
Serum deprivation improves seeding and repopulation of acellular matrices with valvular interstitial cells

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Abstract: Cell-extracted valvular tissues (acellular scaffolds, or aScaffolds) offer unique advantages over synthetic polymers for cardiac valve engineering applications in that they retain extracellular matrix molecules to support cellular ingrowth. The extracellular matrix is important in directing many cellular pathways, such as adhesion, proliferation, migration, differentiation, and survival. However, repopulating this type of scaffold often requires high seeding densities or recurrent cell delivery. The optimization of valvular interstitial cell (VIC) seeding onto aScaffolds is reported herein. VICs (the most prevalent cell type in valve leaflets) have maximal growth in 15–20% serum concentrations on tissue-culture polystyrene. Interestingly, after VIC seeding

onto aScaffolds, a reduction of serum content, from 15% serum to 5% or less, was found to increase significantly the number of adherent cells, as well as induce transfer of VICs from a tissue-culture polystyrene surface to the aScaffold. aScaffolds seeded and cultured with periods of reduced serum levels were shown to support and enhance VIC viability and attachment, as well as accelerate VIC migration into the aScaffold, leading to a uniformly repopulated valve leaflet construct after 4 weeks of static culture. © 2005 Wiley Periodicals, Inc. *J Biomed Mater Res* 75A: 232–241, 2005

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INTRODUCTION

Both mechanical heart valve replacements and non-living xenografts are limited in their ability to grow, remodel, and respond to injury.¹ Tissue-engineered valve prostheses have the potential to overcome these limitations via the reparative activity of the cells incorporated within them. Although many tissue engineering approaches have employed the use of biodegradable synthetic polymers, there is much evidence

that naturally occurring biopolymers composed of extracellular matrix (ECM) proteins have utility as templates for cellular attachment and growth. Uncrosslinked cell-extracted heart valve tissues have numerous desirable qualities: mechanical and hemodynamic properties similar to that of native tissue,² retention of adhesion epitopes that are recognized by cell surface receptors,³ retention of appropriate physiological shape and trilaminar zones of histologically distinct tissue,^{4,5} and reduced immunogenicity.⁶ This type of valve substitute may reduce or eliminate the need for anticoagulative and immunosuppressive treatment, and lead to lower levels of calcification as a result of the removal of cell membrane and nuclear components in the acellularization process.⁶ The ultimate goal of our research is to transform an acellular scaffold (hereafter referred to as an aScaffold for brevity) to a primarily autogenous, viable, and self-repairing tissue through the metabolism of seeded autologous cells during *in vitro* conditioning.

Valvular interstitial cells (VICs) possess the ability to adhere to fibronectin, collagen, hyaluronic acid, and laminin surfaces,⁷ produce large amounts of ECM,⁸

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and participate in ECM remodeling during both valve homeostasis⁴ and wound healing.⁹ In addition, they are highly migratory and contractile,^{10–13} making them good candidates for repopulating a preformed acellular tissue scaffold. Previous studies with cell seeding of VICs onto ECM scaffolds resulted in patchy adhesion^{5,14} or failure of the seeded cells to penetrate the matrix.¹⁵ The role of initial cell seeding density has been explored in the context of cardiac tissue engineering, and the effective cell yield (attached cells compared with total cells initially seeded) is linearly related to initial seeding density.¹⁶ In a tissue-engineering setting, it is desirable to repopulate a xenograft aScaffold with a patient's own cells, and thus produce a living implant that would improve long-term function of the valve through its ability to adapt to changing physiological conditions. In this setting, donor cell availability may be limited and efficient seeding and attachment are of utmost importance.

Growth factor stimulation and integrin receptor binding activate many of the same intracellular signaling pathways, and their collaborative and interdependent signaling has the ability to direct adhesion, migration, proliferation, and tissue development.^{17–19} It has been previously shown that, for certain cell types, such as human endothelial cells^{20,21} and 3T3 fibroblasts,^{22,23} removal of serum induces a time-dependent detachment from tissue-culture substrates, and apoptosis in a subset of detached cells. Interestingly, the application of anti-integrin antibodies²⁰ or integrin receptor substrates²¹ has the ability, in some cases, to block the apoptotic effect of serum withdrawal. Studies of growth factor and hormone depletion suggest that apoptosis might be a default pathway that some cells enter in the absence of extracellular signals, or survival factors, that instruct them otherwise.²⁴ The ability of the ECM to bind and sequester these signaling molecules suggests that matrix proteins have a vital role in the control of growth factor signaling.²⁵ The objective of the work presented herein was to investigate the ability of a tissue-based aScaffold to serve as a promoting three-dimensional (3D) culture environment for VICs by providing a niche filled with cell adhesive ligands and naturally sequestered growth factors that can be released on demand by infiltrating cells. This outside-in signaling provides an important mechanism to guide and control the regeneration of tissues for cell-based therapies. Herein, we examine the ability of the aScaffold to influence VIC function under varied culture conditions, especially low-serum conditions.

