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Attachment of Embryonic Stem Cells-derived Cardiomyocytes in CultiSpher-S Microcarriers by using Spinner Flask

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Abstract

Embryonic Stem (ES) cells have the ability to differentiate under *in vitro* conditions into cardiomyocytes. A transgenic α-myosin heavy chain (α-MHC$^+$) ES cell line was generated, exhibiting puromycin resistance and expressing enhanced green fluorescent protein (EGFP) under control of the α-MHC$^+$ promoter. A puromycin-resistant, EGFP-positive α-MHC$^+$ cardiomyocyte population was isolated with over 92% purity. The cultivation of these cardiomyocytes, in macroporous gelatine microsphere beads in a spinner flask bioreactor has been studied. After we specified the most suitable agitation conditions and the optimal timeframes of cultivation, the average number of cultivated cells per microsphere was optimised. Our study shows that 80 % of microspheres were colonised by cardiomyocytes under optimal conditions. The results of the scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) showed that the population of the beads was not limited to the microcarrier surface, but also invaded the inner surfaces of the microspheres. The present findings demonstrate the successful culture of α-MHC$^+$ cardiomyocytes in macroporous biodegradable microcarriers while maintaining the typical morphological and electrophysiological properties of cardiomyocytes. These findings suggest significant improvement in survival of grafted cardiomyocytes, thus helping overcome current limitations of cell replacement approaches.

Keywords: Cardiomyocyte; embryoid bodies; fluorescence; stem cell; SEM; microcarrier, Cultispher-S.

1. Introduction

The generation of functional cardiomyocytes from embryonic stem cells has several potential applications. Myocardial diseases resulting from damage of cardiac tissue effect millions of people, who may benefit from transplantation of cardiomyocytes. The feasibility of such an approach has already been demonstrated in animal models using several sources of cells (Li *et al.,* 2000, Liechty *et al.,* 2000). Congestive heart failure (CHF) is the resulting condition from any structural or functional cardiac disorder that impairs the ability of the heart to fill with or pump a sufficient amount of blood throughout the body. Cardiac transplantation studies have been conducted using ES cell-derived cardiomyocytes from grafted EBs, although these contained a mixture of different cardiac cell types (Klug *et al.,* 1996; Min *et al.,* 2002). Therefore, many strategies were developed to differentiate early cardiomyocytes from embryonic stem cells. The *in vitro* differentiation of embryonic stem cells and gene expression studies, that result in generation of tissue-specific somatic cells, may represent a powerful tool for general understanding of cellular differentiation and development *in vivo* (Gissel *et al.,* 2005). So far, specific investigations on the generation of cardiomyocytes with high purity and the characterisation of their gene expression signatures have been conducted from a transgenic α-MHC (α-myosin heavy chain) ES cell line (CGR8) exhibiting puromycin resistance enhanced green fluorescent protein (EGFP), under control of α-MHC promoter (Doss *et al.,* 2007). The microcarrier technique is commonly used to grow anchorage-dependent cell lines in suspension cultures. Microcarriers are inert materials with surface characteristics suitable for promoting cell growth including charge, polarity, hydrophobicity and functional groups. An advantage of microcarrier culture systems is the ability to scale up into large volume production units. Microcarrier culture has been used to grow a wide variety of cell types. A wide range of biodegradable materials has been used for preparing microcarriers employed in cell culture, including Gelatin (e.g. Cultispher-S, G & GL) (DelGuerra *et al.,* 2001), D, L polylactic-coglycolic acid (PLGA) (Newman and McBurney, 2004), Cellulose and others (Yang *et al.,* 2007).

In the present study, we cultivated α-MHC$^+$ cardiomyocytes within CultiSpher-S microcarriers by using spinner flask bioreactor and tested their survival. The use of biodegradable microcarriers (CultiSpher-S) as a potential system to increase the survival and to achieve a new protocol can help attach these cells in the biodegradable microspheres.

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2. Materials and Methods

2.1. Generation of ES-derived α-MHC$^+$ Cardiomyocytes

Murine CGR8 ES cells (strain 129; Ampegic 7) were cultured in the absence of feeder cells in Glasgow minimum essential medium (GMEM) (Invitrogen, UK) supplemented with: 10% fetal calf serum (FCS) (Invitrogen, UK), 2 mM L-glutamine (Invitrogen, UK), 100 units/ml leukaemia inhibitory factor (LIF) (Chemicon), 50 μM β-mercaptoethanol (MRE) (Invitrogen, UK) and 100 mg/ml Neomycin (Invitrogen, Germany) in gelatine coated flasks (0.2% gelatine in Phosphate buffer saline), and maintained at 37C° and 5% CO2 (Gissel *et al.*, 2005).

ES cells were passaged four times before induced their differentiation to EBs. To assess this differentiation, the ES cell suspension of 2.5 x 10^5 cells/ml cultivated in Iscove's modified Dulbecco's Medium (IMDM) (Invitrogen, UK) supplemented with 20% FCS, 2 mM/l L-glutamine, 100 µM/l β-MRE, and 1% non-essential amino acids (Invitrogen, UK) (vol/vol). The preparation of EBs was carried out according to the (Gissel *et al.*, 2005) by hanging drops (20 µl/ drop) was made on the lid of 10 cm bacteriological dish containing 5 ml calcium and magnesium-free phosphate-buffered saline (PBS). Hanging drops of EBs were incubated in the corresponding growth medium at 37^{0}C in an atmosphere containing 5% CO_2 for two days. Then, these hanging drops were washed with 20 % IMDM medium and re-seeded in a new sterile dish to get 2 days old EBs and incubated at 37^{0}C for 5 more days to generate 7 days old EBs.

