-mm internal diameter (matched to the diameter of the artery) was placed around the proximal left circumflex coronary artery (LCX). Red-gold microspheres (BioPhysics Assay Laboratory, Worcester, MA) were injected in the left atrium during temporary occlusion of the circumflex artery to determine the exact myocardial territory at risk (“shadow-labeling procedure”). Animals were then allowed to recover for 3 wk (time sufficient for amiodar closure) before SMO implantation. At 3 wk the animals were anesthetized, and a right femoral cutdown was performed before insertion of an arterial sheath for catheterization and blood sampling. A split-thickness skin graft was taken from the back area to produce the SMOs. Coronary angiography was performed to document the amiodar occlusion and to assess the extent of collateral flow to LCX circulation. Microspheres (1.5 × 107) were injected in the left atrium (with a catheter positioned retrogradely in the left atrium) both at rest (samarium) and under pacing at 150 beats/min (lanthanum) for perfusion analysis. After these baseline studies the animals underwent a thoracotomy to expose the LCX myocardium, and SMOs [8 in group 1, 16 in group 2, and none (12 blank injections) in control group 3; n = 6/group] were implanted after the LCX distribution. Three weeks later, the animals were brought back for evaluation. They were anesthetized, a left femoral cutdown was performed, and an arterial sheath was inserted for catheterization and blood sampling. Coronary angiography was performed, and microspheres (lutetium at rest and ytterbium under pacing) were injected as described above. After euthanasia, hearts were harvested, cut at the midventricle level, and then sectioned into systematically identified segments. Samples from the anterior and ischemic left lateral walls were used for histology, molecular studies, in vitro assessment of microvessel reactivity, and microsphere perfusion analyses.

SMO viability. The prepared SMOs were cultured at a concentration of 20 SMOs per 1 ml of serum-free culture medium containing DMEM and 1% PSN Antibiotic Mixture (GIBCO-BRL) in a 24-well Cell Culture Cluster for 2 wk. SMO viability was assessed by incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml; Sigma-Aldrich, St. Louis, MO) at 37°C for 15 min immediately after preparation and every 24 h for in vitro studies and at the time of heart harvest for in vivo experiments. At the end of these studies, samples were collected for RNA and histological analysis.

Molecular studies. The cultured SMOs and myocardial tissue samples from LCX and left anterior descending coronary artery (LAD) distributions were homogenized in TRI reagent (Sigma, St. Louis, MO) buffer, and total RNA and proteins were extracted, following the manufacturer’s instructions.

Histological analysis. SMOs and tissue obtained from the heart, which included the implanted SMOs, were formalin fixed and paraffin embedded. Sections (5 μm) were obtained from all tissue samples, stained with hematoxylin-eosin, and examined microscopically.

Protein extraction and Western blotting. SMOs were homogenized in RIPA solution (Boston Bioproducts, Ashland, MA) and fractionated by 10% SDS-polyacrylamide gels. Protein extracts were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). VEGF, FGF-2, FGF receptor (FGFR), Flk-1, Flt-1, angiotatin, and endothelial nitric oxide synthase (eNOS) signals were detected with anti-VEGF, anti-Flk-1, and anti-Flk-1, anti-FGF-2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), anti-FGFR and anti-angiotatin antibodies (Oncogene Research Products, San Diego, CA), and anti-eNOS antibody (Cell Signaling, Beverly, MA). Immunoblots were visualized by ECL Western blotting detection reagents (Amersham Life Science, Arlington Heights, IL), quantified by densitometry with Image Quant software, and adjusted by the density of β-actin (sample loading control). The data are presented as a percentage of the control value (% control).