During seeding of VICs onto aScaffolds, there is a population of cells that attaches immediately to the aScaffold surface, and another population that takes up residence on the tissue-culture treated polystyrene (TCPS) surface beneath the aScaffold. Studies reporting reversible detachment from TCPS with serum de-

privation prompted the hypothesis that, during cell seeding, the TCPS-resident VICs would show preferential attachment to the aScaffold because of its promoting environment under adverse culture conditions. In the results presented herein, we demonstrate for the first time that temporally limited serum starvation (5% and 1% fetal bovine serum) enhances VIC attachment to the aScaffold. Furthermore, we provide evidence that a reduction in serum content increases the efficiency of cell attachment during seeding via translocation of VICs from the TCPS surface to the aScaffold niche. Finally, the aScaffold serves as a 3D VIC culture platform that actively influences VIC metabolism, proliferation, and migration, in a manner that depends on both the aScaffold's matrix proteins and the local, soluble media signals.

MATERIALS AND METHODS

Acellular scaffolds (aScaffolds)

Porcine hearts were purchased from Quality Pork Processors, Inc. (Austin, MN). Within 24 h of death, aortic leaflets were surgically isolated and washed twice in cold 4°C Dulbecco's phosphate-buffered saline (PBS) (Invitrogen Corp., Carlsbad, CA). Cell extraction was performed via osmotic lysis, detergent extraction (Triton X-100 and sodium cholate), and endonuclease treatment as described elsewhere⁵ with the following minor modifications. All extraction steps were performed in the presence of protease inhibitors (10 μ L per leaflet, Calbiochem Protease Inhibitor Cocktail Set III) in 50-mL Falcon conical tubes outfitted with Aldrich white rubber 24/40 septa (sterilized in ethanol), and purged with prepurified Argon. After acellularization, aScaffolds were punched to fit the wells of 96-well plates and incubated in Turbo™ DNase solution for 3 h at 37°C (30 U/leaflet; Ambion Inc., Austin, TX). Leaflets were washed extensively to remove any remaining DNase. Finally, scaffolds were incubated overnight at 37°C in fetal bovine serum (FBS) (Invitrogen Corp.) and 5 μ g/mL human plasma fibronectin (Chemicon International, Temecula, CA).

Cell culture

VICs were isolated from porcine aortic valve leaflets by sequential collagenase digestion²⁶ and cultured at 37°C in a 5% CO₂ environment in growth media consisting of 15% FBS, 2% penicillin/streptomycin, and 0.2% gentamicin in Media 199 (Invitrogen Corp.). VICs were used between passages 2 and 5 in all experiments.

VIC seeding

VICs were isolated as stated above and cultured at subconfluent densities. A VIC cell suspension was made by

trypsinizing the plate, centrifuging at 1000 rpm for 6 min to obtain a cell pellet, and resuspending in growth media. Cell suspension densities were determined using a Multisizer 3 Coulter Counter (Beckman Coulter Inc., Fullerton, CA). For experiments using low seeding densities, 1600 cells were added per well (5000 cells/cm²); otherwise 25,000 cells/cm² was used. Seedings were performed in tissue-culture-treated 96-well flat-bottom plates (Falcon 3072; Becton Dickinson, Franklin Lakes, NJ). aScaffolds were placed in the center of the wells, and cell suspension was added such that the desired cell number was delivered in 200 μ L of growth media.

Serum deprivation

Initial seeding was performed in growth media (15% FBS) and cells were allowed to attach for 12 h. After this period, the growth media was carefully removed and replaced with Media 199 containing 5% FBS, 1% FBS, or serum-free as specified. Constructs were left in low-serum media for 4-, 8-, or 12-h periods and then returned to growth media until time of assay.

Analysis of VIC attachment

Metabolic activity and viability: MTT assay

The MTT assay is based on the mitochondrial reduction of the tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltertrazolium bromide (MTT). The assay is an indicator of cell viability and is proportional to cell number. The MTT assay was applied as described previously²⁷ with the following modifications: MTT solution (5 mg/mL in RPMI Media 1640; Invitrogen Corp.) was diluted 1:10 in RPMI Media 1640, 200 μ L of which was added to each sample and incubated for 4 h at 37°C. After incubation, the VIC/aScaffold constructs were moved to microcentrifuge tubes containing 200 μ L of acidic isopropanol (0.04N HCl; Sigma-Aldrich). These tubes were vortexed for 10 min to solubilize the dye, and then the solution was transferred to a 96-well plate and the absorbance measured at 550 nm, blanked with isopropanol from unseeded controls (Wallac Victor² Multilabel Counter; PerkinElmer). For VICs on TCPS, 200 μ L of isopropanol was added to each well after removal of dilute MTT solution, and incubated for an additional 5 min at 37°C before reading at 550 nm.

Effective cell yield

Effective cell yield was calculated by dividing of the amount of DNA in the VIC/aScaffold construct by the amount of DNA in the control treatment (VICs plated on TCPS with no serum deprivation), and represents the percentage of total cells seeded that attached to the aScaffold. DNA was quantified using the PicoGreen[®] dsDNA assay and lambda DNA standard curve (Molecular Probes, Eu-

gene, OR) according to the manufacturer's instructions. aScaffold samples were prepared for DNA quantification by removing the sample from culture, rinsing briefly in PBS, and then digesting in papain protease solution (Worthington Biochemical Corp., Freehold, NJ) for 2 h at 60°C. Papain solution consisted of 10 U enzyme/mL PBE buffer (100 mM Na₂HPO₄, 10 mM ethylenediaminetetraacetic acid, pH 6.5) with 10 mM L-cysteine freshly added. VICs on TCPS were prepared for assay by rinsing the wells in PBS, then adding 100 μ L of PBE buffer and sonicating (W-380 Ultrasonic Processor; Ultrasonics, Inc.) for 1 min.

