The Thoracic Surgery Directors Association (TSDA) Resident Research Award, sponsored by Medtronic, Inc, was established in 1990 to encourage resident research in cardiothoracic surgery. Abstracts submitted to The Society of Thoracic Surgeons (STS) Program Committee representing research performed by residents were forwarded to the TSDA to be considered for this award. The abstracts were selected by the TSDA Executive Committee consisting of Edward D. Verrier, President, Douglas J. Mathisen, President-Elect, Jeffrey P. Gold, Secretary/Treasurer, Gordon N. Olinger, MD, Immediate Past President, John H. Calhoon, Councillor-at-Large, and Richard J. Shemin, Councillor-At-Large.

The twelfth TSDA Resident Research Award was given to Tjörvi E. Perry, MD, a cardiac surgery resident at the Children's Hospital in Boston, Massachusetts. He received a monetary award of $2,500 and an engraved desktop award.

The TSDA, with support by Medtronic, Inc, makes this award annually, using the above selection procedure. The resident author of the selected study is recognized at the STS meeting.

Bone Marrow as a Cell Source for Tissue Engineering Heart Valves

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Background. **This study was designed to assess the feasibility of using ovine bone marrow-derived mesenchymal stem cells to develop a trileaflet heart valve using a tissue engineering approach.**

Methods. **Bone marrow was aspirated from the sternum of adult sheep. Cells were isolated using a Ficoll gradient, cultured, and characterized based on immunofluorescent staining and the ability to differentiate down a specific cell lineage. Two million cells per centimeter squared were delivered onto a polyglycolic acid (PGA), poly-4-hydroxybutyrate (P4HB) composite scaffold and cultured for 1 week before being transferred to a pulse duplicator for an additional 2 weeks. The tissue-engineered valves were assessed by histology, scanning electron microscopy, and biomechanical flexure testing.**

Results. **Cells expressed SH2, a marker for mesenchymal stem cells, as well as specific markers of smooth muscle cell lineage including α-smooth muscle actin, desmin, and calponin. These cells could be induced to differentiate down an adipocyte lineage confirming they had not fully committed to a specific cell lineage. Preliminary histologic examination showed patchy surface confluency confirmed by scanning electron microscopy, and deep cellular material. Biomechanical flexure testing of the leaflets showed an effective stiffness comparable to normal valve leaflets.**

Conclusions. **Mesenchymal stem cells can be isolated noninvasively from the sternum of sheep and can adhere to and populate a PGA/P4HB composite scaffold to form "tissue" that has biomechanical properties similar to native heart valve leaflets. Thus, bone marrow may be a potential source of cells for tissue engineering trileaflet heart valves, particularly in children with congenital heart disease.**

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Surgically significant congenital heart disease occurs in approximately 0.4% of live births per year in the United States. A substantial proportion of these defects involve stenosis or atresia of the right ventricular outflow tract and pulmonary valve. Currently, homografts are the preferred replacement device in our center for reconstituting blood flow from the right ventricle to the pulmonary artery in these cases [1]. Aortic or pulmonary valve homografts, however, have limitations, including limited availability and suboptimal durability [2, 3]. Of particular importance to the pediatric population, homografts and all other currently available conduits and valves have no ability to grow.

Tissue engineering is an evolving science joining engineering and biology in an attempt to develop replacement tissue. Cells derived from the wall of the carotid artery have been successfully used to bioengineer func-

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tional cardiovascular structures in animals [4, 5]. However, a less invasive source for cells applicable for tissue engineering heart valves would be preferable clinically.

Friedenstein and colleagues [6] were the first to describe stromal cells derived from bone marrow, and demonstrated the feasibility of isolating and expanding bone marrow stromal cells ex vivo. They also demonstrated the capacity for these cells to differentiate to osteocytes after implantation. More recently, it has become evident that mesenchymal stem cells can be isolated consistently from bone marrow, expanded ex vivo, and differentiated to various cell phenotypes including osteocytes, chondrocytes, adipocytes, and myocytes [7]. In this study, we describe the use of a cell population derived from ovine bone marrow for tissue engineering a trileaflet heart valve.

