

Development of a Human Cardiac Tissue-Based Angiogenesis Model

James M. Lewis, M.D.,* Catherine T. Anthony, Ph.D.,* Lynn H. Harrison, M.D.,†‡
T. Bruce Ferguson, M.D.,†‡ H. Andrew Heck, M.D.,†‡ Forrest Rubenstein, M.D.,†‡
Douglas A. Balster, M.D.,§ and Eugene A. Woltering, M.D.,*‡¹

*Department of Surgery Sections of Surgical Endocrinology, Louisiana State University Health Sciences Center (LSUHSC);
†Department of Cardiothoracic Surgery; ‡Veterans Administration Medical Center, New Orleans, Louisiana; §Department of Pediatrics,
Ohio State University, Columbus, Ohio

Submitted for publication August 21, 2005

Background. Previous angiogenesis models use animal tissues such as the chicken chorioallantoic membrane (CAM) or the rabbit cornea. These models may not accurately reflect the mechanisms responsible for human angiogenesis.

Materials and methods. We hypothesized that fragments of human myocardial tissue would develop an angiogenic response from the cut edges of vessels contained within the tissue. To test these hypotheses, we obtained human atrial appendage tissue at the time of cardiac bypass. Fragments of atrial tissue were then incorporated into fibrin thrombin clots. Tissue fragments were observed, and the percent of wells that developed neovessel invasion into the clot was calculated (%I). The subsequent growth of cardiac-derived microvessels was rated and scored over time (Angiogenic Index).

Results. There were 20 human atrial appendages plated ($n = 24$ to 60 wells/specimen) and evaluated in this model. Out of the 20, 16 (80%) atrial appendages developed an angiogenic response in the majority (>50%) of wells plated. Neovessel growth was progressive over 14 to 16 days in culture in all specimens tested. The mean angiogenic index of all specimens was 8.59 ± 0.91 .

Conclusions. This human cardiac tissue-based assay might be useful to screen compounds designed for use in human trials or provide highly vascularized cardiac tissue for autotransplantation. Additionally, the assay provides the foundation to study an individual patient's cardiac tissue and its response to angio-

genesis stimulators or inhibitors. This may allow the development of patient-specific therapies designed to enhance revascularization or repair of injured cardiac muscle. © 2006 Elsevier Inc. All rights reserved.

Key Words: angiogenesis; heart; revascularization; *in vitro*; human.

INTRODUCTION

Angiogenesis, the formation of new blood vessels, occurs normally during embryogenesis and pathologically in a variety of diseases [1–9]. Angiogenesis rarely occurs in healthy adults, except during certain phases of the human female reproductive cycle. A variety of natural and synthetic inhibitors of angiogenesis have been tested in *in vitro* and *in vivo* models in an effort to find a non-toxic, effective inhibitor of angiogenesis [10, 11].

An estimated 12 million people in the United States have coronary artery disease, with approximately one million people suffering new or recurrent heart attack each year, at an estimated 40% mortality [12]. The role of therapeutic angiogenesis in patients with coronary artery disease is currently under investigation [12–15]. Angiogenic responses in myocardial tissue may be critical after periods of infarction or relative ischemia. We hypothesized that fragments of human myocardial tissue embedded in a fibrin/thrombin matrix would develop an angiogenic response in a repeatable and controlled fashion. This new model, the *Human Cardiac Angiogenesis Model (HCAM)*, might be useful for evaluation of agents designed to stimulate or inhibit angiogenesis in myocardial tissue.

