Cardiac muscle tissue engineering: toward an in vitro model for electrophysiological studies

N. Bursac, M. Papadaki, R. J. Cohen, F. J. Schoen, S. R. Eisenberg, R. Carrier, G. Vunjak-Novakovic, and L. E. Freed. Cardiac muscle tissue engineering: toward an in vitro model for electrophysiological studies. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H433–H444, 1999.—The objective of this study was to establish a three-dimensional (3-D) in vitro model system of cardiac muscle for electrophysiological studies. Primary neonatal rat ventricular cells containing 70% or higher fractions of cardiac myocytes were cultured on polymeric scaffolds in bioreactors to form regular or enriched cardiac muscle constructs, respectively. After 1 wk, all constructs contained a peripheral tissue-like region (50–70 µm thick) in which differentiated cardiac myocytes were organized in multiple layers in a 3-D configuration. Indexes of cell size (protein/DNA) and metabolic activity (tetrazolium conversion/DNA) were similar for constructs and neonatal rat ventricles. Electrophysiological studies conducted using a linear array of extracellular electrodes showed that the peripheral region of constructs exhibited relatively homogeneous electrical properties and sustained macroscopically continuous impulse propagation on a centimeter-size scale. Electrophysiological properties of enriched constructs were superior to those of regular constructs but inferior to those of native ventricles. These results demonstrate that 3-D cardiac muscle constructs can be engineered with cardiac-specific structural and electrophysiological properties and used for in vitro impulse propagation studies.

myocyte; impulse propagation; electrophysiology; three-dimensional

CULTURED CARDIAC MYOCYTES offer many advantages for developmental, physiological, and pharmacological studies of cardiac tissue because they allow for direct cell manipulation and control of environmental parameters without interference from the compensatory feedback mechanisms that exist in vivo. Compared with monolayer cultures, it has been suggested that three-dimensional (3-D) multilayered cultures of cardiac myocytes more closely resemble intact cardiac tissue with respect to cellular differentiation (8) and electrical properties (38, 39). Three-dimensional cardiac myocyte cultures could thus be used for in vitro studies of cardiac tissue development and function and, if sufficiently large and functional, for in vivo cardiac repair.

Impulse propagation studies in cultures of cardiac myocytes can improve our understanding of the electrophysiological behavior of normal and pathological cardiac tissues. Such studies are currently performed in one-dimensional cardiac strands and two-dimensional (2-D) isotropic, anisotropic, and photolithographically patterned monolayers using optical mapping techniques (9, 10, 27). Impulse propagation studies cannot be performed in 3-D myocyte aggregates (17, 30) because of their small size (100–300 µm) and isopotential nature. Other 3-D cultures of cardiac myocytes grown on microcarrier beads (1, 31), collagen fibers (1), synthetic, biodegradable polymeric templates (3, 12), or in collagen gels (8) have not yet been evaluated electrophysiologically.

The goal of the present work was to establish a 3-D in vitro model system for impulse propagation studies in cardiac muscle using tissue engineering principles. This approach relies on the use of primary cells in conjunction with biodegradable polymer scaffolds (13, 18) and tissue culture bioreactors (11, 12). The polymer scaffold provides a 3-D substrate for cell attachment and tissue formation, whereas the mixing of culture medium in the bioreactor promotes mass transfer of nutrients and gases to the forming tissue. Primary neonatal rat ventricular cells were cultured on polymer scaffolds in bioreactors to form tissue constructs, which were characterized histologically, biochemically, and electrophysiologically and compared with neonatal and adult rat ventricular tissues.

MATERIALS AND METHODS

All experiments involving animals were performed according to the Institutional Committee on Animal Care of the Massachusetts Institute of Technology, which follows federal and state guidelines.

Cardiac myocyte preparation. Primary cultures of cardiac myocytes were prepared by enzymatic digestion of ventricles obtained from neonatal (2 day old) Sprague-Dawley rats (Taconic), as previously described (44). Briefly, ventricles (n = 50, 5 litters in 3 independent studies) were incubated with 0.1% trypsin overnight and dissociated in four to five sequential steps using 0.1% collagenase. Isolated cells were resuspended in culture medium [DMEM, supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 10 mM HEPEs, all obtained from Gibco-BRL].