Microscopy

Cells attached to aScaffolds were visualized by using laser scanning confocal microscopy (Pascal LSM 5; Carl Zeiss Inc.). Briefly, aScaffolds were removed from culture and incubated in growth media containing 0.5 μ L of calcein AM/mL and 2 μ L of ethidium homodimer/mL (Live/Dead[®] Cell-Mediated Cytotoxicity Kit; Molecular Probes) at 37°C for 30 min. Samples were then rinsed in PBS to remove excess dye before imaging. Samples were imaged under a 10 \times water objective and held stationary with the use of a Series 20 Chamber Platform (Warner Instruments, Hamden, CT).

Characterization of VIC proliferation

Proliferation was determined by monitoring changes in DNA content over a 5-day culture period using the PicoGreen[®] dsDNA assay as described in the previous experimental section. Each sample was lyophilized, weighed, and digested by papain protease before DNA quantification. DNA levels were normalized by aScaffold mass to account for differences in the scaffold size. Seeded aScaffolds that received a 12-h period of serum deprivation (5% FBS) were compared with those seeded and cultured in growth media without serum deprivation.

Characterization of VIC migration into ascaffolds

To assess the extent to which VICs penetrated the interior of the aScaffolds, samples were sectioned and examined histologically at 2- and 4-week time points. Two conditions were explored. First, VICs were seeded in growth media and maintained in growth media (replaced every 2 days) with no serum deprivation. Second, VICs were seeded in growth media and allowed to attach for 12 h. Media containing 5% FBS was then added for 12 h, after which growth media was returned. These samples were also recurrently exposed to serum deprivation postseeding (5% FBS), for a period of 36 continuous hours every 7 days of culture. Five percent FBS was chosen for this recurrent periodic exposure to maximize adherent VIC viability and migration potential. For analysis, samples were removed from culture, rinsed in PBS, and fixed overnight in 4% paraformaldehyde and 14% sucrose.

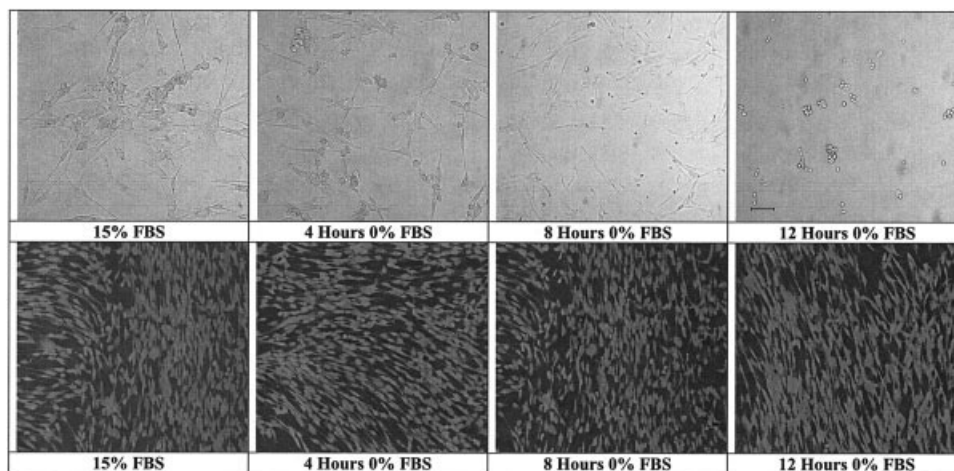


Figure 1. Morphology of VICs in serum-free media as a function of time and substrate; scale bar represents 100 μm . Top row: VICs seeded on TCPS imaged with phase contrast microscopy. Bottom row: VICs seeded on aScaffolds imaged with Live/Dead[®] staining with laser scanning confocal microscopy. Images are 2D projections of 3D files.

Samples were then equilibrated in Histo Prep[™] (Fisher Scientific) for 30 min and sectioned at 20- μm thickness (Leica CM1850 Cryostat). Sections were blocked and hydrated in 1% bovine serum albumin (Sigma-Aldrich) solution for 15 min before the application of propidium iodide nucleic acid staining solution (500 nM propidium iodide in dH_2O ; Sigma-Aldrich) for 30 min. After staining, samples were rinsed thoroughly with PBS. RNase treatment was not used. Images were taken on an inverted fluorescent microscope (Nikon Eclipse TE300).

Statistics

Data are presented as mean \pm standard deviation. At minimum, three samples were represented for each data point. Data were compared using a two-tailed, unpaired *t* test, and *p* values <0.05 were considered statistically significant.

RESULTS

aScaffold characterization

DNA levels in aScaffolds were determined postextraction to confirm acellularization. One sample leaflet was taken from each extraction vessel and cryosectioned (10- μm thickness). Cell removal was considered complete when sections exhibited no propidium iodide staining, and levels of DNA were <5 ng/mg as measured by the PicoGreen[®] dsDNA assay. For each subsequent analysis of DNA content, this background level of DNA was subtracted from the results. Unextracted porcine leaflets had a starting DNA concentration of 842 (± 191) ng DNA/mg tissue. Sections stained with Masson's trichrome have the appearance

typical of detergent-treated collagen,⁵ and lack observable cytoplasmic or nuclear staining (results herein).

