An in vitro beating heart model for long-term assessment of experimental therapeutics

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Aims Within the framework of studies aiming at regenerative medicine for cardiovascular disease, we have developed an in vitro model to analyse human embryonic stem (ES) cell engraftment into the myocardium.

Methods and results This model is based on organotypic rat ventricular slices maintained in culture at the air–medium interface on semi-porous membranes. Survival and differentiation of human cardiomyocytes derived from ES cells were then assessed for several months. In addition, we observed that ventricular tissue slices not only exhibited normal histology, but also rhythmic contractions till the end of the experiments (up to 3 months). Similar results were obtained using ventricular slices obtained from two human foetuses at 8 and 9.5 weeks of age. Calcium transients were associated with the beating frequency, and the pattern was modulated in a dose-dependent manner by epinephrine.

Conclusion Our data suggest that the organotypic heart slice culture on semi-porous membranes is a relevant in vitro heart model for long-term histological and physiological studies.

KEYWORDS Heart slice cultures; Cell therapy; Human embryonic stem cells

1. Introduction

Development of cell therapy products to substitute for cardiomyocytes lost due to cardiovascular diseases has been a major field of research over the past 10 years. A variety of cells have been contemplated, including skeletal myoblasts,1–6 mesenchymal stem cells from bone marrow,7–10 and most recently embryonic stem (ES) cell-derived cardiomyocytes11–13 or cardiac committed stem cells.14 However, functional results have been controversial, calling for a reassessment of the mechanisms of action of the transplanted cells. It has, in particular, been difficult to determine whether a significant proportion of cells did engraft, whether some cells differentiated into fully mature cardiac myocytes, or whether such a differentiation process led to functional integration into the recipient heart.14,15

The precise answers to these questions have been difficult to provide for technical reasons, as data were derived from in vivo experiments, in the absence of in vitro models that would allow long-term assessment of cells transplanted into heart tissue.

Preliminary attempts at designing such a model have been made16–18 on the basis of organotypic heart slice cultures designed to evaluate the toxicity of xenobiotics.19 In their conditions, however, thin slice preparations of cardiac tissue could only be experimentally utilized for <2 weeks in culture due to poor viability.16 Another, more complex model has been recently designed by decellularizing the heart while preserving the extra-cellular matrix and reseeding it with cardiac or endothelial cells.20

We have reconsidered this important issue within the framework of studies aiming at substitutive cell therapy to the heart using cardiomyocytes derived from human ES cells. We have sought long-term survival of heart tissue slices, with good histological preservation, by applying to the heart the techniques designed for the so-called ‘chronic’ brain slices,21–23 in which we managed recently to assess implanted cells for several months.24,25

2. Methods

All experiments were performed in strict accordance with the recommendations of the European Ethical Committee (EEC) (86/609 EEC), the French National Ethical Committee (87/848), and by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) for the care and use of laboratory animals. The human heart tissue was obtained following elective abortion, with specific parental consent for scientific use as an ancillary to an ongoing clinical protocol aiming at foetal neural grafting in patients with Huntington’s disease (‘MIG-HD’, ref. NCT00190450). This investigation conforms to the principles outlined in the Declaration of Helsinki.
2.1 Organotypic ventricular slice cultures, set up, and characterization

Cultures were prepared from ventricular sections of 3-day-old rats (n = 30, Charles River, France) and from 8- and 9.5-week-old human foetuses. The hearts were removed and placed in phosphate-buffered saline (PBS), the atria were removed, and the ventricles sagitally sliced at 1 mm thickness using a rodent heart matrix (Harvard Apparatus, UK) (see supplementary material online, Figure S1). The heart slices were immediately transferred to a Millicell-CM 0.4 µm membrane (Millipore, France), and the insert was placed into a well containing 1 mL of medium in 6-well plates. The culture medium consisted of Dulbecco’s modified Eagle’s/F12 medium (DMEM/F12) supplemented with 20% knockout serum replacer (KSR), 1% non-essential amino acids, 2 mM L-glutamine, 0.1% β-mercaptoethanol, and 0.1% penicillin/streptomycin (Invitrogen, France). Heart slices were maintained for 30-80 days at 37 °C in a humidified atmosphere containing 5% CO2. Medium was changed three times a week. Slices were observed under an inverted microscope every other day.