METHODS

To test these hypotheses tissue from 20 human atrial appendages was anonymously obtained at the time of cardiopulmonary bypass,

¹ To whom correspondence and reprint requests should be addressed at The James D. Rives Professor of Surgery and Neuroscience, Chief, Section of Surgical Endocrinology and Surgical Oncology, Director of Surgical Research, Louisiana State University Health Sciences Center, New Orleans, LA 70112. E-mail: ewolte@lsuhsc.edu.

with prior approval of the Louisiana State University Health Sciences Center Institutional Review Board. Cardiac specimens and their angiogenic response were tested in a fibrin-thrombin clot based angiogenesis assay [16, 17]. Briefly, the atrial appendage specimens were obtained and placed in refrigerated tissue culture medium. There were then 1 mm³ tissue fragments created using sharp dissection. These architecturally intact cardiac muscle fragments were then placed into wells in a standard 96-well plate (Corning Inc., Corning, NY). The tissue allocation was completed within 3 h of surgery to optimize cellular viability. Each well had been preloaded with a human thrombin solution (0.05 IU in 1.0 μ l/well) (Sigma Chemical Co., St. Louis, MO), which was allowed to evaporate to dryness before use.

After the placement of the tissue fragments into the bottom of each thrombin-containing well, the tissue was covered with 100 μ l of a clot-forming medium. This medium consisted of fibrinogen (3 mg/ml) and 0.5% Σ -amino caproic acid (Sigma Chemical Co.) added to nutrient medium. Nutrient medium consisted of medium-199 and an antibiotic/antimycotic solution (100 U penicillin, 100 U streptomycin sulfate, and 0.25 μ g amphotericin/ml). This mixture was allowed to clot by incubating overnight in a 6% CO₂, 94% air atmosphere at 37°C in a humidified incubator. The cardiac fragment-containing clot was then supplemented with 100 μ l of a nutrient medium containing 20% fetal bovine serum (GibcoBRL). Total well volume was 200 μ l. Nutrient medium was changed every 3 to 5 days.

Evaluation of Angiogenesis

Visual evaluation of all wells was performed on an inverted phase microscope at multiple levels of magnification. Tissue fragments were graded using two criteria: 1) initiation of sprouting (% initiation) and 2) degree of sprouting or response, angiogenic index (AI). Initiation of an angiogenic response was defined as the development of three or more sprouts around the periphery of the disk. The AI was defined using a semi-quantitative visual rating system devised in our laboratory. Briefly, each tissue fragment was visually rated for the development of vessel sprouting in all four quadrants. Each tissue quadrant was given a 0 to 4 rating, depending on the number of sprouts (density) and the lengths of the sprouts. Scores of all four quadrants were summed and the AI expressed as a numerical rating from 0 to 16. Previous experience using this system in a human placental vein model showed an excellent correlation between observer scores and more objective ratings, such as vessel length (mm) or a total vessel surface area (mm²) determined by digital image analysis [17, 18]. Multiple unbiased observers grading a series of identical wells had nearly identical AI scores.

Histology

We have previously demonstrated that fragments of human placental veins develop an angiogenic response in the fibrin-thrombin clot model. In that study we used transmission electron microscopy to confirm that these neovessel sprouts were endothelial in nature and confirmed these findings with immunohistochemical stains for factor VIII [18]. In these current experiments, the wells with the highest angiogenic indices were fixed and evaluated by standard histology. The nutrient media was removed and a 10% paraformaldehyde solution added for 1 h. This solution was removed and replaced with fresh paraformaldehyde for an additional hour. The paraformaldehyde was again removed, and normal saline added to the wells. Routine histology sections were oriented en face, demonstrating the full thickness cardiac myocardium specimen, and fibrin/thrombin clot containing the myocardial-derived neovessels.

RESULTS

Initiation

To evaluate the onset of the angiogenic response (initiation) in the 20 human cardiac muscle specimens,