Material and Methods

Cell Isolation and Expansion

Bone marrow was aspirated into a heparinized syringe from the sternum of adult sheep using a standard 14-gauge bone marrow aspiration needle. We aspirated no more than 5 mL at a time in an attempt to avoid contamination from peripheral venous blood being drawn back into the sternum. A total of 10 mL of bone marrow in 20 mL phosphate-buffered saline was transferred to a 50-mL conical frit tube containing a Ficoll density gradient, and centrifuged at 2700 rpm at room temperature for 15 minutes. The mononuclear cell layer was subsequently isolated and plated on fibronectin-coated tissue culture plates (Falcon, Beckton-Dickinson, Franklin Lakes, NJ). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) with 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY), 1% L-glutamine, penicillin, and streptomycin (Life Technologies, Grand Island, NY). Cells were expanded ex vivo in a humidified incubator at 37°C, 5% CO_2/air. Cells were passaged at 70% to 80% confluence.

Cell Characterization

Cells were characterized on the basis of indirect immunofluorescent staining for phynotypic markers, and potential to differentiate along a specific cell lineage. Early passage cells were double labeled using primary monoclonal antibodies against SH2 (endoglin), a marker for mesenchymal stem cells [8, 9]; α-smooth muscle actin, calponin, and desmin, specific markers of cells of myogenic lineage; and goat antihuman CD31/PECAM-1, a specific marker for mature endothelial cells. Fluorescein-conjugated secondary antibodies were used to label SH2, α-smooth muscle actin, calponin, and desmin; Texas Red-conjugated secondary antibodies were used to label PECAM-1/CD31.

In an effort to assess the differentiation potential of cells derived from bone marrow, cells were treated in adipogenic induction media containing 0.5 mmol/L methyl isobutylxanthine, 1 μmol/L dexamethasone, 10 μg/ml insulin, and 100 μmol/L indomethacin in DMEM with 10% FBS for 3 days. After 3 days in induction media, cells were placed in mesenchymal stem cell (MSC) maintenance media (DMEM, FBS, GPS) for 24 hours. The cells were then subjected for a second and third time to induction media followed each time by culture in maintenance media [7]. Mature smooth muscles derived from the wall of a carotid artery underwent the same protocol as a control.

Scaffold Design and Assembly

A biodegradable 21-mm heart valve scaffold was constructed from two sheets of nonwoven polyglycolic acid (PGA) mesh (supplied by Albany International Research, Mansfield, MA) dip-coated in a 1% solution of poly-4-hydroxybutyrate (P4HB) (supplied by Tepha, Cambridge, MA) wrapped around a two-piece heart valve mold as previously described by Hoerstrup and coworkers [5].

Cell Delivery to Scaffold, and Ex Vivo Conditioning of Tissue-Engineered Heart Valve

Cells were manually delivered onto a PGA/P4HB composite trileaflet heart valve scaffold in a concentrated cell suspension ($2 \times 10^6/cm^2$ scaffold) from a pipette in 100-μL aliquots. After cell attachment, the cell-polymer constructs were cultured for 1 week under static conditions. The constructs were then transferred to a pulsatile flow simulator [10] for 3 weeks to provide shear stress and mechanical signals to the developing tissue. After the ex vivo conditioning period, the trileaflet heart valves were examined. Longitudinal sections were taken from the middle portion of each of the three leaflets and assessed by histology, scanning electron microscopy (SEM), and biomechanical flexure testing, respectively.

Histology

A representative portion of leaflet and corresponding conduit wall was cryopreserved and stained with hematoxylin and eosin for overall morphology.

Scanning Electron Microscope

Tissue samples were immersed in 0.1% glutaraldehyde for 1 hour. Samples were then transferred to 0.1% formalin for 24 hours. Following fixation, samples were dehydrated overnight in 100% alcohol. For viewing under the SEM, specimens were air-dried and coated with 5 nm of gold using a Polaron E5150 SEM Coating Unit. A JEOL-35 SEM was used to view and photograph each sample.