Serum deprivation: Effects on VIC morphology and viability

In 2D subconfluent culture on TCPS, there was an observable change in VIC morphology over a 12-h incubation in serum-free RPMI 1640. Figure 1 depicts these changes, which included retraction of cytoskeletal processes, rounding, and detachment followed by cells floating upward in the media. Floating cells were collected at 4, 8, and 12 h of serum deprivation and replated in 15% FBS growth media. Many of the replated VICs were able to reattach although fewer cells attached from the 12-h deprivation group. All replated cultures were able to reach confluency within 7 days.

Morphological changes were also evaluated in VICs seeded on aScaffolds, and the cells that attached to the aScaffold surface are shown in Figure 1. Two days after seeding VICs on aScaffolds, the VIC/aScaffold constructs were incubated in serum-free media for 4, 8, or 12 h, and in the last half hour of that period incubated in serum-free media containing Live/Dead[®] stain. Cells adherent to the aScaffold surface showed no signs of detachment or changes in morphology. VICs remained elongated, spread, and viable after 12 h of serum-free conditions. Serum-free media was chosen to maximally challenge the aScaffold-adherent VICs, and best illustrates the protective effect of the ECM substrate during serum deprivation. Detachment of VICs from TCPS was also observed under 1% and 5% serum conditions, although to a lesser extent compared with serum-free conditions. Serum levels of 1% or 5% were used in all subsequent seeding exper-

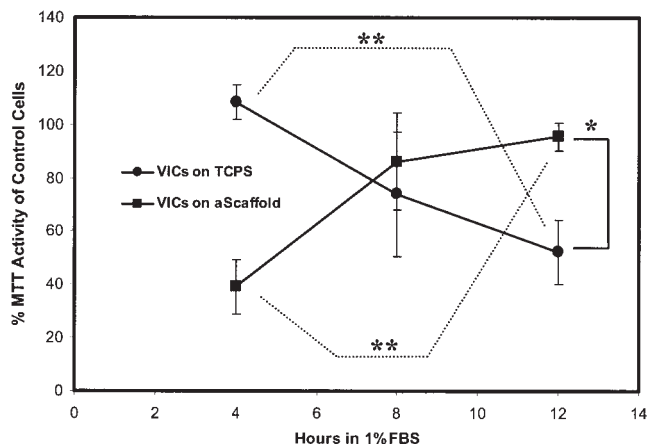


Figure 2. Metabolic activity of VICs in low-serum media as a function of time and substrate, as determined by the MTT assay. VICs were seeded on aScaffolds or in monolayer culture on TCPS, in media containing 15% FBS, and given 12 h for attachment. Samples were then incubated in media containing 1% FBS for up to 12 h, and then returned to media containing 15% FBS. Constructs were assayed 3 days postseeding. Data are represented as percent activity of VICs seeded on TCPS cultured in 15% FBS media with no serum deprivation. * $p < 0.05$; ** $p < 0.005$.

iments to protect viability of cell populations while still exploring the cellular response to reduced serum environments.

Serum deprivation: Effects on metabolic activity

VICs seeded on both TCPS and aScaffolds were given a 12-h attachment period in growth media, then incubated for 4, 8, or 12 h in low serum (1% FBS), and then returned to 15% FBS growth media. Metabolic activity was analyzed using the MTT assay 3 days after initial seeding. Figure 2 shows the results of these experiments. Data are presented as percent metabolic activity of VICs plated on TCPS that did not receive serum deprivation. There is a significant loss of metabolic activity for VICs seeded on TCPS at the 12-h time point ($p < 0.005$). In contrast, VICs seeded on aScaffolds exhibited an increase in metabolic activity with increasing time in 1% serum, and this increase was significant at the 12-h time point ($p < 0.005$). In addition, the two treatments differed significantly at the final time point ($p < 0.05$). The lower activity of the VICs on aScaffolds at 4 h is a result of the small fraction of seeded cells that attach initially to the aScaffold, as is further characterized in experiments below.

Effective cell yield

The following experiment was conducted to understand the increase in MTT metabolism of VIC/aScaf-

fold constructs after serum starvation, and the focus was to determine whether the cell population residing beneath the seeded aScaffold was a factor. After seeding the aScaffolds, there are two populations of adherent cells: one on the aScaffold and the other on the TCPS beneath. After seeding and 12 h for attachment, one set of aScaffold samples was transferred to fresh TCPS wells before low-serum treatment (1% FBS), and was therefore separated from its population of TCPS-adherent VICs. The other set remained in the wells in which they were seeded for the duration of the experiment. VICs seeded at the same density on TCPS represented the control. After serum deprivation, the VIC/aScaffold constructs recovered in growth media for 12 h before DNA content was assayed. Figure 3 shows the divergence in effective cell yield of these two treatments. After 12 h of incubation in media with 1% FBS, there was a significantly larger effective cell yield in the untransferred samples (VIC source on TCPS) than those transferred to fresh wells (no VIC TCPS source) ($p < 0.005$).