Two experimental groups were established as follows (Fig. 1A): 1) a regular group of ventricular cells isolated as described above and 2) an enriched group with a higher fraction of cardiac myocytes, prepared from the regular group by centrifugation at 600 rpm for 5 min, followed by two preplatings, 75 min each (Fig. 1A); cells that remained unattached after the second preplating were used. Cell yields were ~6 × 10⁵ and 5 × 10⁶ cells/ventricle for the regular and...
Fig. 1. A: model system for tissue engineering. Cells from neonatal rat ventricles were seeded onto polymer scaffolds and cultured for 7 days to form regular and cardiac myocyte-enriched constructs. B: electrophysiological setup. Tissue constructs were studied using an extracellular microelectrode array (inset) under controlled environmental conditions in a 37°C/5% CO₂ perfused chamber. Stimulation was bipolar, and extracellular recordings were unipolar with reference to an Ag-AgCl electrode placed 3.5 cm away from the microelectrode array.
enriched group, respectively. Cell viability was 91 ± 3%, as assessed by trypan blue exclusion.

Monolayer studies. Cells from the regular and enriched groups were cultured in monolayers at a cell density of 1.3 x 10^6 cells/cm^2 in 12-well dishes, T75 flasks, and on glass coverslips to assess spontaneous contractions and biochemical and immunohistochemical parameters, respectively. After 2 days of static culture, monolayers were placed on an orbital shaker set to 75 rpm. Medium was completely replaced on day 3 and by 50% on day 5. Spontaneous contractions were assessed by videomicroscopy, by manually counting the number of beats per minute using five randomly selected fields (0.3 x 0.4 mm^2 each) per plate and six plates per experimental group, on days 3, 5, and 7. Cells in T75 flasks were removed after 7 days by a 5-min incubation with 0.05% trypsin-EDTA ( Gibco BRL) and counted, and a suspension of 2 x 10^6 cells/ml was stored at -20°C for determination of DNA and protein contents and lactate dehydrogenase (LDH) activity per cell. Cells on glass coverslips were fixed with HistoChoice (Amresco) for immunohistochemical analysis.

3-D tissue culture studies. Cells from the regular and enriched groups were cultured on polygalactin (PGA) scaffolds, which are highly porous (97%) meshes of randomly entangled 13-mm fibers formed as 5- x 2-mm (diameter x thickness) disks (Fig. 1A; Ref. 13). Briefly, scaffolds were prewetted in culture medium, positioned on thin stainless steel wires using segments of silicone tubing, and fixed to a silicone stopper placed in the mouth of a spinner flask (8 scaffolds per flask) (12). Flasks were filled with 120 ml of culture medium, placed in a humidified 37°C, 5% CO2 incubator for 24 h. Scaffolds were inoculated with cells (8 x 10^6 cells per scaffold). Culture medium was replaced by 100% on day 3 and by 50% on day 5. Cell-polymer constructs (n=22, from 3 independent studies) were harvested after 7 days for morphometric, histological, biochemical, and electrophysiological assessments.

Ventricular tissues. To verify the analytical methods, evaluate the developmental state of cardiac myocytes in constructs, and establish baseline values for parameters studied in engineered constructs that were not readily found in the literature, two control groups were examined. Adult ventricles (n=10) were obtained from 3- to 4-mo-old Sprague-Dawley rats following anesthesia by intramuscular injection of 65 mg/kg ketamine and 5 mg/kg xylazine (Sigma). Hearts were rapidly removed, and ventricular sections were excised from 1 mm below the atrioventricular groove to 1-2 mm above the apex. For electrophysiological studies, full-thickness pieces of the ventricular wall (9 x 7 mm^2, 2-4 mm thick) were then prepared by making two longitudinal cuts parallel to the base-apex line. Neonatal ventricles (n=10, from 3 litters) were obtained from 2-day-old rats following decapitation. For electrophysiological studies, full-thickness pieces of the ventricular wall (6 x 4 mm^2, 1.5-2.5 mm thick) were prepared by bisecting the ventricle. Smaller pieces of the adult and neonatal ventricles (7-13 mg wet wt) were used for biochemical and histological assessments. The properties of neonatal and adult ventricles were compared with those of constructs without a priori assumption that the engineered tissue resembled either of the native ventricular tissues.