Biomechanical Flexure Testing

Thin strips were removed from the central portion of the leaflet of the tissue-engineered heart valve specimen. Five black graphite markers (approx. 100 micron) were embedded into one edge of each tissue-engineered leaflet. The specimen was placed across two static pins in a three-point bending apparatus designed for assessing soft biomaterials [11]. A third pin depressed the center of the leaflet in a controlled manner, while images were acquired. The displacement of the tissue and bending bar were use to determine the load. The effective stiffness of the leaflet was calculated by measuring the change in relation of each

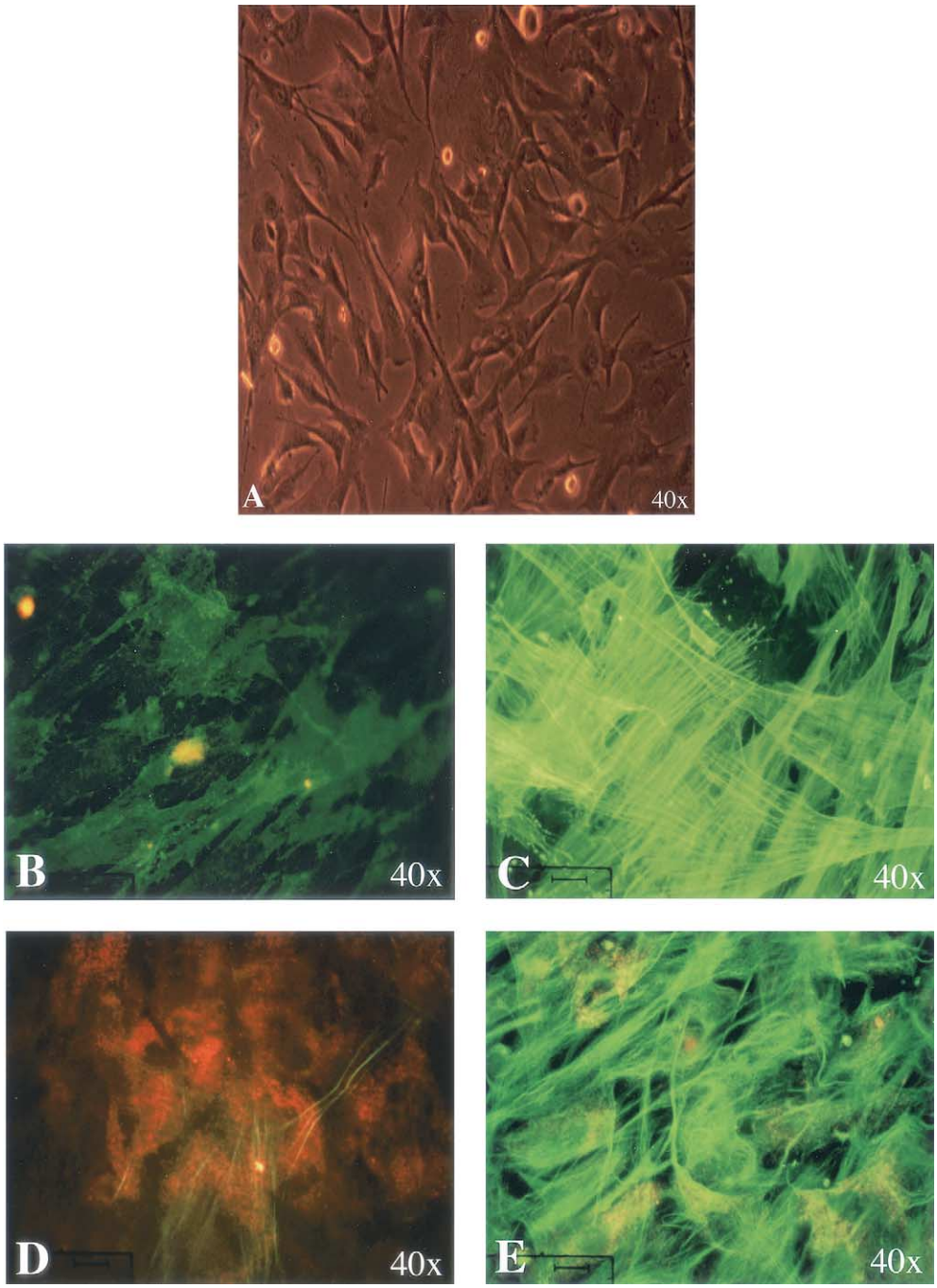

Fig 1. (A) Light microscope photograph of early passage bone marrow-derived mesenchymal stem cells. The mesenchymal stem cells were double labeled with primary antibodies against SH2 (endoglin), α-smooth muscle actin, calponin, desmin (fluorescein-conjugated secondary antibodies), and CD31/PECAM-1 (Texas Red-conjugated secondary antibodies) (×40 before 30% reduction). (B) Cells stained uniformly positive for SH2 (endoglin) and negative for CD31/PECAM-1 (×40). (C) Cells stained uniformly positive for α-smooth muscle actin and negative for CD31/PECAM-1 (×40). (D) A subset of cells stained positive for calponin and negative for CD31/PECAM-1 (×40). (E) Cells stained uniformly positive for desmin and negative for CD31/PECAM-1 (×40).