Rate and rhythm of heart slice beating were characterized in parallel as well as the localization of the most apparent contractions. Further functional characterization of the contractile function of ventricular slices was carried out on 23 rat and five human heart slices, at 30 days after plating, using a stereomicroscope and a temperature-controlled stage set at 37 °C. During these sessions, each slice was incubated with 10, 100 nM, then 1 µM epinephrine (Sigma, France). Beat frequency was monitored 3 min after β-adrenergic stimulation.

Additional analysis was performed on three rat heart slices at 30 days after plating using a confocal laser scanning microscope LSM510 (Zeiss), following incubation with 5 µM fluo-4 AM (Invitrogen) for 20 min at 37 °C.

2.2 Intra-slice cell implantations

Human ES cells (SA01, Cellartis, Sweden) were maintained on mitomycin C-inactivated mouse embryonic fibroblast feeder cells (STO, ATCC) in DMEM/F12 medium supplemented with 20% KSR, 1% non-essential amino acids, 2 mM L-glutamine, 0.1% β-mercaptoethanol, and 4 ng/mL basic fibroblast growth factor (Invitrogen). Undifferentiated human ES cells were harvested and resuspended in the medium used for organotypic slice culture at a density of 1 × 10⁵ cells/mL. An aliquot of 0.1 µL of this cell suspension was then injected into heart slice cultures, using a 0.5 µL Hamilton syringe fixed to the arm of a micromanipulator. Penetration of human ES cells into rat heart slices was quantified by counting human cells every 25 µm layer from the slice surface. Five sections of five different heart slices were analysed.

The contraction rate of heart slices was monitored daily using a Zeiss-Lumar V12 stereomicroscope.

2.3 Gene expression

Total RNA was extracted from heart slice cultures using TRIZOL Reagent (Invitrogen), according to manufacturer’s protocol. RNA (1 µg) was reverse-transcribed using SuperScript II RNase H-Reverse Transcriptase (Invitrogen). cDNA was used as a template for gene expression analysis of human cardiac markers. Real-time quantitative PCR was performed using a LightCycler with SYBR Green I master (Roche Diagnostic, France). The following primer pairs were used: human-Oct-4 forward: 5'-CTT-GCT-GGA-GAA-GTG-GGT-GGA-GGA-A-3' and reverse: 5'-CT-GAG-TGG-TTT-CGG-GGA-3'; human-Nanog forward: 5'-GGA-GAA-AAC-ACC-CTT-3' and reverse: 5'-GGC-GGA-GAAC-ACC-CTT-3'; human-Gata4 forward: 5'-TTT-TCA-ATT-TTG-CAG-AGT-3' and reverse: 5'-GGC-GGT-GAT-TCA-GAG-3'; human-β myosin heavy chain (β-MHC) forward: 5'-GGC-GGA-GAT-TCT-GGA-GGA-3' and reverse: 5'-GT-GG-CTG-AGC-GAC-AGG-AT-3'; human-atrial natriuretic peptide (ANP) forward: 5'-TGT-TGC-CAT-GTT-ATG-AT-3' and reverse: 5'-GAG-AGG-GGA-AGT-CAC-3'; human ubiquitin C (UbC) forward: 5'-ATT-TGG-GTC-GGG-CTT-3' and reverse: 5'-CT-CGT-CAC-ATT-CGT-GGT-3'; and rat ubiquitin C (UbC) was used as an internal control forward: 5'-TGC-CAC-TCT-ATG-GTG-AT-3' and reverse: 5'-GAA-AAC-TAA-GAC-ACC-TCC-3'. Amplification PCR reaction was performed with an initial denaturation step at 95 °C for 5 min, followed by 50 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 15 s.

Quantification of gene expression was based on the DeltaCt method and normalized on human UbC. Melting curve analysis was used to determine the specificity of PCR products, which was confirmed using gel electrophoresis run.