In vitro assessment of coronary microvessel reactivity. After cardiac harvest, epicardial coronary arteries (70–150 μm in diameter and 1–2 mm in length) originating from branches of the LAD and LCX were dissected and examined in isolated organ chambers as described previously (14, 26). The responses to sodium nitroprusside (SNP, 1 nM–100 μM), an endothelium-independent cGMP-mediated vasodilator, as well as to adenosine 5′-diphosphate (ADP, 1 μM–10 μM) and VEGF (1 fM–1 nM), two endothelium-dependent receptor-activated microspheres (BioPhysics Assay Laboratory, Worcester, MA) were injected in the left atrium during temporary occlusion of the circumflex artery to determine the exact myocardial territory at risk (“shadow-labeling procedure”). Animals were then allowed to recover for 3 wk (time sufficient for amiodar closure) before SMO implantation. At 3 wk the animals were anesthetized, and a right femoral cutdown was performed before insertion of an arterial sheath for catheterization and blood sampling. A split-thickness skin graft was taken from the back area to produce the SMOs. Coronary angiography was performed to document the amiodar occlusion and to assess the extent of collateral flow to LCX circulation. Microspheres (1.5 × 107) were injected in the left atrium (with a catheter positioned retrogradely in the left atrium) both at rest (samarium) and under pacing at 150 beats/min (lanthanum) for perfusion analysis. After these baseline studies the animals underwent a thoracotomy to expose the LCX myocardium, and SMOs [8 in group 1, 16 in group 2, and none (12 blank injections) in control group 3; n = 6/group] were implanted after the LCX distribution. Three weeks later, the animals were brought back for evaluation. They were anesthetized, a left femoral cutdown was performed, and an arterial sheath was inserted for catheterization and blood sampling. Coronary angiography was performed, and microspheres (lutetium at rest and ytterbium under pacing) were injected as described above. After euthanasia, hearts were harvested, cut at the midventricle level, and then sectioned into systematically identified segments. Samples from the anterior and ischemic left lateral walls were used for histology, molecular studies, in vitro assessment of microvessel reactivity, and microsphere perfusion analyses.

SMO viability. The prepared SMOs were cultured at a concentration of 20 SMOs per 1 ml of serum-free culture medium containing DMEM and 1% PSN Antibiotic Mixture (GIBCO-BRL) in a 24-well
icant difference in visible collateral vessel development and collateral flow among the three groups.

In vitro experiments. RNA isolation and MTT tests (data not shown) revealed that all SMOs remained viable in culture. Histological analysis indicated that their microstructure was unaffected (Fig. 1). The expression of basic FGF remained relatively high, with no significant difference between the hypoxic and normoxic conditions, while the expression of FGFR remained low under both conditions. In contrast, while the expression of \textit{flk-1} was constant during the entire length of culture under normoxic conditions (1.2 \times), under hypoxic conditions \textit{flk-1} expression significantly increased (2.5 \times) after a 72-h period of culture. As with \textit{flk-1}, an increase in \textit{flt-1} expression (2.1 \times) was visible after 72 h of culture under hypoxic conditions. No such increase was detected under normoxic conditions (1.1 \times). The expression of angiostatin was found to remain relatively constant under both hypoxic and normoxic conditions, and no significant difference was noted.

Safety of SMO implantation. The procedure was safe and resulted in no adverse events. None of the SMO-treated animals developed treatment-related complications such as arrhythmia, infection, or bleeding.

SMO viability in vivo. SMOs were collected from the hearts of nine pigs after completion of the experimental protocol 3 wk after implantation. All SMOs harvested were viable as evidenced by MTT and RNA testing, and histological analysis showed that they preserved the basic skin microstructure. Moreover, the presence of erythrocyte-filled capillaries invading the dermis of SMO implants was observed (Fig. 2).

Microvessel reactivity. Figure 3 shows the relaxation curves in response to increasing concentrations of vasodilators after preconstriction with the thromboxane/prostaglandin endoperoxide analog U-46619. Partial endothelial dysfunction in the circumflex distribution of control animals was noted with impaired vasorelaxation to VEGF, an endothelium-dependent vasodilator ($P < 0.01$ vs. LAD territory; Fig. 3A). However, the response to ADP, another endothelium-dependent vasodilator, was not significantly different. The altered microvascular response to VEGF induced by ischemia in the control group returned to normal after implantations of SMOs in both treated groups (Fig. 3, B and C). Relaxation to the endothelium-independent agent SNP was unaffected by ischemia or treatment modality, as expected.

Isotope-labeled microsphere evaluation of myocardial perfusion. The results of ILM assays in determining circumflex myocardial perfusion are shown in Figs. 4 and 5. Three weeks after ameroid placement, at the time of SMO implantation, baseline circumflex coronary flow was similar among the three groups, both at rest (group 1 (8 SMOs): 0.381 \pm 0.023, group 2 (16 SMOs): 0.348 \pm 0.026, control group 3 (12 blank injections): 0.395 \pm 0.032 ml/min/g; $P = 0.44$) and under pacing (group 1: 0.349 \pm 0.018, group 2: 0.347 \pm 0.032, group 3: 0.314 \pm 0.019 ml/min/g; $P = 0.53$) (Fig. 5). The ratios of blood flow in the circumflex territory to the LAD territory in the post- vs. pretreatment settings were 1.02 \pm 0.06 (group 1, 8 SMOs) vs. 1.06 \pm 0.03 (group 2, 16 SMOs) vs.