graphite marker to the next over increasing loads. The moment–curvature relationship for beams undergoing large displacement was used to determine the effective bending stiffness, E_{eff}, over the entire loading curve:

$$M = E_{eff} I$$

Where κ is change in specimen curvature, I is the second moment of inertia, M is the bending moment, and E_{eff} is the effective tissue stiffness [12]. To determine the value for E_{eff} over the entire bending path, M/I was plotted against κ so that the slope of the curve at each point was equivalent to E_{eff}.

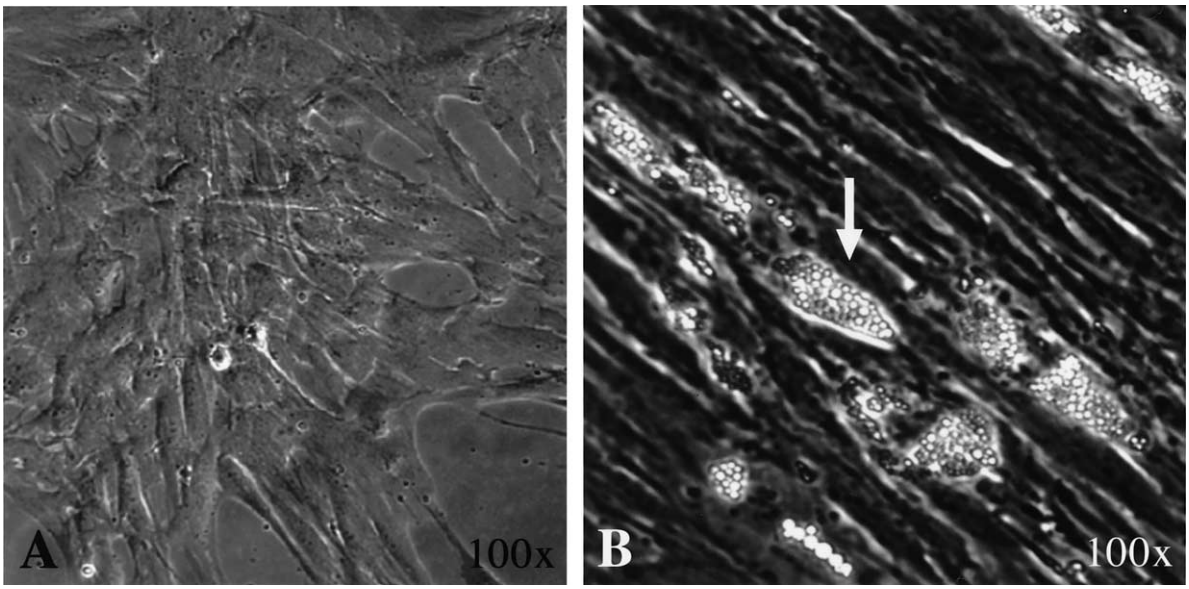

*Fig 2. (A) Light microscope photograph of early passage bone marrow-derived mesenchymal stem cells in their undifferentiated state (×100). (B) Light microscope photograph of the mesenchymal stem cells containing intracellular vacuoles (*arrow*) after induction with adipogenic cell culture medium (×100).*

Results

Cell Isolation and Expansion

A total of 10 mL of bone marrow was obtained from the sternum of each adult sheep. Ten to 15 million cells were obtained from each sample. Cells attached to culture plates over a 4- to 5-day period, and proliferated to confluence within 10 days. Cells were passaged at 70% to 80% confluence, and expansion continued successively in larger cell culture plates.