Histological and immunohistochemical assessments. Cells on glass coverslips were incubated for 30 min with mouse antiseric tropomyosin monoclonal antibody (clone CH1, Sigma) diluted 1:100 in PBS containing 0.5% Tween 20 and 1.5% horse serum and then for 30 min with a secondary antibody (Vectorstain), diluted 1:200. Coverslips were then incubated with avidin-biotin complex reagent and 3,3’-diaminobenzidine (Sigma). Ten randomly selected fields (0.3 x 0.4 mm^2 each) from six coverslips from each group were analyzed using videomicroscopy and NIH Image 1.60 software to estimate cardiac myocyte fraction as a percentage of cell area stained positively for tropomyosin.

Ventricles and 7-day constructs were fixed in 2% glutaraldehyde for 10 min, rinsed in PBS, and immersed in 10% neutral buffered Formalin (Sigma). Samples were embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H+E) for general evaluation and Masson’s trichrome stain for collagen assessment. Immunohistochemical staining for tropomyosin was used to assess the fraction of cardiac myocytes in constructs. Sections were incubated with 1 mg/ml trypsin (Sigma) at 37°C for 15 min and 0.3% hydrogen peroxide for 30 min, blocked with horse serum for 30 min, and incubated with antisarcomeric tropomyosin as described above. A humidified chamber was used for all incubation steps. Sections were counterstained with Mayer’s hematoxylin (Sigma) and coverslipped using glycerol mounting media (Sigma). Specificity of staining for tropomyosin was confirmed by staining for sarcomeric α-actin, another myocyte-specific protein, using otherwise identical methodology. Construct macroscopic architecture was assessed from stained tissue sections using videomicroscopy and NIH Image 1.60 software.

Transmission electron microscopy. Samples were fixed in Karnovsky’s reagent (0.1 M sodium cacodylate with 2% paraformaldehyde and 2.5% glutaraldehyde, pH 7.4), post-fixed in 2% osmium tetroxide, dehydrated in ethanol in propylene oxide, and embedded in PolyBed 812 (Polysciences). Samples were cut at 60 nm, stained with lead citrate and uranyl acetate, and examined using a transmission electron microscope (J EOL-100CX, J EOL).

Media analysis. Physiological ranges of PO2 (115–130 mmHg), PCO2 (48–55 mmHg), and pH (7.21–7.33) were maintained for the duration of cultivation, as measured by a blood gas analyzer (IL 1610, Instrumentation Laboratory). Glucose and lactate concentrations were measured using a glucose/lactate analyzer (2300 StatPlus, YSI). The activity of LDH in the culture medium was monitored using a LDH-L reagent kit (Chiron Diagnostics). Media samples were sonicated using a Sonic Dismembrator (Vibra-Cell, Sonics and Materials), and absorbance was measured at 340 nm (Spectronic 1001+, Milton Roy) against cell-free medium. An LDH activity of 1 U/l corresponded to 3,600 cells in monolayers.

DNA and protein assays. DNA and protein assays were performed on engineered constructs and native ventricles using modifications of previously described methods (7). Samples were homogenized in buffer (1.0 mM ammonium hydroxide/2% Triton X-100, 0.04 mg/ml wet wt) for 1 min. For the DNA assay, homogenates were incubated at 37°C for 10 min, diluted with assay buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.00), and centrifuged. DNA contents of supernatants were determined using a spectrophotometer (PTI) and calf thymus DNA as a standard (7). DNA contents measured for regular and enriched monolayers were comparable (7.1 ± 0.2 pg/cell) and consistent with published values (7).