Transfer of VICs between two surfaces

To further explore the potential for VICs to transfer between TCPS and the aScaffold, VICs were directly seeded onto TCPS at low densities (to avoid transfer attributed to overconfluence), were given 12 h to at-

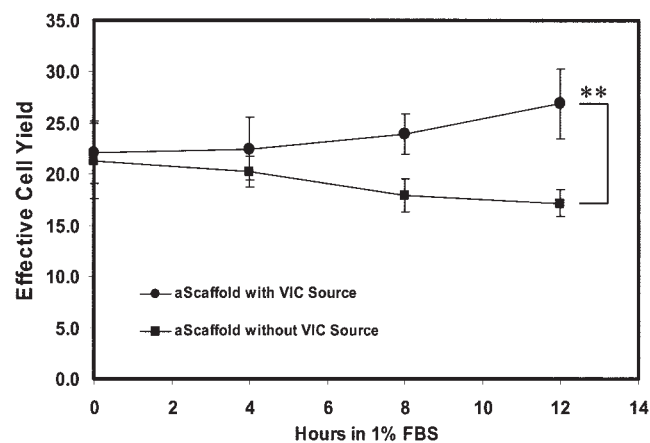


Figure 3. Effective cell yield as a function of aScaffold treatment. Effective cell yield is the percentage of seeded cells that attach to the aScaffold. *aScaffold with VIC source* refers to aScaffolds that were left in the well in which they were seeded for the duration of the 1% FBS treatment. *aScaffold without VIC source* refers to aScaffolds that were moved to fresh wells 12 h after seeding, before incubation in 1% FBS media. *VIC source* indicates the presence of a population of VICs adherent to the TCPS below the VIC/aScaffold construct. Treatments were given 12 h of recovery in growth media (15% FBS) after serum deprivation. DNA levels were determined by the PicoGreen[®] dsDNA assay, and normalized by aScaffold mass. ** $p < 0.005$.

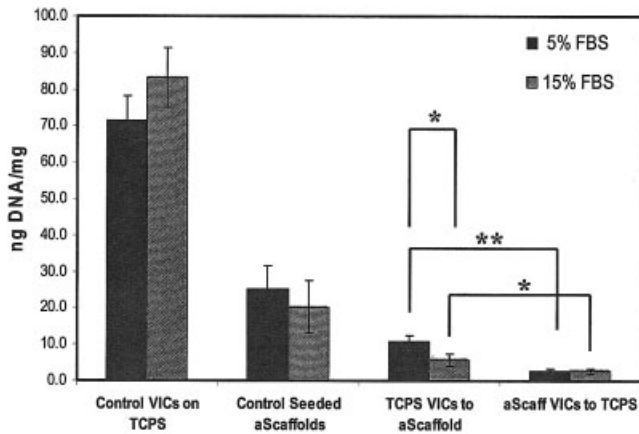


Figure 4. Transfer of VICs between TCPS and aScaffolds. *Control VICs on TCPS* are monolayer VICs directly seeded onto TCPS. *Control seeded aScaffolds* is the population of cells that attach to an aScaffold after direct seeding. *TCPS VICs to aScaffold* represents a fresh aScaffold when placed in a well previously seeded with VICs, and the cell population that developed on the aScaffold was monitored. *aScaffold VICs to TCPS* represents aScaffolds that were seeded with VICs and then transferred to a fresh TCPS well, and the cell population that developed on TCPS was monitored. DNA was determined by PicoGreen® assay and normalized by aScaffold mass. Five percent serum was applied 12 h postseeding, for 12 h. Assay was performed 36 h after cell seeding. * $p < 0.05$; ** $p < 0.005$.

tach, and then a fresh aScaffold was added and allowed to float above the seeded TCPS surface. The cell population that developed on the aScaffold surface was monitored. Conversely, aScaffolds were seeded and given 12 h to attach, then moved to fresh TCPS wells. The cell population that developed on the TCPS surface was monitored. Total experimental time was limited to 36 h to minimize effects caused by proliferation: 12 h for attachment, 12 h for serum deprivation (5% FBS), and 12 h for recovery. Figure 4 shows the results of these experiments. For both low-serum and growth media, there were significantly more VICs transferred from TCPS to aScaffolds than vice versa, as analyzed by DNA content ($p < 0.05$), although low serum induced significantly more transfer of cells from TCPS to aScaffolds than growth media ($p < 0.005$). Although not statistically significant, the trends for the control VICs on TCPS matched what was seen in Figure 2, where there were fewer cells after low-serum treatment. Similarly, the control seeded aScaffolds showed a trend of increasing cell number with low-serum treatment. These trends were probably not significant because of the low seeding density used. Interestingly, VICs indirectly seeded onto fresh aScaffolds (via transfer from TCPS), that were also serum-deprived, had more than half the effective cell yield of control seeded aScaffolds in growth media, indicating that the cells are able to transfer from the TCPS to the aScaffolds very effectively.