Cell Characterization

Early passage cells stained uniformly positive for SH2 (endoglin), α-smooth muscle actin, and desmin. A subpopulation of cells stained positive for calponin. Cells stained uniformly negative for PECAM-1/CD31 (Fig 1).

After the first treatment in adipogenic induction media, lipid vacuoles became evident within cells. An increasing number of cells began to develop the vacuoles with each induction regimen (Fig 2). Mature smooth muscles derived from the wall of a carotid artery underwent the same protocol, but did not develop intracellular vacuoles.

Evaluation of Tissue-Engineered Heart Valves After Ex Vivo Conditioning

On gross examination, the valves' tissue was evenly distributed tissue over the entire construct when compared with unseeded heart valve scaffolds (Fig 3). The leaflets coapted uniformly in the closed position. Scanning electron microscopy showed a confluent cell layer on the surface of the leaflets and conduit wall (Fig 4). Histology showed patchy cell confluence on both sides of the construct. Cells were found throughout the full thickness of the tissue-engineered leaflets and conduit wall. Residual polymer fibers were dispersed throughout the constructs (Fig 5). Three-point bending studies demonstrated an effective stiffness comparable to the effective stiffness found in fresh native pulmonary leaflet (Fig 6).

Comment

These initial findings suggest that bone marrow could offer an alternative, less invasive source for cells for tissue engineering heart valves. We have shown that cells derived from bone marrow, and staining positively for SH2, α-smooth muscle actin, desmin, and calponin can be isolated and expanded ex vivo. These cells attach and proliferate on biodegradable PGA/P4HB porous scaffold to form tissue. Mechanical flexure testing using three-point bending studies indicated that tissue formed from cells derived from bone marrow has mechanical properties that resemble that of native valve leaflet tissue.

Although these initial results are encouraging, several issues remain to be addressed. Although bone marrow cells have been demonstrated to possess the potential to differentiate into various cell types of mesodermal lineage, a precise mechanism by which these cells and their environment control differentiation is not fully understood. Long-term function and durability of a tissue-engineered heart valve made from immature bone marrow-derived stromal cells is contingent on differentiation of those cells to an appropriate cell type either during the ex vivo period or after implantation. Standard cell culture techniques were used as described in the methods section of this report. Mesenchymal stem cells were cultured in DMEM cell culture medium, FBS, and antibiotics. A

