Development of an Operator-Independent Method for Seeding Tissue-Engineered Vascular Grafts

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The manual seeding of cells onto a biodegradable scaffold by pipetting is an effective method of cell seeding. However, the widespread use and ultimate clinical utility of this technique is limited by operator variability. This study was conducted to evaluate an operator-independent vacuum-seeding method for use in an upcoming clinical trial. Using bone marrow-derived mononuclear cells, we achieved seeding comparable to manually seeded scaffolds in terms of cellular attachment, distribution, and viability in vacuum-seeded grafts at vacuum pressures of -25 to -50 mmHg. In conclusion, we describe an operator-independent seeding method for use in the clinical setting.

Introduction

WE PERFORMED THE FIRST clinical trial evaluating the use of tissue-engineered vascular grafts (TEVGs) in humans. In this pilot study we evaluated the use of TEVGs as conduits for use in congenital heart surgery, where their growth potential could be used to its fullest potential. Results of this pilot study demonstrated no graft-related mortality with acceptable safety and efficacy and the added benefit of growth potential, making them the first human-made vascular graft with growth potential.^{1,2}

In this pilot study, our TEVG was assembled as described by Shin'oka *et al.* (2005). Briefly, our TEVG was assembled by pipetting a concentrated suspension of autologous bone marrow-derived mononuclear cells (BM-MNCs) of $\sim 300,000$ cells/cm² onto a biodegradable scaffold fabricated from a knitted polyglycolide fiber tube coated with a 50:50 copolymer sponge of L-lactide and ϵ -caprolactone. The seeded scaffold was then statically incubated in autologous serum for 2 h before implantation. While the technique proved to be both safe and effective in our initial pilot study, its ultimate clinical utility was limited by inter- and intra-operator variability.^{3,4} The development of an operator-independent method for seeding autologous BM-MNCs, which achieves similar cellular viability, distribution, and attachment to the scaffold as previous methods, is an important next step in the translation of this technology from the bench to the clinic.

Several seeding techniques, including rotational,^{5,6} centrifugal,^{7,8} magnetic,^{9,10} and electrostatic,^{11–13} have been developed to optimize cell seeding, but as of yet no technique as far as we know has been able to fulfill all the requirements of an ideal, clinically useful seeding system and achieve approval by the Food and Drug Administration (FDA) for use in TEVGs. Vacuum seeding first emerged in the early 1990s, but has generally consisted of a cell suspension-filled scaffold under extra-luminal vacuum pressure.^{14–17} These external vacuum pressure systems require infused intra-luminal media and more recently dynamic rotation of the scaffold during seeding. In this investigation we present an intra-luminal vacuum seeding technique that does not require rotation for use with BM-MNCs and the achievement of a rapid, operator-independent, and self-contained cell seeding technology capable of clinically viable construction of TEVGs with similar cellular distribution, attachment, and viability to that of manually seeded grafts.

Materials and Methods

Scaffold source, characteristics, and preparation

A biodegradable scaffold fabricated from a knitted polyglycolide fiber tube coated with a 50:50 copolymer sponge of L-lactide and ϵ -caprolactone was constructed as previously described.¹ The seeded scaffold was divided transversely into three equally sized sections and each section sent for

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additional studies. The sections were randomly assigned. One section was used to measure the cell attachment and seeding efficiency by DNA assay, the second section was used to evaluate cell viability by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, and the third section was used to determine cell distribution by histology.

Cell source (BM-MNC isolation)

The bone marrow for this study was purchased from Cambrex. Total unfractionated human bone marrow (BM) was filtered through 100- μ m filters, and collected in sterile 50-mL tubes. Remaining coagulated BM in filters was swirled and any collected BM was added to the 50-mL tubes. Mononuclear cells were isolated by density gradient centrifugation using Histopaque-1077 (Sigma). The serum layer was collected in sterile 50-mL tubes and saved for cell culture. Next, the buffy coat layer (containing BM-MNC) was removed and placed in 15-mL tubes. Cell suspensions were spun down at 2000 rpm for 10 min. After centrifugation, the supernatant was removed and the cell pellet resuspended in 10 mL of phosphate-buffered saline (PBS). The cells in solution were spun down at 2000 rpm for 10 min. Finally, the supernatant was removed and the cells resuspended in 1 mL of saved autologous serum.