The effect of proliferation

As apparent in Figure 3, when culture time is kept short (<36 h), there is not a significant increase in the effective cell yield of seeded aScaffolds exposed to 1% FBS (top series). In contrast, if seeded aScaffolds treated with serum deprivation are given 4 days to proliferate, this difference in cell number becomes significant, as seen in Figure 5(a) ($p < 0.05$). Here, VICs seeded on aScaffolds that received 12 h in 5% FBS were compared with VICs seeded and maintained in either growth media (15% FBS) or low serum (5% FBS) for the duration of their culture. Quantification of DNA showed the same trends as MTT assay data depicted in Figure 5 (data not shown). TCPS VICs in

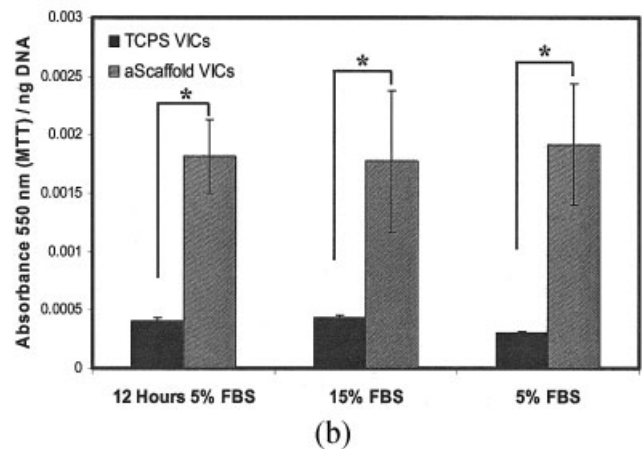
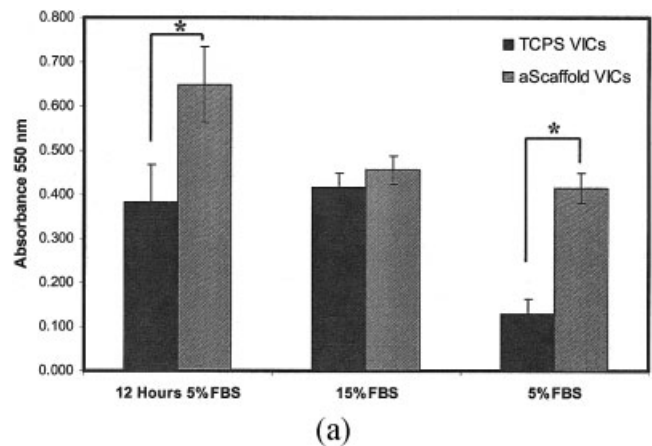


Figure 5. (a) Metabolic activity/viability as a function of media conditions used for VIC seeding as determined by the MTT assay. The first condition was seeded in 15% FBS, allowed 12 h for attachment, and then incubated in 5% FBS for another 12 h before return to 15% FBS media. For the second and third conditions, VICs were seeded on aScaffolds or TCPS in media containing 15% FBS and 5% FBS, and maintained in those serum conditions until time of assay. Samples were assayed 4 days after seeding. * $p < 0.05$. (b) Metabolic activity as determined by MTT assay, normalized by DNA content, and as a function of media conditions used for seeding. Samples were assayed 4 days after seeding. * $p < 0.05$.

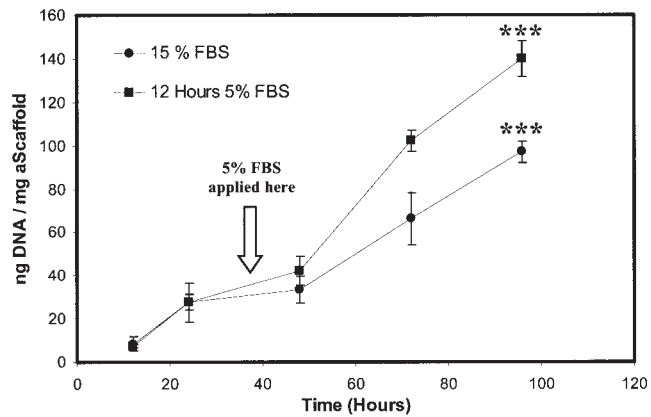


Figure 6. Proliferation of VICs on aScaffolds as a function of time and serum conditions. VICs were seeded on aScaffolds in media containing 15% FBS, and allowed 12 h for attachment. One sample set was then incubated in media containing 5% FBS for 12 h, and then returned to media containing 15% FBS. Samples were removed from culture at various time points and assayed for DNA content, which was normalized by aScaffold mass. *** $p < 0.001$.

5% FBS had significantly lower MTT activity when compared with TCPS VICs in growth media ($p < 0.05$), but aScaffold VICs in 5% FBS maintained activity compared with aScaffold VICs in growth media.

By normalizing absorbance given by the MTT assay to DNA content, it is evident that VICs adherent to aScaffolds have significantly higher metabolic activity per cell than TCPS counterparts, and this is shown in Figure 5(b). Interestingly, the serum conditions seem to have little effect on the metabolic activity of the viable cells, whereas the substrate, either aScaffold or TCPS, has a dramatic effect.

Proliferation and repopulation

Serum deprivation (5% FBS) of VIC/aScaffold constructs led to the immediate translation of the proliferation curve to higher values of cell number than VIC/aScaffolds constructs that were maintained in 15% FBS (Fig. 6). The increases in cell number are small initially, but the proliferation of these additional cells leads to significantly more attached cells for serum-deprived samples at time points >48 h. After VIC adhesion to the aScaffold surface, there is rapid proliferation until the surface is confluent and multiple cell layers can be seen by confocal microscopy. After confluence is reached, the VICs continue to proliferate, as well as begin their migration to the scaffold interior.