For protein assays, the viscosity of homogenates was reduced by several passages through a 26-gauge needle. After centrifugation, protein concentration was measured in the supernatant using a Bio-Rad DC protein assay kit and a microplate spectrophotometer (MR5000, Dynatech). Regular and enriched monolayers had comparable protein contents (290 pg/cell), resulting in protein-to-DNA ratios of 41 mg/mg that were consistent with published values (29).
Metabolic activity assays. Metabolic activities of cells within constructs and ventricular tissues were assessed by the uptake and enzymatic reduction of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma). Samples (2–15 mg wet wt) were rinsed with PBS and incubated with MEM (GIBCO-BRL) without phenol red and 0.5 mg/mL MTT for 4 h on an orbital shaker at 37°C and 60 rpm. Medium was replaced with an equal volume of 0.1 N HCl in absolute isopropanol and pipetted directly through the constructs to solubilize the resulting formazan crystals. After 10 min of incubation at 37°C, the absorbance was read at 570 nm, using a microplate spectrophotometer.

Electrophysiological assessment. An electrophysiological system was custom-designed to enable stimulation and recording of unipolar extracellular potentials in constructs and ventricular tissues under controlled environmental conditions using a linear array of microelectrodes (Fig. 1A). A cylindrical Plexiglas chamber was tightly fitted inside an electrically grounded brass casing placed on a 37°C heater (VWR). The brass case distributed the heat evenly through the chamber and served as an electrostatic shield. The chamber was gassed with a prewarmed mixture of 5% CO2 in air and filled with 50 ml of culture medium (DMEM with 15 mM HEPES, 4.5 g/l glucose), which was recirculated (at 60 ml/min for constructs and 120 ml/min for ventricular tissues) using a pulseless gear pump (Cole-Parmer). Temperature and pH were maintained at 37 ± 0.1°C and 7.32 ± 0.02, respectively.

A photomicrograph of the microelectrode array is shown in Fig. 1B. All microelectrodes were made of insulated tungsten wire and had uninsulated tips with diameters of 50 ± 6 µm (Microprobe). Two electrodes for bipolar stimulation were positioned 200 µm apart and connected to a programmable cardiac stimulator (SEC-3102, Nihon Kohden). Eight recording electrodes were positioned 500 µm apart in a linear array, 1.5 to 5 mm from the stimulating site. Exact distances between electrodes were measured using a microscope and NIH 1.60 image analysis software. Shielded cables connected recording electrodes to bioelectric amplifiers (AB.601G, NIH). A reference Ag-AgCl electrode (WPI) was placed 1.5 to 5 mm from the stimulating site. Exact distances and fitted by linear regression. All recorded signals were amplified and band-pass filtered between 0.3 and 1,000 Hz. The unfiltered noise level was 35 µV, peak to peak, with virtually no 60-Hz component. Analog recordings were digitized at a sampling rate of 3 kHz using a 16-bit analog-to-digital board (AT-MIO-16X, National Instruments), real-time displayed using LabView data acquisition software, and stored and analyzed using MATLAB (The Mathworks).

Activation times at each recording electrode were determined as the minima of five-point derivatives (2) of the low-pass filtered signals. The stimulus-activation time intervals at each electrode (conduction times) were plotted against the corresponding distances and fitted by linear regression. The conduction velocity of a propagated beat was calculated as the inverse slope of the best linear fit (16). The peak-to-peak (p-p) amplitudes of the responses were determined from linearly detrended signals around the activation times. Recording sites with very low or fractionated (polyphasic) activity were ignored.

For each tissue sample, p-p amplitudes at each electrode and conduction velocities were averaged from recordings made during the initial 20 min of pacing at 60 beats/min (i.e., over at least 200 beats). Conduction velocity, maximum amplitude, and average amplitude were calculated, respectively, as the averages of conduction velocities, maximum p-p amplitudes, and all p-p amplitudes from all samples within a group. The maximum and average amplitudes, respectively, represented local and spatially averaged properties of constructs or ventricles.