2.4 Immunohistochemistry

Heart slices fixed in 4% paraformaldehyde for 2 h at 4 °C were cut into 10 µm thick sections. The cryosections were washed with PBS and then incubated for 1 h at room temperature with a saturating solution consisting of 5% normal goat serum, 5% normal horse serum in PBS 0.6% Triton X-100 (Sigma). Slides were incubated overnight at 4 °C with the following primary antibodies used at 1:400: anti-human nuclei mouse monoclonal (HNA, Chemicon, France), anti-connexin 43 mouse monoclonal (CX43, Chemicon), anti-rat CD31 mouse monoclonal (BD Pharmigen, France), anti-tropinin I rabbit polyclonal (cTNI, Chemicon), anti-sarcomeric actinin mouse monoclonal (Sigma), anti-desmin mouse monoclonal (Chemicon), anti-human alpha-actinin mouse monoclonal (Chemicon), and anti-Ki67 mouse monoclonal (Chemicon). After three washes with PBS, the slides were incubated with a 1:500 dilution of fluorescent-conjugated secondary antibodies for 1 h at room temperature. Secondary antibodies used were: Alexa-Fluor 488 goat anti-rabbit IgG and Alexa-Fluor 555 goat anti-mouse IgG (Molecular Probes, France). The specificity of all secondary antibodies was tested by incubation of only the secondary antibody without a pre-primary antibody incubation. All secondary antibodies were negative for non-specific staining in our immunostaining condition. After three washes, the slices were incubated with 1/10000 DAPI and then the slides were mounted by using Fluoromount-G (Clinisciences, France). The slides were observed under an epifluorescence microscope (Zeiss Imager Z1), and images were processed using the Axiovision software.

2.5 Statistical analyses

Statistical analysis was performed using analysis of variance (ANOVA) and Student’s t-test. For time course of cell differentiation, statistical analysis was performed using ANOVA and Dunnett’s test.

A value of P ≤ 0.05 was considered statistically significant.

3. Results

Macroscopic analysis of the organotypic rat heart slices plated at the air-medium interface on semi-porous membranes demonstrated good overall preservation over time (Figure 1A). Immuno-labelling of cardiomyocytes using tropinin I and sarcomeric alpha-actinin antibodies confirmed the regular organization of the cells with little, if any, tissue disruption (see supplementary material online, Figures S1B and S2). Connexin 43 immunolabelling also revealed a dotted orientation distribution (Figure 1C), in keeping with that observed in histological sections of the heart. CD31 immunostaining additionally demonstrated the
3.1 Human embryonic stem cells differentiate into cardiomyocytes following intra-slice transplantation

Sixty days after transplantation of undifferentiated human ES cells into organotypic rat heart slices, HNA-positive human cells were readily observed (Figure 2B). These cells often clustered in several layers at the surface of the slices, but also dispersed in the tissue itself, intermingling with rat cells or accumulating around blood vessels. The large majority of human cells (81 ± 2.3%) was located within 250 µm from heart slice surface (see supplementary material online, Figure S3). Microscopic observation of the cardiac matrix appeared intact, which may have prevented the human ES cells from dispersing completely throughout the tissue.

The human cells expressed markers of differentiated cardiomyocytes, including troponin I (Figure 2B) and human-specific desmin (Figure 2C) and alpha-actinin (Figure 2D), for both, of which no cross-reactivity was observed in the surrounding rat tissue.

The expression of human specific genes was then monitored over time at 10, 20, 30, 40, 50, and 60 days after injection of undifferentiated human ES cells into rat heart slices using real-time PCR (Figure 3). We observed a downregulation of the undifferentiated stage markers Oct-4 and Nanog over the first weeks of culture (Figure 3A). Starting after 10 days and peaking at 1 month, the gene expression of Gata4 and Mef2c increased, indicating cardiac mesoderm differentiation (Figure 3B). After 2 months, cells expressed MLC2a, β-MHC and ANP, and three cardiac markers (Figure 3C).

In addition to human ES-derived cardiomyocytes and intermingled with them, anti-Ki67 immunolabelling—co-registering with HNA—revealed a significant proportion of human cells that were still proliferating up to 2 months after implantation (Figure 2E).

In contrast, immunostaining with endo- and ectodermal markers was negative. There was neither teratoma in heart slices transplanted with human ES cells.