Seeding methods

Manual seeding ($n=8$). All seeding was performed by a single operator. Biodegradable scaffold measuring 18 mm in diameter was removed from its sterile bag using sterile forceps and placed in a labeled 150 mm cell culture dish. BM-MNC solution containing 40×10^6 cells was pipetted into the scaffold. First, 2.5 mL was pipetted in the internal lumen. Next, 2.5 mL was pipetted over the outer lumen. BM-MNCs were manually pushed into the scaffold wall by compressing scaffold multiple times by hand with sterile gloves. Manual compression proceeded until the entire scaffold transitioned from dry to wet state. Subsequent to seeding, the scaffold was placed in a sterile cell culture dish to partially dry for 5 min and allow for maximal cell adhesion. The seeded scaffold was transferred to a sterile container and submerged in 25 mL of previously saved autologous plasma diluted with 125 mL of sterile PBS. The seeded scaffold was placed in the incubator for 2 h before being removed for evaluation.

Vacuum seeding. Suction tubing was connected to two 70-mL specimen traps, which in turn were connected to a pressure regulator connected to house suction via suction tubing and placed inside the hood. The vacuum-seeding device was assembled in the hood by attaching the outer perforated sleeve to the inner suction core (Fig. 1). The biodegradable scaffold was introduced over the outer perforated section of the vacuum-seeding device. The scaffold was secured onto the outer perforated sleeve by wrapping a transparent adhesive dressing called tegadermTM around both edges creating an airtight seal. Tubing was used to connect the vacuum device to the proximal specimen trap (Fig. 2). The vacuum device with the secured scaffold was introduced into a 100-mL sterile graduated cylinder containing 70 mL of PBS. The vacuum was turned on to prewet the scaffold in an effort to decrease its intrinsic hydrophobicity. The specimen trap solutions were discarded and reconnected. BM-MNC

FIG. 1. Vacuum-seeding device. Outer portion depicted with multiple perforations, inner portion with screw-on cap. Stainless steel sleeves can be selected for different size grafts.

cells were suspended in 70 mL of PBS, using a 100-mL sterile graduated cylinder. The vacuum device with the prewetted scaffold was introduced into the graduated cylinder containing the cell suspension. The vacuum was turned on for the duration of cell seeding (~ 30 s). Subsequent to seeding, the scaffold was removed from the seeding device and placed in a cell culture dish to partially dry for 5 min and allow for maximal cell adhesion. The seeded scaffold was transferred to a sterile container and submerged in 25 mL of autologous plasma diluted with 125 mL of PBS. The seeded scaffold was placed in the incubator for 2 h before being removed for evaluation.

Pressure effects on vacuum seeding

The effects of varying vacuum pressures ([1] 625 mmHg [V625], $n=8$; [2] 250 mmHg [V250], $n=3$; [3] 50 mmHg [V50], $n=3$; [4] 25 mmHg [V25]) on cell seeding was analyzed using MTS assays. Vacuum pressure was controlled using a regulator.

FIG. 2. Schematic representation of vacuum-seeding device. A graduated cylinder containing cell suspension is aspirated and collected in specimen traps.

Cell attachment

Scaffold cellularity was determined by measuring DNA content with the PicoGreen detection assay. The Quant-iT PicoGreen dsDNA reagent is an ultrasensitive fluorescent nucleic acid stain for the rapid quantification of double-stranded DNA in solution. When combined with lysed cells, this stain binds dsDNA and the resultant signal can be read using a fluorescence microplate reader. Once a standard curve of cell number as a function of fluorescence was generated using known quantities of human BM cells, this method enabled quick and efficient determination of the number of cells effectively seeded onto a scaffold before implantation.