Migration

Movement of VICs into the aScaffold's interior is a process that occurs over weeks, but is accelerated by

serum deprivation. Figure 7 shows aScaffold cross-sections stained with propidium iodide cultured with either 15% FBS growth media, or a schedule of 5% FBS serum deprivation. The serum-deprived samples received 12 h of 1% FBS 12 h after seeding, but also were cultured in 5% FBS for 36 continuous hours every 7 days of culture (recurrent low-serum exposure). After 2 weeks of culture, there were fewer cells that migrated into the aScaffolds when seeding was performed in growth media, as compared with cells that migrated the full thickness of the aScaffold when seeding included serum starvation. At 4 weeks of total culture, this difference was even more pronounced, and serum-deprived samples had a cross-sectional density similar to that of the native porcine leaflet.

DISCUSSION

Naturally occurring biopolymeric scaffolds have been used for many years to provide a matrix for cell adhesion, growth, and proliferation; the advent of tissue engineering techniques has only increased their attractiveness for this purpose. In contrast to synthetic polymers, aScaffolds have the innate ability to incite angiogenesis,²⁸ recapitulate complex physiological shapes and mechanics,⁴ and provide biological cues for cell migration—all without significant modification. Moreover, being composed of natural polymers such as collagen and elastin, aScaffolds are biodegradable. This feature allows aScaffold remodeling to be dynamically coupled with cellular synthesis and degradation of ECM components.

Usually, two approaches have been taken in the application of ECM matrices to tissue engineering. The first approach is to implant the acellular tissue *in vivo* with the hope of attracting host cells into the scaffold.²⁹ These cells would then populate the scaffold, proliferate, and differentiate to recapitulate the function of the lost tissue. The other approach, used in this study, is to first precondition the aScaffold *in vitro* with a chosen population of cells. When autologous cells are used, the biocultured organ is returned to the patient as a living implant, able to remodel the matrix from its native xenogenic form into a state that is immunogenically compatible with the host.

Many methods have been used to improve cell seeding. These have included the use of dynamic seeding conditions, such as those in stirred reactors and spinner flasks. Dynamic media conditions have the advantage of increased external mass transport but are also known for producing turbulent eddies that can damage both cells and constructs. Seeding in a bioreactor allows control of flow regime, shear stress, pulsatile forces, and can be scaled up to accommodate multiple samples.³⁰ The choice of seeding vessel can affect cell

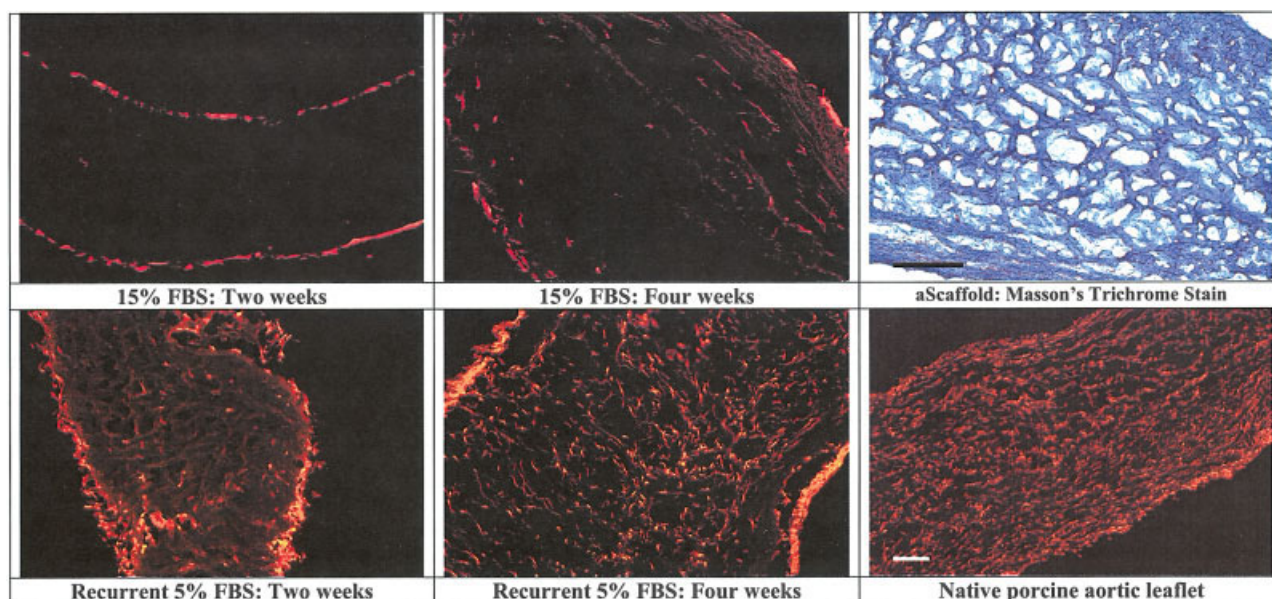


Figure 7. Migration of VICs into aScaffolds as a function of culture conditions and time. Images are cross-sections of aScaffolds, 20 μm in thickness, stained with propidium iodide at 10 \times original magnification; scale bar equals 100 μm . Top row: VICs seeded on aScaffolds in growth media (15% FBS) and maintained in growth media for the duration of culture. Bottom row: VICs seeded on aScaffolds that received a 12-h period of 5% serum 12 h after seeding, and also received 36 continuous hours of 5% FBS every 7 days of culture (recurrent low-serum exposure). Unseeded aScaffold (20 \times original magnification) stained with Masson's trichrome exhibits no cellular staining (upper right corner); scale bar equals 100 μm .