Statistics. Data were calculated as means ± SE and analyzed using either a paired t-test or one-way ANOVA applied at a rate of 60 beats/min, starting at a pacing voltage of 0.1 V, which was then increased in 0.1-V increments until the sample was captured (i.e., until each pacing impulse was followed by a recorded tissue response). The corresponding pacing voltage, defined as the excitation threshold, represented the lowest stimulus that produced a stable propagation (for at least 1 min at a rate of 60 beats/min) over the length of the recording array. For the next 20–30 min, the sample was continuously paced at 60 beats/min using pacing amplitudes 1.5 times higher than the excitation threshold, and responses were recorded every 4–5 min for a period of 1 min. The pacing rate was then increased every 5 min by 30 beats/min, and responses at each rate were recorded for the last 40 s, similar to the protocol in Ref. 37. The maximum pacing frequency at which the sample could be captured for at least 5 min was defined as the maximum capture rate. After reaching the maximum capture rate, stimulation was stopped for 10 min and then reapplied at 30 and 60 beats/min for 5 min each to check for reproducibility of the recorded waveforms. At the end of the experiment, double and triple extrastimuli and rapid stimulation at frequencies above the maximum capture rate were applied in an attempt to induce arrhythmia.

Table 1. Morphometric and biochemical parameters in 7-day constructs

<table>
<thead>
<tr>
<th>Group</th>
<th>Construct Weight and Dimensions</th>
<th>Cell Number in Construct, 10^6</th>
<th>Cell Number per Construct Lost During Cultivation, 10^6</th>
<th>Glucose Consumption Rate per Construct, g·l^{-1}·day^{-1}</th>
<th>Lactate Production Rate per Construct, g·l^{-1}·day^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular</td>
<td>35.45 ± 3.33</td>
<td>20.14 ± 0.82</td>
<td>5.36 ± 0.19 (20.95 ± 0.65)</td>
<td>5.37 ± 0.95* (2.51 ± 0.11*)</td>
<td></td>
</tr>
<tr>
<td>Enriched</td>
<td>31.08 ± 1.71</td>
<td>22.92 ± 0.58*</td>
<td>4.63 ± 0.31 (17.68 ± 1.12)</td>
<td>2.11 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 constructs. Parenthetical values are cumulative lactate dehydrogenase activity per construct, in U/l. Regular and enriched groups were prepared as defined in MATERIALS AND METHODS. *Statistically significant difference between regular and enriched groups.
followed by Fisher’s protected least significant difference post hoc test. To determine time-dependence trends for beating rates in monolayer cultures, a univariate repeated-measures ANOVA was used. Differences were considered statistically significant when \( P < 0.05 \). All calculations were performed using SuperANOVA III for Macintosh.

RESULTS

Monolayer cultures. After 24 h of culture, cardiac myocytes from both the regular and enriched groups started to contract spontaneously and by day 3–4 formed synchronously contracting networks. Rates of contraction decreased significantly between culture days 3 and 7 (\( P < 0.05 \)) in monolayers from both groups. At day 7, enriched monolayers had significantly higher cardiac myocyte fractions and contraction rates than regular monolayers (60.5 ± 1.5 vs. 43.8 ± 0.5% of the culture area, \( P < 0.04 \), and 169 ± 8 vs. 132 ± 10 beats/min, \( P < 0.01 \)), which is consistent with previous reports (22).

Construct morphology. After 7 days of culture, cell-polymer constructs appeared discoid (~5 × 1.3 mm (diameter × thickness); Table 1). The peripheral zone was 50–70 µm thick (Fig. 2A) and consisted of more cell layers in the enriched than in the regular group (7 ± 1 vs. 5 ± 1 layers, respectively). Cells in this outermost zone formed a continuous, 3-D tissue-like structure by attaching to other cells, spreading along the randomly oriented PGA fibers, and forming bridges between the fibers (Fig. 2, A and C). Distinct cardiac bundles, spatially oriented groups of cells (>100 µm in size), and interstitial collagen septa were not observed. Randomly oriented cells in the peripheral zone exhibited a variety of shapes, from elongated cells spread on the polymer fibers to round unattached cells, as assessed histologically. The majority of the cells expressed the muscle-specific proteins sarcomeric tropomyosin (Fig. 2, C and D) and sarcomeric \( \alpha \)-actin (data not shown). Immediately below the peripheral zone was a 60- to 70-µm-thick region consisting mainly of cells that did not express tropomyosin. At the construct center, cells were sparsely distributed and either elongated, expressing tropomyosin, or round, with pyknotic nuclei and acidophilic cytoplasm (Fig. 2B).