TABLE 1

Atrial appendage	D.I.C.*	N	Day initiated	%I†	AI‡ \pm SEM
1	15	58	6	98	14.74 \pm .34
2	16	49	5	90	12.08 \pm .78
3	14	60	6	40	2.52 \pm .48
4	14	60	4	75	6.55 \pm .69
5	14	60	7	37	1.77 \pm .4
6	14	60	5	88	8.12 \pm .68
7	14	59	2	95	12.37 \pm .64
8	15	60	4	95	9.05 \pm .51
9	15	60	3	98	13.6 \pm .46
10	15	60	3	97	12.68 \pm .61
11	14	30	3	87	10.4 \pm .93
12	15	29	6	27	1.48 \pm .58
13	14	24	4	46	2.96 \pm .85
14	14	30	5	73	7.6 \pm 1.1
15	14	30	4	97	10.57 \pm .96
16	14	30	6	73	8.2 \pm 1.1
17	15	30	3	80	10.37 \pm 1.1
18	15	28	6	61	5.04 \pm 1.2
19	14	40	5	83	10.15 \pm .98
20	14	30	2	87	11.63 \pm 1.3

Outlines the percent of wells developing an angiogenic response in culture and the subsequent growth (angiogenic index, 0–16 scale) of neovessels in this assay system. Data on 20 human cardiac specimens evaluated in the fibrin-thrombin clot angiogenesis assay are presented. Day initiated represents the first day on which visible neovessel sprouts were observed

* D.I.C. = Days In Culture.

† %I = Percent Initiation; percent of wells demonstrating neovessels / the number wells plated was evaluated.

‡ AI = Angiogenic Index \pm Standard Error of the Mean.

the percent of wells demonstrating neovessels / the number of wells plated was evaluated (Table 1). All 20 specimens elicited an angiogenic response. For any one specimen an angiogenic response was observed in 27 to 98% of the wells plated. Average initiation rate for all specimens was 73%. As a comparison, 50% initiation of controls in placental vein disk assays is considered successful [16]. Onset of initiation for the 20 samples ranged from 2 to 7 days.

AI

All atrial appendage specimens were evaluated using our semi-quantitative AI (a 0–16 scale) as described above (Table 1). The mean AI \pm SEM of the 20 specimens was 8.59 \pm 0.91 and is similar to values obtained with the human placental vein assay [16]. The AI in individual human cardiac specimens ranged from a low of 1.77 \pm 0.4 to a high of 14.74 \pm 0.34.

Histology

Proliferating cells were factor VIII positive, confirming the presence of endothelial cells (neovessels) within the fibrin/thrombin clot (Figs. 1, 2).

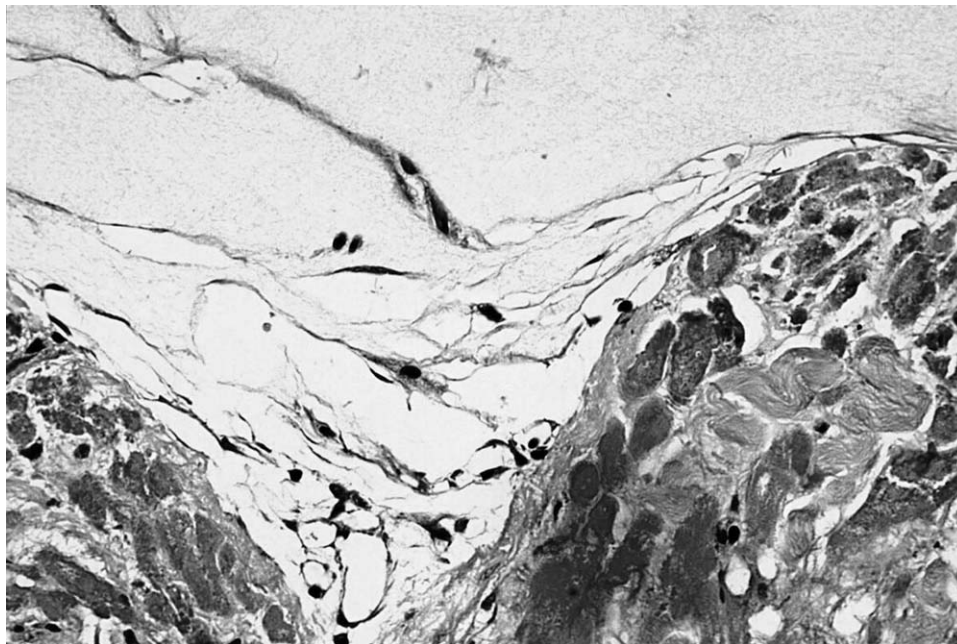


FIG. 1. Figure 1 demonstrates standard hematoxylin and eosin staining of an angiogenic human atrial appendage fragment in a fibrin-thrombin clot. The central area demonstrates the angiogenic vessels that invaded the clot (20 \times).