shape, cell distributions, and metabolic activity.¹⁶ Different stages of tissue development may require different regimes of culture conditions, adding complexity to the choice of conditions. In this study, we focused on a biochemical approach, specifically the manipulation of serum conditions, to optimize cell seeding in static culture. These results could also be extended to complement existing bioreactor approaches. Our research indicates that any reduction in serum content, from 15% to 5%, 1%, or serum-free, results in an enhancement of VIC seeding onto these acellular matrices. These findings may be substrate-dependent, and further studies are needed to assess the extension of this work to other types of matrices.

Cell attachment to a material provides intracellular signals via cell surface receptors that allow the cell to respond to changes in its environment. In this manner, surface receptors, such as integrins, selectins, and immunoglobulins, are important for both signal transduction and tissue architecture. There are signaling molecules that are synergistically activated by both growth factor receptors and integrin receptors,¹⁹ and other growth factor receptors that require integrin-mediated adhesion for activity.³¹ The cross-talk between these receptor types indicates that substrate attachment can be modulated by growth factor signals,¹⁸ and offers explanation to the serum-dependent attachment of VICs to TCPS. Short RGD (arginine-glycine-aspartic acid) peptides in the ECM proteins collagen, fibronectin, and vitronectin have been shown to rescue apoptosis in serum-deprived bron-

chial epithelial cells via integrin binding.²¹ The presence of these proteins in acellular tissue suggests that integrin-mediated adhesion can overcome the loss of soluble growth signals. In addition, aScaffolds contain numerous ECM components that have the ability to bind and store growth factors, thus locally delivering signaling molecules in a sustained manner.²⁵ aScaffolds are preincubated in FBS and fibronectin before seeding, and are likely hosting many ECM-associated serum factors. VIC adhesion to TCPS is largely a function of interactions with serum proteins that are non-specifically adsorbed to the plastic surface. The difference in the abilities of TCPS and aScaffolds to sequester soluble serum proteins likely contributes to the behaviors of VICs on these surfaces when FBS is withdrawn. VICs seeded on aScaffolds retain elongated morphology and attachment during serum deprivation, whereas subconfluent VICs on TCPS undergo rounding, retraction, and detachment.

We have shown that exposure of a seeded aScaffold to limited serum deprivation increased seeding efficiency, but only when there was a source of cells on the culture plate bottom. Populations of VICs that detach during low serum maintain viability, as evidenced by their ability to attach after replating in growth media. The VICs that lose TCPS attachment during low serum have the ability to transfer to the aScaffold ECM substrate and add to the resident population of VICs there. During culture, there is also some direct contact of the aScaffold with the bottom of the culture plate, allowing VIC transfer and preferen-

tial attachment of the adhesive protein-based substrate. Over a short time course, the increase in cell number as a result of serum deprivation is not significant, but it becomes significant when those additional cells are allowed to proliferate. We have also shown that MTT data normalized by DNA content (metabolic activity per cell) are significantly higher for VICs on aScaffolds than VICs on TCPS, and that this difference is a function of culture substrate and not of serum conditions. As a result, the use of a monolayer VIC MTT calibration curve would have led us to overestimate the number of VICs attached to aScaffolds.

The production of a viable tissue-engineered implant depends not only on cell attachment, but also on cell migration and repopulation of the neotissue. We have demonstrated that a schedule of serum deprivation not only enhances cell attachment, but dramatically changes the manner in which these cells migrate into and repopulate the scaffold interior. These uniquely seeded constructs are approaching the cellularity of native leaflets after only 4 weeks of static culture. In contrast to the manner in which many growth factor receptor systems are down-regulated upon ligand binding, these serum-starved VICs might be sensitized to serum components during deprivation, and therefore more responsive when serum is returned. Alternatively, there could be antagonistic signaling molecules at high enough levels in 15% serum that, when absent, increase the proliferative and migratory propensity of VICs. The next step is to conduct studies to determine what specific factors contained in serum, or combinations of factors, cause this remarkable modulation of VIC behavior.

CONCLUSION

Effective cell seeding and population of tissue-engineered constructs are vital for their performance as viable, living implants. This study has demonstrated that modulation of serum conditions after cell seeding is a simple yet effective method to increase the proportion of seeded cells that gain attachment. Cell yields were improved by the transfer of VICs from the bottom of the culture plate to the proteinaceous aScaffold during serum deprivation. When cultured in low serum, VICs detach from the plastic surface, float in the media, and regain attachment on the aScaffold surface. VICs attached to the aScaffold surface resist detachment during serum deprivation; hence, there is a net increase in attached cells on the construct with exposure to low-serum media. Acellular tissue matrices seeded with this method were shown to enhance VIC viability and attachment, as well as accelerate VIC migration, leading to a repopulated construct in 4 weeks of static culture. Modulation of serum condi-

tions both after cell seeding and during aScaffold culture has numerous desirable outcomes, and represents a significant step toward using a patient's own cells in the creation of a tissue-engineered heart valve.

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