3.2 Heart slices in culture show spontaneous rhythmic contractions over months

Repeated stereomicroscopic observation to check preservation of tissue slices revealed that heart slices exhibited spontaneous and regular rhythmic contractions during the entire duration of the experiments (see supplementary material online, Videos 1–3). This phenomenon happened whether slices had received cell grafts or not and was thus considered an intrinsic functional property of the heart slice itself in our technical set up.

Contractions were most often visible macroscopically as rhythmic alternation of one or several portions of the slice. More rarely, the entire piece of tissue would rise partially from the stage during contraction (see supplementary material online, e.g. Video 3). The areas where contractions were observed macroscopically also varied from one observation session to another.

Each contraction lasted less than a few 100 ms. The contraction rates were regular within the portion of the slice over time during an observation session, whereas two areas of the same slice could beat asynchronously. Beating rates were low, below 60 bpm, and in general between 30 and 40 bpm. The monitoring of spontaneous beating frequency of an organotypic rat heart slice at regular intervals over 81 days showed very little contraction rate change over time. Beating rate was usually recorded at 37°C, but a decrease in temperature down to 25°C had no visible impact on the frequency of contraction.

3.3 Rhythmic contractions of organotypic heart slices on semi-porous membranes are associated with calcium waves and modulated by epinephrine

Physiological mechanisms underlying heart slice beating were explored by loading organotypic cultures with Fluo-4 AM ester. A confocal analysis of the temporal changes in fluorescence intensity showed rhythmic calcium transients in different regions of the heart slices (see supplementary material online, Video 2). Quantification of these calcium oscillations showed that their rhythm was similar to that of the contractions observed macroscopically. Calcium waves were regular, not only over time but also in amplitudes at each local spot of analysis, where cells were excited in synchrony (Figure 4A).

Cardiomyocytes in the organotypic heart slices also responded to pharmacological manipulation, as demonstrated by applying epinephrine to the culture medium. This treatment increased spontaneous beating frequencies in a dose-dependent manner from 10 nM to 1 µM (Figure 4B). In parallel, the confocal analysis of calcium currents in heart slices loaded with fluo-4 AM showed a dose-dependent increase in Ca2+ transient amplitude and frequency with epinephrine (Figure 4C).
3.4 Organotypic human heart slice cultures at air–medium interface on semi-porous membranes

One millimetre thick organotypic heart ventricular slices were obtained from two human foetuses at 8 and 9.5 weeks of gestational age and plated at air–medium interface on semi-porous membranes. In keeping with the results obtained with rat tissue, human organotypic heart slices exhibited preserved histological markers of cardiomyocytes and blood capillaries. In parallel, regular rhythmic contractions were observed up to the end of the observation period (60 days) at a beating rate comparable with that of rat slices (between 30 and 40 bpm, see supplementary material online, Video 3). The addition of epinephrine to the culture medium at 10, 100 nM, and 1 μM also increased spontaneous beating frequency of human heart slices in a dose-dependent manner (Figure 4D).

4. Discussion

The main outcome of this study is the set up of an organotypic heart tissue culture method that allows long-term histological and physiological studies, including the human. This result was obtained by adapting to the heart the technique that allowed chronic organotypic brain slices by maintaining them at the air/medium interface on semi-porous membranes. The organotypic heart slice system is a useful substrate to analyse after implantation long-term cardiomyocyte differentiation of human ES cells. Preservation of cell excitability and intercellular connections was associated with long-term persistency of regular rhythmic contractions and calcium transients. This continuous physiological activity makes organotypic heart slices on semi-porous membranes suitable, in addition, for pharmacological studies.