DISCUSSION

Angiogenesis occurs in normal tissue, such as endometrium, during the adult reproductive cycle, and pathologically in tissues with both malignant and non-malignant diseases [1–9]. Angiogenesis after myocardial ischemic events may be critical for recovery and continuing survival. Recent studies that attempted to manipulate cardiac angiogenesis have demonstrated promising results. Schumacher *et al.* demonstrated angiographically that recombinant human FGF-1 injected into patients undergoing elective CABG resulted in increased capillary networks around injection site at 12-weeks post-operatively [13]. Three-year follow up demonstrated persistent angiographic findings, and improved LVEF as compared to controls [14]. In one small trial four of six patients with severe angina pectoris who received intramyocardial injection of phVEGF-A165 via thoracotomy showed improved angina, myocardial tissue velocity, and perfusion [15]. Patients injected with intravenous Ad5-FGF4 have demonstrated improved exercise treadmill tests at 4 and 12 weeks compared to placebo treated patients [12].

The model presented here is a modification of our human placental vein angiogenesis assay [16–19]. The assay allows for a rapid evaluation of angiogenesis, using human tissue fragments of placental veins. Previous investigations have documented the observed cell proliferation as endothelial in nature, and consistent with neovascularization [17]. We have used this placental-based assay to evaluate the effect of drugs [16, 17] and plant extracts [18, 19]. Recently, we have extended the model



FIG. 2. Figure 2 is a photomicrograph demonstrating immunohistochemical localizing of factor VIII on the endothelial tubes invading the fibrin-thrombin clot matrix (20 \times). (Color version of figure is available online.)

to include a variety of animal [21], and unpublished data investigating bovine retinal angiogenesis and human tissues [20]. Our most recent studies suggest that the observed *in vitro* angiogenesis indices mimic observed clinical responses (data not yet published investigated in our laboratory). Although far from definitive, these most recent data do suggest that clinical responses may parallel angiogenic responses. Realizing the potential usefulness of a cardiac model system, the initial study presented here demonstrates that a significant angiogenic response can be elicited using the cardiac atrial appendage. In addition, because the proliferation is factor VIII positive, the cells are endothelial and therefore indicative of neo-vascularization. As this was an initial study, we did not request other cardiac sources (ventricular). Ethical considerations (IRB) must be addressed for future studies to obtain "normal" tissue with which to assess the angiogenic potential of normal *versus* diseased cardiac tissue. If validated, this model system holds the potential to expand several aspects of cardiac care. The HCAM model might be useful for testing possible therapeutic agents that are thought to enhance cardiac myocardial tissue revascularization. This human tissue-based model has the advantage that the myocardial tissue used is architecturally intact; containing vessels, nerves, and supporting stromal elements. Using the HCAM in *in vitro* studies could be a stepping-stone to translational *in vitro/in vivo* experiments. The efficacy of angiogenic stimulants could be assessed in our assay, and the results used to design *in vivo* protocols. In our experience this human cardiac-based angiogenesis model is inexpensive, predictable, and reproducible. There does appear to be wide variation among individual patient angiogenic potentials. This might suggest inherent patient characteristics, the influences of patient personal habits, an inherent homogeneity of the atrial appendage tissue, or other factors that may be critical for the rapid development of cardiac neovessels. Being able to characterize and predict these subtle patient-dependant differences might aid in determining which patients would benefit most from pro-angiogenic therapy. These concepts are under investigation in our laboratory.

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