Histological evaluation

Seeded scaffold sections were cut into 5 mm (length) rings, rinsed in PBS, and then fixed in 10% neutral buffered formalin. Samples were rinsed in PBS and dehydrated through graded ethanol washes. Samples were then preinfiltrated and embedded with catalyzed Technovit 7100 glycolmethacrylate (Energy Beam Sciences). Ten-micron sections were cut using a Leica 2165 microtome (Leica Microsystems) and stained with Lee's Methylene Blue. Specimens were analyzed under a light microscope; cells from five different sections were counted and averaged.

Cell viability

Viability of mononuclear cells from human BM was identified and quantified by 7-aminoactinomycin D staining for flow cytometry and trypan blue staining.

Subsequent to seeding MTS was used to quantify cell viability. MTS is bioreduced by cells into a colored formazan product that is soluble in tissue-culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. This colorimetric assay was used for determination of proliferation and viability of cells. A section of the seeded scaffold was placed in single well of a 96-multiwell plate in a solution of 200 μ L of media supplemented with 50 μ L of MTS solution. After an additional 4 h of incubation in the multiwell plate, the absorbance was read at 490 nm.

Cell retention

A perfusion bioreactor was used to mimic physiologic flow and evaluate the effects of flow on cell adhesion to seeded scaffolds.¹⁸ Scaffolds were seeded either manually or vacuum seeded at a vacuum pressure of 50 mmHg and incubated for 2 h in autologous serum. After 2 h the autologous serum was replaced with Dulbecco's modified Eagle's medium (Gibco) and seeded scaffolds were allowed to incubate for an additional 2 h. After incubation a segment of seeded scaffold was attached to a custom-made bioreactor chamber and secured using #2 silk suture. Using WIZ peristaltic roller pump (ISCO), PBS was circulated through 1/8-inch-diameter silicon tubing (Saint Gobain) and through the attached graft at a rate of 40 mL per minute to generate a force of 1 dyn/cm². After 15 min of constant flow, the graft was removed from the chamber and divided into 1 cm segments for further analysis by DNA assay.

Statistical analysis

All data are expressed as mean $\pm$ SD. The data for cell attachment and cell viability were analyzed by the analysis of variance with Fisher protected least significant difference (PLSD) test as *post hoc* test. The data for cell distribution were analyzed by the *F*-test. The data for cell retention were analyzed by Student's *t*-test. $p < 0.05$ was considered to be statistically significant.

Results

Cell attachment

The scaffold was cut into 1 mm² sections. DNA assay showed no statistically significant difference in seeding efficiency when scaffolds were seeded with 40×10^6 cells by either manual or vacuum methods ($p = 0.12$). Likewise, DNA assay showed no statistically significant difference in attached cell number per square millimeter when scaffolds were seeded by either manual or vacuum methods ($p = 0.13$) (Fig. 3).

FIG. 3. Seeding efficiency of scaffolds (a) and average number of attached cells per square millimeter scaffold (b) detected by DNA assay. Data are presented as mean $\pm$ SD from three to eight independent experiments. SD, standard deviation; NS, no statistically significant difference.

FIG. 4. Average number of attached cells per HPF (a) and images of HPFs from manually seeded scaffold (b) and vacuum-seeded scaffold at pressures of V25 (c), V50 (d), V250 (e), and V625 (f). Data are presented as mean $\pm$ SD from three to seven independent experiments. HPF, high-power field; NS, no statistically significant difference.

Cellular distribution

Cells from fixed sections of seeded scaffold were counted and the average number of cells per high-power field at 400 times magnification was calculated. No significant difference in the number of cells was seen in the manual versus vacuum-seeded scaffolds ($p=0.11$) (Fig. 4).

Cellular viability

Before seeding, cell viability was calculated to be $96.6\% \pm 3.10\%$ through FACS analysis and $93.8\% \pm 3.77\%$ through trypan blue staining. Subsequent to seeding MTS assay showed a significant difference in cell viability between the manually seeded scaffolds and vacuum-seeded scaffolds ($p=0.038$). *Post hoc* analysis revealed a significant difference between manually seeded scaffolds and vacuum-seeded scaffolds at pressures of -625 mmHg ($p=0.0074$) and -250 mmHg ($p=0.0097$). However, there was no significant difference in cellular viability between manually seeded scaffolds and vacuum-seeded scaffolds at vacuum pressures of -25 mmHg ($p=0.18$) and -50 mmHg ($p=0.17$) (Fig. 5).