Attempts at setting-up organotypic heart slice cultures have been made in the recent past by several authors, in a search similar to ours for models allowing testing of experimental therapeutics. Standard histological staining in combination with immunohistological analysis for cardiac markers demonstrated that, originally, slices were structurally intact. The function of ion channels and receptors could be preserved, in contrast to the observation made on dissociated cardiomyocytes in culture. The main limitation in previous models was the survival time of the tissue, which only allowed for short-term experimental evaluation (<2 weeks). Physiological recordings were even less encouraging, as they revealed a complete loss of network activity within 3–4 days. Comparison of these
data with the vast literature concerning the much more studied organotypic brain slice cultures led us to surmise that, as was the case for the nervous system, the poor preservation of heart tissue over time in those studies was related to the immersion of the slice in the culture medium. Long-term preservation of organotypic brain slices has been readily obtained by replacing immersion by techniques that allowed direct oxygenation of the slices, either using slow rotation to create continuous changing of the liquid–gas interface, or by placing slices at the air–medium interface on a semi-porous membrane. We chose to develop the latter technique because it was suitable for subsequent intra-slice cell implantation. Our results confirmed the long-term preservation of the organotypic slices also for the heart in those air–medium interface conditions. It is interesting to underline the fact that this long-term culture system preserved not only individual cells, but even more the pluricellular structures they form in the tissue. This was exemplified by the overall organization of well-aligned cardiomyocytes potentially linked by gap junctions, the preservation of membrane-bound connexins, the presence of a dense network of blood vessels—although without blood—and the demonstration of calcium waves spreading along local arrays of excitable cardiomyocytes. However, Cx43 was localized not only at intercellular contacts but more widespread in the cells, possibly a consequence of injury at the time of slice preparation.

The original goal of the set-up of chronic heart slices was the search for an in vitro heart substrate to analyse grafted cells. The rat heart slices allowed us to address this issue quite successfully, as human ES cells implanted at a fully undifferentiated stage not only survived and integrated with the rat tissue but also readily differentiated into cardiomyocytes. This latter result is of particular interest as it was obtained without any priming of the cells to be implanted with cytokines that push ES cells towards the cardiomyogenic lineage. Embryoid bodies generated from stem cells primed with TGF-beta, and BMP2 demonstrated an increased potential for cardiac differentiation, in vitro. In vivo, transplantation of stem cells into heart also resulted in cardiac differentiation, provided that TGF-beta/BMP2 signalling was intact, but this capacity remained limited unless cardiomyocytes had been previously damaged. Organotypic heart slices seem, therefore, to replicate the latter situation as they provide a similarly appropriate environment for cardiomyogenic}

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**Figure 3** Gene expression analysis of human cardiac markers. (A) Real-time polymerase chain reaction for Oct-4 and Nanog, (B) Gata4 and Mef2c, (C) myosin light chain 2a, β-myosin heavy chain, and atrial natriuretic peptide at different time points after implantation of undifferentiated human embryonic stem cells into rat heart slices. *P ≤ 0.05 and **P ≤ 0.001 when compared with human embryonic stem cells at day 0 (Dunnett’s test after analysis of variance). Data are mean ± SEM.
Figure 4 Characterization of spontaneous contraction of rat and human heart slices. (A) Ca^{2+} spiking within selected ROI of a heart slice loaded with Fluo-4. Recordings are expressed as DF/F0, where F0 is the lowest level of fluorescence. (B) Functional effects of epinephrine on heart slices in organotypic cultures. Dose–response increase in frequency of rat slice beating. **P ≤ 0.01 and ***P ≤ 0.001 when compared with untreated controls (Student’s t-test after analysis of variance). Data are mean ± SEM, n = 23. (C) Ca^{2+} spiking recorded after loading with Fluo-4 in rat heart slice cultures at rest and during application of 1 μM epinephrine. (D) Increase in frequency of human heart slice beating during epinephrine application vs. control. Mean ± SEM, n = 5.
differentiation, indicating the preservation of the cardiac paracrine pathway required for therapeutic benefit of stem cell transplantation in the diseased heart.

As an unexpected outcome of this study, preservation of spontaneously functional excitable networks was maintained for several months in the heart slices. The rhythm of the contractions was slow, in keeping with extranodal pacemakers in the ventricular slices. Notwithstanding that limitation, the slice model appears as a promising substrate for physiological analysis. It indeed displays regular contractions associated with calcium waves that spread over networks of cardiomyocytes that respond readily to the activation of β-adrenergic receptors. This will allow for functional studies looking for the effects of drugs on cardiomyocytes, whether for pharmacological or for pharmacotoxicological purposes.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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