Cross striations were present in cells in the peripheral zone of the constructs as well as in neonatal and adult ventricles, as assessed immunohistochemically.
The presence of subcellular elements characteristic of cardiac myocytes, including myofila-
ments with well-defined sarcomeres, z-lines, glycogen granules, and mitochondria in the outermost layer of constructs, was demonstrated by transmission electron microscopy (TEM). Cell-to-cell connections were demonstrated by the presence of desmosomes and intercalated disks. Construct composition. After 3 days, the respective numbers of viable cells present in enriched and regular constructs were 66 and 57% of those seeded at time 0, as calculated from medium LDH levels. LDH release between culture days 3 and 7 was one-third of that between days 0 and 3, indicating that the cell death rate decreased with cultivation time. At 7 days, cell numbers in enriched and regular constructs were 38 and 47% of the respective numbers seeded at time 0, as determined by the DNA content of constructs. For comparison, 7-day cell monolayers from both groups contained 61 ± 6% of the initially plated cells. The number of cells seeded at time 0 (8 million per PGA disk) could be accounted for by summing cell numbers in constructs at day 7 (determined from DNA content) and in the medium over 7 days (calculated from cumulative LDH activity/construct) (Table 1), implying that no significant cell proliferation occurred during the cultivation period. Glucose consumption and lactate production rates were higher in enriched than in regular constructs (P < 0.005, Table 1), whereas the lactate-to-glucose molar ratios were similar for both construct groups (1.00 ± 0.20 and 1.30 ± 0.11, respectively).

Ventricular tissues from neonatal and adult rats had respectively six- and threefold higher DNA contents per unit wet weight (an index of cellularity) than engineered constructs from either group (P < 0.01, Fig. 4A), which is consistent with the relatively acellular appearance of the construct centers (Fig. 2B). Relative cell size, assessed from the ratio of total protein to DNA, was comparable for cells in constructs, neonatal ventricles, and monolayers and lower than for cells in adult ventricles (P < 0.01) (Fig. 4B). This finding was consistent with the relative cross-sectional areas of cells in constructs and neonatal and adult ventricles observed histologically (Fig. 2, D–F, respectively). The MTT conversion per unit DNA (an index of metabolic activity) was similar for constructs and neonatal ventricles and was slightly higher in adult ventricles (Fig. 4C).

Construct electrophysiology. Spontaneous, macroscopic contractions of engineered constructs were visually observed in flasks between days 2 and 4 of cultivation, which indicated the presence of intercellular communication. At day 7 the majority of constructs and native ventricles exhibited transient spontaneous beating lasting for 1–10 contractions (Fig. 5A), which may have resulted from reentrant or triggered activity (4). Electrical stimulation resulted in impulse propagation in the peripheral cardiac tissue-like zone of the constructs. In contrast, impulses failed to propagate when the electrodes were advanced toward the central acellu-
lar region of the constructs. All 7-day constructs were electrically excitable and could be captured over a wide range of pacing frequencies (up to 270 beats/min, Fig. 5, B–D). Step increases in construct pacing frequency resulted in transient decreases in conduction velocity to steady-state values (data not shown). Rapid stimulation induced short tachyarrhythmias with rates close to the maximum capture rates in 3 of 6 enriched constructs, 2 of 6 regular constructs (Fig. 5E), 1 of 10 adult ventricles, and 0 of 10 neonatal ventricles. A separate experiment showed that constructs remained electrically excitable for up to 4 wk of culture (data not shown).