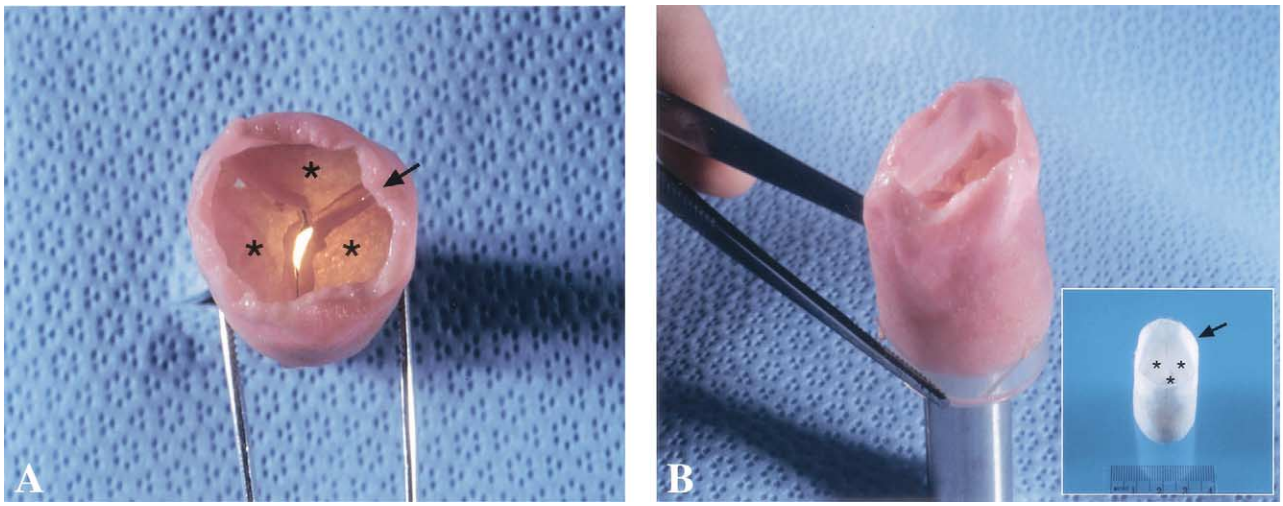

Fig 3. (A) Gross appearance of a tissue-engineered heart valve conduit as seen from above (arrow *pointing to distal edge of conduit) and leaflets* (asterisks*) made from cells derived from bone marrow. (B) Tissue-engineered heart valve from side and* (inset*) unseeded polyglycolic acid/poly-4-hydroxybutyrate heart valve scaffold* (arrow *pointing to distal edge of conduit;* asterisks *on leaflets).*

specific protocol for differentiating MSCs to myocytes was not implemented, yet cells expressed markers of myogenic lineage before delivery onto the heart valve scaffold. Although cells may very well be affected by both chemical and physical cues, more studies are required to better understand the mechanisms and signals that direct the differentiation of bone marrow-derived MSCs.

Initial micromechanical evaluation of leaflets tissue engineered from cells derived from bone marrow demonstrated an effective stiffness comparable to that of