Evaluation of cell retention under venous flow

Seeded cell retention was evaluated after 15 min perfusion by media in a bioreactor at physiological venous flow conditions (Fig. 6). DNA assay showed no significant difference between the number of retained cells on manually seeded scaffolds ($p=0.66$). Likewise, DNA assay showed no significant difference between the number of retained cells on vacuum-seeded scaffolds ($p=0.66$).

Discussion

The seeding of porous scaffolds is a key step in the construction of TEVGs. In animal studies, unseeded grafts are

subject to higher failure rates compared with seeded grafts when implanted as inter-positional grafts.^{19–22} While manual seeding techniques have proven successful in the laboratory and the clinic, they introduce the possibility of inter-operator variability as well as intra-operator variability and result in variance in the quality of seeded grafts. Since the act of pipetting media onto a scaffold and ensuring it saturates both the lumen and outer circumference requires both training and skill an ideal seeding system would eliminate the need for this step. Moreover, an automated system of preparing TEVG has often been cited as key development in the scaling

FIG. 5. Average number of live cells per square millimeter scaffold (MTS assay). Data are presented as mean $\pm$ SD from three to eight independent experiments (* $p=0.0074$, ** $p=0.0097$).

FIG. 6. Average number of attached cells per square millimeter scaffold after exposure to 15 min of simulated venous flow or 15 min of static culture, in manually seeded scaffolds (a), and vacuum-seeded scaffolds (b). Data are presented as mean $\pm$ SD from five to eight independent experiments. NS, no statistically significant difference.

up the technology.^{3,4} In preparation for the first clinical trial evaluating the use of TEVG in the United States, we were requested by the FDA to develop an operator-independent method for seeding BM-MNC on to the scaffold. Herein, we report the use of vacuum seeding in the attainment of an operator-independent seeding method that achieves similar cellular viability, distribution, and attachment to the scaffold as that seen in manually seeded scaffolds. For the purposes of comparison a single operator performed all of the manual seeding described in this article so as to eliminate the concern of inter-operator variability from our experiments. Our studies show no statistically significant difference between manually seeded scaffolds and scaffolds seeded at vacuum pressures of -25 mmHg and -50 mmHg. Only at the significantly higher vacuum pressures of -250 mmHg and -625 mmHg did we observe a reduction in cell viability compared to manually seeded grafts. Presumably, this reduction in viability results from increased shear stress induced by the higher vacuum pressures. While previous operator-independent vacuum seeding systems have been

reported by Soletti *et al.* (2006, 2010) and Nieponice *et al.* (2008) our method differs in that we employ an intra-luminal vacuum force and thereby eliminate the need for scaffold rotation and intra-luminal infused flow of media. The relative simplicity of our device makes it a better choice for clinical applications and aided in receiving FDA approval as part of an investigational device exception investigating the use of TEVG in congenital heart surgery.

Several seeding methods have been developed over the past 25 years, including rotational,^{5,6} centrifugal,^{7,8} magnetic,^{9,10} and electrostatic,^{11–13} that are capable of achieving significantly higher cell attachment to scaffolds than that possible using a manual seeding method. However, the *in vivo* effect of higher cell attachment to scaffolds is unknown. Since vascular remodeling occurs through a primarily inflammatory process,²³ greater seeded cell densities could alter the inflammatory process, vascular neotissue formation, and graft function. Until studies on the *in vivo* effects of increased seeded cell attachment are conducted the ideal seeded cell attachment of TEVGs will remain unknown. As manually seeded grafts are the only grafts to have been tested in clinical trial and shown efficacy, they remain the standard of quality in terms of seeded cell viability, distribution, and attachment.

To summarize in this study we describe an operator-independent vacuum-seeding method for TEVGs for use in the clinic that achieves seeding characteristics indistinguishable from those found in manual seeding methods.

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Disclosure Statement

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