Representative examples of impulse propagation in an enriched construct, a neonatal ventricle, and an adult ventricle are shown in Fig. 6, A–C, respectively. Propagating extracellular waveforms in constructs and native tissues showed fairly smooth, biphasic shapes with distinct downward deflections that enabled confident determination of activation times and implied nondecremental, macroscopically continuous propagation without wave collisions (34). Notches in extracellular waveforms occasionally observed in constructs (see E3 in Fig. 6A) may have reflected asynchronous excitation in adjacent groups of cells caused by the presence of empty space, polymer fibers, and/or necrotic tissue (36). Conduction times in ventricles and constructs increased linearly with distance over 5 mm (Fig. 6D).

Fig. 4. Cellularity, hypertrophy, and metabolic activity indexes of constructs and ventricles. A: DNA content per unit wet weight (ww). B: protein per unit DNA. C: MTT conversion per unit DNA. Data represent means ± SE of 5 samples. *Statistical difference between constructs and native ventricles; †statistical difference between neonatal and adult ventricles.

Fig. 5. Electrophysiological recordings. A: short transient spontaneous beating at a rate of 70 beats/min, which lost regularity after 6 s. B, C, and D: steady-state responses after ≥4 min of pacing at rates of 80, 150, and 200 beats/min, respectively. E: short, 5-s tachyarrhythmia at ~190 beats/min, apparently induced by 4 rapid stimuli at 250 beats/min. S and R indicate the stimulus spike and the construct response, respectively. All tracings were recorded from enriched constructs using the same electrode.
Table 2. Electrophysiological parameters in 7-day constructs and native ventricles

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Excitation Threshold, V</th>
<th>Conduction Velocity, cm/s</th>
<th>Maximum Amplitude, mV</th>
<th>Average Amplitude, mV</th>
<th>Maximum Capture Rate, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constructs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regular*</td>
<td>6</td>
<td>2.70 ± 0.24</td>
<td>9.35 ± 0.27</td>
<td>0.52 ± 0.05</td>
<td>0.26 ± 0.09</td>
<td>111.7 ± 9.5</td>
</tr>
<tr>
<td>Enriched*</td>
<td>6</td>
<td>2.97 ± 0.30</td>
<td>11.89 ± 0.46†</td>
<td>0.90 ± 0.14</td>
<td>0.43 ± 0.14</td>
<td>175.0 ± 21.3†</td>
</tr>
<tr>
<td>Ventricles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neonatal</td>
<td>10</td>
<td>0.74 ± 0.20</td>
<td>21.82 ± 1.48</td>
<td>31.91 ± 3.53</td>
<td>18.34 ± 4.31</td>
<td>475.0 ± 25.0</td>
</tr>
<tr>
<td>Adult</td>
<td>10</td>
<td>1.34 ± 0.17‡</td>
<td>31.69 ± 4.44‡</td>
<td>25.82 ± 2.81</td>
<td>14.62 ± 3.59</td>
<td>281.2 ± 21.0‡</td>
</tr>
</tbody>
</table>

Data represent means ± SE; n = no. of constructs or ventricles. *Significant difference between constructs and ventricles; †significant difference between enriched and regular constructs; ‡significant difference between neonatal and adult ventricles.

Fig. 6. Impulse propagation (representative 66 ms) in a construct from the enriched group (A), neonatal ventricle (B), and adult ventricle (C). The extracellular waveform is shown propagating from the electrode closest to the stimulus site (E1) toward the furthest electrode still in the sample (E6). Simultaneous deflections at the beginning of traces (S) represent stimulus artifacts. Amplitude ranges for each electrode were adjusted to best display recorded response waveforms. D: plot of conduction times vs. distances relative to E1, which was assigned the coordinates (0,0). Conduction velocities were calculated as inverse slopes of best fit lines.
of previously reported in vivo and ex vivo epicardial mapping studies (6, 46, 48). Bipolar point stimulation and unipolar recording (16, 25) in the custom-designed test chamber (Fig. 1B) did not adversely affect samples with respect to their electrical properties (waveform shapes were stable) or structure (no apparent tissue damage was observed histologically). Automated data analysis was facilitated by the high average signal-to-noise ratios (of ~10 and 470 for constructs and native ventricles, respectively). Whereas 1- to 5-V amplitude, 1-ms duration electrical pulses were sufficient to induce impulse propagation in slices of ventricles and in the peripheral zone of 7-day constructs, it was difficult to overdrive 7-day confluent monolayers of neonatal cardiac myocytes even when using stimuli of twice this amplitude and duration. In addition, impulse propagation in monolayers could not be assessed using extracellular electrodes because of fractionation and low amplitudes of recorded waveforms. These findings may be due to 3-D electrotonic interactions between cells (9) and relatively high cell density around the stimulating and recording electrodes in 3-D constructs compared with 2-D monolayers.