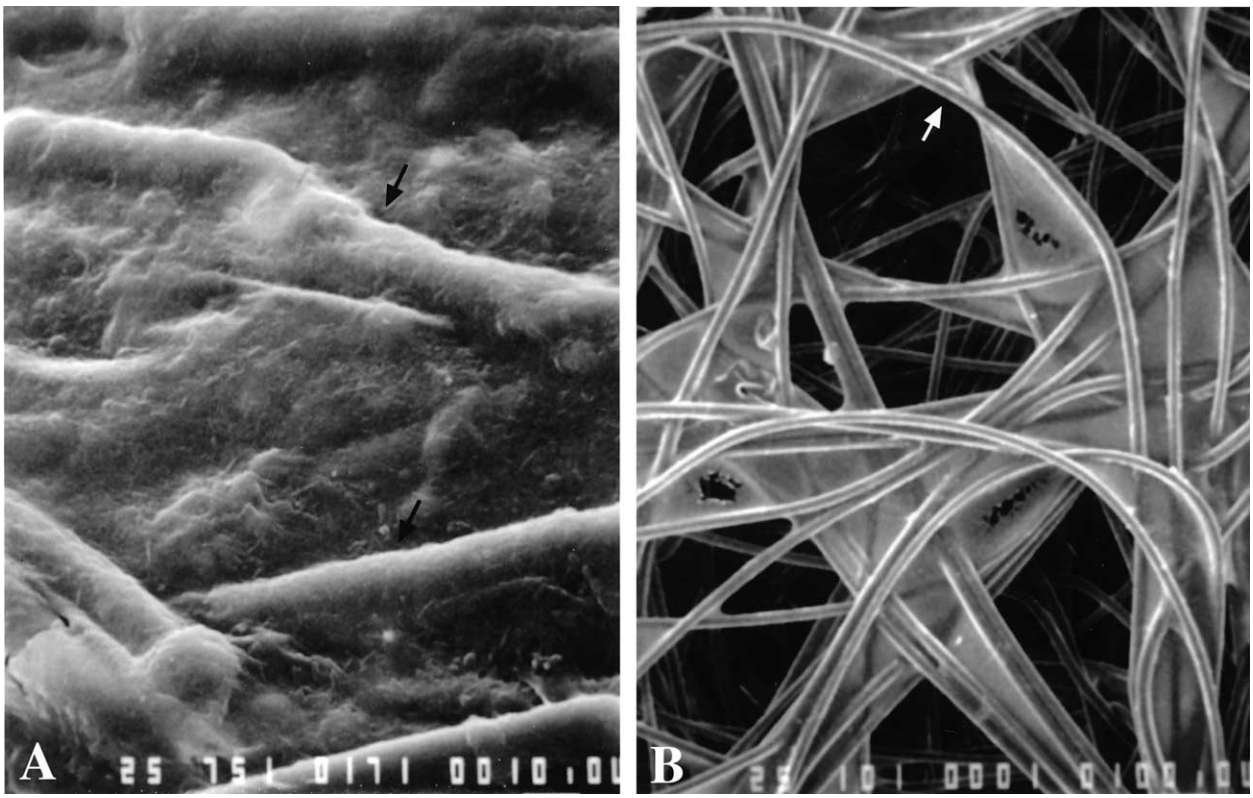

Fig 4. (A) Scanning electron microscopy of the surface of a tissue-engineered heart valve, and residual polyglycolic acid fibers (arrows). *(B) Unseeded polyglycolic acid fibers* (arrow) *coated with poly-4-hydroxybutyrate.*

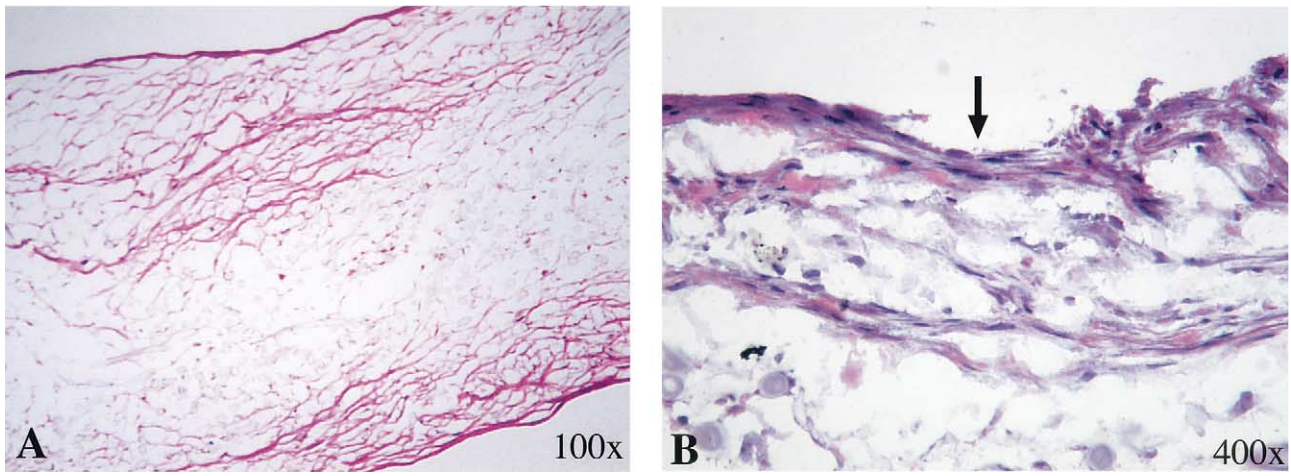

Fig 5. (A) Histologic staining of a tissue-engineered heart valve showing cell confluence on both sides of the construct, as well as cells dispersed through the full thickness (×100 before 25% reduction). (B) Histologic staining of the surface (arrow) *of a tissue-engineered heart valve (×400 before 25% reduction).*

native tissue, and lends support for the use of these cells for tissue engineering semilunar heart valves. Currently, however, micromechanical testing of tissue-engineered heart valve function remains a destructive outcome measure, and may not be wholly representative of in vivo behavior. We have recently been able to assess the mechanical function of tissue-engineered heart valves in a nondestructive manner using ultrasound and magnetic resonance imaging [13]. We anticipate that assessment of an increasing number of functional outcome measures will add validation to our protocol for tissue-engineered heart valves.

The feasibility of using cells derived from ovine bone marrow to tissue engineer heart valves ex vivo has been demonstrated. Future research will be directed toward implantation of tissue-engineered heart valves made from bone marrow cells into the pulmonary position of an animal model in an effort to evaluate short- and long-term durability.