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**Fig. 1.** Histological section from a skin-derived microorgan (SMO) cultured for 72 h in serum-free medium. The culture preserves the basic skin microstructure. Note that, as expected, capillaries (*) are void of cells.

**Fig. 2.** Histological sections from intramyocardial SMO implants in a porcine model of chronic myocardial ischemia 3 wk after implantation (A and B). The sections illustrate that SMO implants remain viable and preserve the basic skin microstructure. In addition, new red blood cell-filled small capillaries that vascularize the dermis of the implanted SMO were identified (amplified areas C–E). Note that the implanted microorgans look normal, with no signs of inflammation or tissue deterioration. In contrast to capillaries in Fig. 1, new red blood cell-filled small capillaries are found in the dermis of the implanted SMOs.
1.02 ± 0.10 (control group 3, 12 blank injections) at rest (P = 0.89) and 1.36 ± 0.13 (group 1) vs. 1.26 ± 0.14 (group 2) vs. 1.04 ± 0.10 (group 3) under pacing (P = 0.20). When all treated animals were considered as a single group (predefined analysis plan) and compared with control pigs, the perfusion ratios remained not different at rest (1.02 ± 0.10 control pigs vs. 1.04 ± 0.10 treated pigs; P = 0.83), but there was a significant increase in perfusion ratios under pacing associated with SMOs implantations (1.04 ± 0.06 control pigs vs. 1.31 ± 0.09 treated pigs; P = 0.03; Fig. 5).

Endothelial cell density. Figure 6 shows the density of CD31+ capillary endothelial cells in the left lateral wall of pigs from all treatment groups 3 wk after treatment. There was a tendency toward increased cell density in groups 1 and 2 compared with controls (group 3 (controls): 52 ± 5, group 1 (8 SMOs): 78 ± 12, group 2 (16 SMOs): 72 ± 7; P = 0.09; Fig. 6A). When all treated pigs were compared with controls, endothelial cell density was significantly higher in pigs that had undergone SMO implantations (75 ± 7 vs. 52 ± 5; P = 0.03; Fig. 6B).

Western blot analyses. Densitometry of angiogenic and endothelial protein levels in the ischemic territory is displayed in Fig. 7. Comparisons of CD31, VEGF, FGF-2, FGFR1, and eNOS expression among the three groups were significant for VEGF and eNOS [P values (ANOVA) were 0.21, 0.03, 0.10, 0.42, and 0.02, respectively]. These were explained by a significant increase in VEGF expression in animals treated with 8 SMOs versus control animals (P = 0.02) and a significant decrease in eNOS expression in animals treated with 16 SMOs versus those treated with 8 SMOs (P = 0.03).
DISCUSSION

Several studies confirming the potential of growth factor-based therapeutic angiogenesis to promote the development of new vessels in animal models of myocardial or peripheral ischemia have been published over the last decade (8, 13, 14, 16, 18, 20, 24, 26). Many of these studies have brought to light the biological and technical pitfalls of protein or gene therapy for angiogenesis. Despite important advances in knowledge and technical development, initial human experience with therapeutic angiogenesis has been less successful than experience from animal models would have predicted (12, 19, 28).

The molecular mechanisms governing the endogenous angiogenic process are complex and involve a multitude of genes whose expression must be coordinated to produce adequate amounts of closely interacting and interdependent proteins, with appropriate timing. For example, studies have demonstrated that VEGF and angiopoietin-1 are expressed sequentially during embryogenesis, each controlling specific and complementary functions that lead to the formation of mature blood vessels. Angiogenic processes also seem to be closely intertwined with other important physiological regulatory determinants of vascular biology, such as endothelial function.