The inferior electrophysiological properties of constructs compared with native ventricles (Table 2) can be attributed to differences in their macroscopic tissue architecture. In particular, the relatively high excitation thresholds (24) and low response amplitudes were associated with low construct cellularity (Fig. 4A). Low maximum capture rates and conduction velocities in constructs probably resulted from decreased cell coupling, the presence of intercellular defects, and geometric current-to-load mismatches (due to tissue discontinuities) (9, 26). Other mechanisms that could contribute to inferior construct electrophysiological properties include cell depolarization, reduced excitability, and slower repolarization resulting from injury during isolation and/or cultivation (32, 39). Intracellular recordings would be necessary to test the proposed mechanisms.

Compared with enriched constructs, lower conduction velocities, maximum capture rates, and amplitudes in regular constructs probably resulted from 1) the higher fraction of noncardiomyocytic cells, which would be expected to form high-resistance junctions with cardiac myocytes (28) and act as passive current sinks (9), and 2) the thinner cardiac tissue-like zone (Table 1). Lower maximum capture rates in the regular than enriched constructs could also be due to the relatively longer duration of cellular action potentials (as previously observed in fibrotic compared with normal cardiac tissue; Ref. 42).

Neonatal and adult ventricular tissues did not exhibit spontaneous beating ex vivo in a previous (39) or the present study. In contrast, enzymatically isolated ventricular cardiac myocytes cultured in monolayers are known to revert to a less differentiated phenotype, depolarize, and regain spontaneous contractile activity for as yet unknown reasons (39). In the present study, visible spontaneous contractions in constructs ceased after 4 days of cultivation. This finding might be attributed to gradual depolarization and decoupling of cardiac myocytes due to injury during cultivation. However, it is more likely that the cultivation of cardiac myocytes on 3-D biomaterial scaffolds in tissue culture bioreactors (Fig. 1A) promoted differentiated cellular phenotype and function. In support of this hypothesis, Sperelakis (38) showed that 3-D aggregates composed of electrically differentiated cardiac myocytes did not contract spontaneously but responded to electrical stimulation.

The aim of the present study was to demonstrate basic cardiac-specific features in constructs and to evaluate construct structure and electrophysiological properties on a macroscopic (tissue) level, rather than on a cellular level. In ongoing work, we are expanding our electrophysiological studies to include whole cell clamp and sharp microelectrode intracellular recordings and assessment of the spatial distribution of the gap junctional protein connexin 43 (23). We are also attempting to culture constructs with a thicker cardiac tissue-like zone by direct perfusion of constructs during cultivation (to improve mass transfer) and by coculturing cardiac myocytes with microvascular endothelial cells (as a first step toward inducing vascularization).

In conclusion, cardiac-specific features of engineered cardiac muscle constructs were demonstrated structurally and electrophysiologically and were related to the cellular composition of constructs. The 3-D multilayer structure in conjunction with macroscopic impulse propagation in engineered constructs can offer advantages for in vitro studies of cardiac muscle. In addition, structurally and functionally improved 3-D engineered cardiac muscle constructs could be eventually applied in vivo. To date, attempts to regenerate cardiac tissue have involved the injection of different muscle cell types (33, 43) or small tissue fragments (19) into the heart. Implantation of cardiac muscle constructs with a defined shape instead of isolated cells could potentially improve the efficiency and localization of tissue repair.