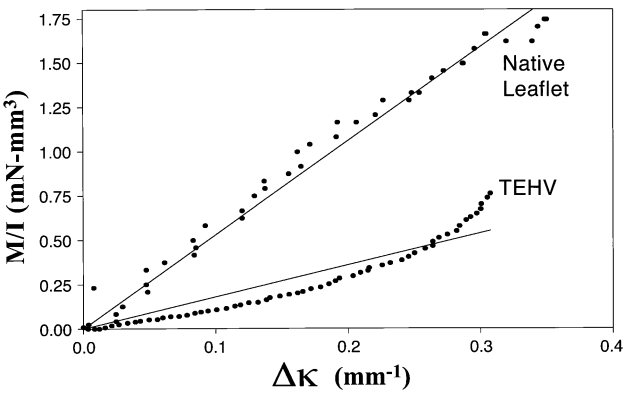

Fig 6. The effective stiffness of a tissue-engineered heart valve compared with the effective stiffness of a native leaflet. Unseeded polyglycolic acid/poly-4-hydroxybutyrate polymer has lost most of its strength after 4 weeks of in vitro conditioning. (TEHV = *tissue-engineered heart valve.)*

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DISCUSSION

DR ALEXANDER KADNER (Zurich, Switzerland): Which experiments did you perform to analyze your extracellular matrix? Did you perform any immunohistochemistry staining for collagen typing, et cetera? Furthermore, which control experiments did you perform to exclude any osteogenic differentiation of your isolated cell population?

DR PERRY: We did not conduct any studies to look specifically at what type of extracellular matrix proteins were in the tissue. We certainly need to determine that either biochemically or with histology, staining specifically for collagen, elastin, and glycosaminoglycans.

In terms of control experiments to exclude osteogenic differentiation, that is a fundamental question that we will need to address. In what exact state of differentiation these cells are when we implant them, we do not know yet. When we deliver them onto the scaffold, they are most likely in some type of undifferentiated state, because prior to the cell delivery they stain positive for SH2, a marker for undifferentiated mesenchymal stem cells. We have not performed any immunohistochemistry to determine if the cells are still SH2 positive on the scaffold before we implant them, but we need to do so. It would certainly be unfortunate if we implanted a heart valve made from undifferentiated bone marrow-derived mesenchymal stem cells, only to find the cells differentiating uncontrolled into one of many cell types of mesodermal lineage, such as bone, cartilage, or fat. So if we are going to use bone marrow as a cell source, we need to determine that the cells are in their mature, differentiated state before they are implanted, or that we have some control of their differentiation after implantation.

DR PAUL KURLANSKY (Miami Beach, FL): I was wondering what the polymer scaffold consists of and for how long does it need to maintain its integrity in order to provide an adequate scaffold for the ingrowth of the cells and the development of a functional tissue?

DR PERRY: The polymer that we used for this study is a combination of polyglycolic acid (known as PGA), and poly-4-hydroxybutyrate (known as P4HB). What we have seen over the last maybe 8 months, is that the PGA does not retain its mechanical strength long enough for tissue to take over the mechanical strength required prior to implantation. One of the options we are considering is to work with a different polymer that has a longer degradation profile so the tissue is able to form before the polymer loses its mechanical strength.

DR CHARLES B HUDDLESTON (St. Louis, MO): I am not sure I completely understood what level of differentiation the cells have achieved. In the histologic slides, the valve looks normal, but I gather from your stained slides that there is no direct proof that endothelium has formed, or did I misunderstand?

DR PERRY: We did not stain for endothelium, but we never seeded endothelial cells onto the construct.

DR HUDDLESTON: But the notion is that these cells could potentially differentiate into endothelial cells, correct?

DR PERRY: Yes.

DR HUDDLESTON: Because there is some indirect evidence that some of the cells differentiated into smooth muscle components?

DR PERRY: To date, no one, as far as I am aware, has been able to differentiate an undifferentiated mesenchymal cell into an endothelial cell in vitro.

DR HUDDLESTON: Is there any reason to believe that such differentiation could not happen?

DR PERRY: No. Theoretically, endothelial progenitor cells exist in the bone marrow, but the problem is that they are in such small concentrations. Moreover, a marker to identify and isolate an endothelial cell in its progenitor state is not available.