Several studies have emphasized the important relationship between the release of NO and the regulation of VEGF-mediated blood vessel growth and development. VEGF enhances the expression of eNOS in native and cultured endothelial cells, an effect that may be important in the process of VEGF-induced angiogenesis (2). eNOS expression is increased in proliferating compared with confluent endothelial cells (1). Moreover, inhibitors of eNOS suppress angiogenesis, and the proliferative response to VEGF is decreased in the presence of eNOS inhibitors (31). A recent study examining the roles of VEGF receptor types 1 (VEGFR-1) and 2 (VEGFR-2) has reported that VEGF-stimulated NO release is inhibited by the blockade of VEGFR-1. VEGFR-1, in turn via NO-dependent mechanisms, inhibits VEGFR-2-mediated endothelial cell proliferation and promotes formation of capillary networks in human umbilical vein endothelial cells (3). Consequently, it has been suggested that VEGFR-1 functions as a signaling receptor. It promotes endothelial cell differentiation into vascular tubes. This occurs in part by limiting NO-dependent VEGFR-2-mediated endothelial cell proliferation. NO appears to be a molecular switch for endothelial cell differentiation. Altogether these results illustrate the importance of preserving a physiological approach to orchestrate a successful angiogenic response.

In view of these challenges, SMOs may offer several advantages over conventional single growth factor administration strategies for therapeutic angiogenesis. The results from in vitro
SMOs that could be safely harvested as well as implanted without risking significant damage to the skin or the myocardium. Nonetheless, regardless of the numbers implanted, the fact that intramyocardial SMO incorporations were associated with increased endothelial cell density and improved perfusion, overexpression of eNOS expression in the ischemic territory of animals that had 16 SMOs implanted was due to elaboration of readily bioavailable NO by the SMOs. Alternatively, this “dose” of SMOs could have been too high and resulted in deleterious effects on endothelial function. It is possible that SMO implantation resulted in the restoration of endothelial function by the multitude of angiogenic cytokines and mediators secreted and thereby contributed to increased effectiveness of growth factors. “Repaired” endothelium became capable of adequate response to factors such as VEGF, the response to which is dependent on endothelial NO availability. This mechanism would be reminiscent of endothelial function normalization and restoration of the angiogenic response to VEGF following oral l-arginine supplementation in a porcine model of ischemia with hypercholesterolemia-induced endothelial dysfunction (29).

Apart from the endothelial dysfunction present in most patients considered for therapeutic angiogenesis, and the limitations of single growth factor approaches employed thus far, previous clinical trials may have been flawed by problems pertaining to the route and duration of delivery. Protein therapy allows for a more controlled regulation of expression and offers the possibility of multiagent and repeated administration, but the duration of expression is limited. Gene therapy on the other hand, provides longer expression; however, regulations of expression levels as well as multiagent and repeated administrations are more difficult to achieve. Systemic injections of growth factors carry the theoretical risk of exposing nontarget tissues to the biological effects of the systemic levels of growth factors, with the potential hazards of stimulating tumor development. Prior work from our group (16–18) has shown that intramyocardial delivery is the most favorable approach and results in local biological effects. SMOs may offer significant advantages over previously attempted treatment options. Derived from nonimmunogenic autologous cells, they can be implanted directly within the ischemic myocardium and its bordering zones, where they remain viable at least for the 3-wk period examined in our study protocol. The appearance of small neovessels in the vicinity of the implanted SMOs penetrating the dermis suggests that the implanted microorgans are capable of long-term viability and sustained release of growth factors. In addition, SMOs have the potential for adapting their angiogenic protein secretion levels in response to the biological modifications of their environment over time. These properties are speculative at this moment and need to be confirmed in further experiments.

The absence of a dose-response effect of SMOs on perfusion and endothelial cell density are important limitations of our study. The absence of any prior in vivo study on the effect of SMOs on the myocardium prompted an empirical determination of the number of SMOs required for implantation. We chose to implant either 8 or 16 SMOs in an area of ~20 cm², ensuring that implantations were also done in the bordering zones between the ischemic and nonischemic territories. We used these parameters to determine the adequate numbers of SMOs that could be safely harvested as well as implanted without risking significant damage to the skin or the myocardium. Nonetheless, regardless of the numbers implanted, the fact that intramyocardial SMO incorporations were associated with increased endothelial cell density and improved perfusion.
is a proof of concept that self-functioning microorgans can be used as a novel and promising therapeutic alternative for ischemic diseases.

In conclusion, this study demonstrates that skin microorgans remain viable after autotransplantation in the myocardium and improve myocardial perfusion in a porcine model of ischemia. This improvement in perfusion is associated with increased expression of VEGF and normalization of microvascular reactivity in the ischemic territory. Implantation of skin microorgans may thus constitute a novel approach to therapeutic angiogenesis. Additional studies are required to confirm the potential use of skin microorgans for the treatment of patients with end-stage CAD who are not candidates for conventional revascularization techniques, or as adjunct therapy at the time of coronary artery bypass grafting.

REFERENCES