On day 8 the beating clusters were observed and α-MHC$^+$ cardiomyocytes were formed. These cells were treated with 4µg/ ml puromycin (Sigma Aldrich, Germany) as described by (Doss *et al.*, 2007) and cultivated in the same conditions for 6 days. The α-MHC/EGFP$^+$ beating cardiomyocytes were examined under a fluorescence microscope and documented through the videos and pictures (Axiovert 10, Zeiss, Germany) (Fig. 1, left).

2.2. Preparation of CultiSpher-S Mircrocarriers

CultiSpher-S microcarriers beads (diameter 130 – 380 µm) were obtained from Percell Biolytica (Astrop, Sweden) and were prepared according to the manufacturer's instructions. They represent macroporous gelatine beads in which the cardiomyocytes have the ability to use the interior surface. Briefly, for their preparation dry microcarriers (100 mg dry CultiSpher-S/5 ml) were swollen and hydrated in calcium and magnesium-free PBS for 2 hours at room temperature. Without removing the PBS, the microcarriers were sterilized by autoclaving at 121^{0}C, 15 psi (about 1 bar) for 15 min. Consequently, PBS was removed and the microcarriers were washed twice in IMDM medium. The sterile CultiSpher-S were counted by haematocytometer and divided in aliquots over small bacteriological dish (3 cm) where each one contained 200 microspheres prior to use, as shown in Figure 2 C.

2.3. In Vitro Expansion of α-MHC$^+$ Cardiomyocytes in CultiSphere-S

The 14 days old of beating α-MHC$^+$ cardiomyocytes were dissociated with collagenase B (Roche, Mannheim, Germany) (1 mg/ml calcium and magnesium free PBS), and incubated at 37^{0}C in 5%CO_2 for 30-45 minutes (Figure 2.d). After dissociation the cells suspension was centrifuged (1000 rpm for 7 min.) Then the supernatant was removed and the single dissociated cardiomyocytes were re-suspended in the 1 ml culture medium IMDM supplemented with 20% FCS, 1% non-essential amino acids (vol/vol), 2 mM/l L-glutamine, and 100 µM/l β-MRE (Figure 1, left). Dissociated cells were mixed with known numbers of hydrated CultiSpher-S microcarriers in siliconized sterile spinner flask filled with IMDM medium plus 20 % FCS, and incubated for four days at 37 °C, 5 % CO_2, over the magnetic stirrer at stirring rate of 40 rpm for 4 days. The entrapment of the cells was observed before and after the stirring of the cells and Cultispher-S microcarriers. Cell entrapment was monitored using a fluorescence microscope (Axiovert 10, Zeiss, Germany).

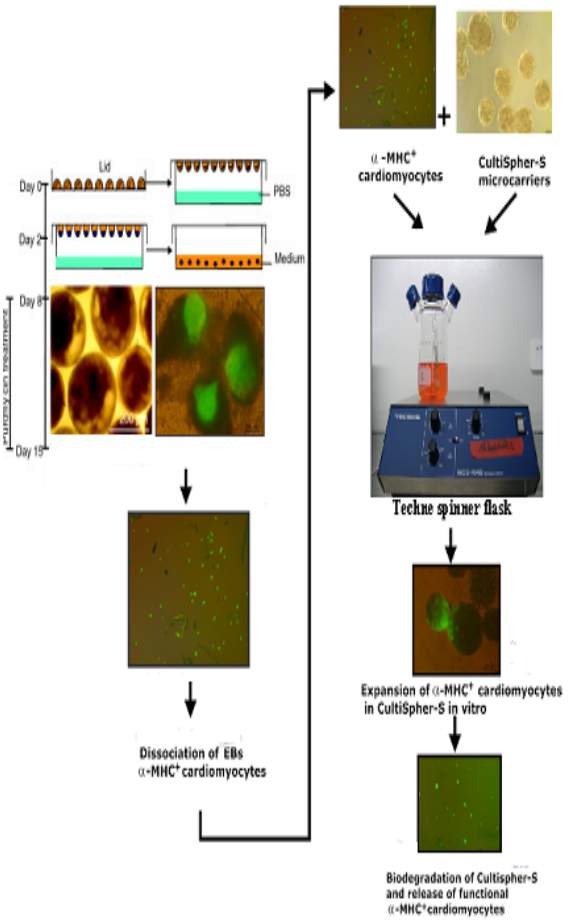

Figure1. Diagrammatic illustration of Techne spinner flask over magnetic Stirrer rotating the suspension of CultiSpher-S and cardiomyocytes at rate 30 rpm. Protocol used for generating ES cell-derived cardiomyocytes (left) and expansion of α-MHC+ cardiomyocytes in CultiSpher-S beads *in vitro* (right).

The 7 days old α-MHC$^+$ cardiomyocytes entrapped in CultiSpher-S were transferred into 15 ml Falkon tubes and were washed with calcium and magnesium-free PBS without centrifugation. The CultiSpher-S microspheres carrying α-MHC$^+$ cardiomyocytes were incubated in the presence of collagenase B (2 mg/ml) for 2 hours until the complete dissociation of the microcarriers, the cells were counted by using both the haemocytometer (Thoma Optic Labor, Germany) and vital stain the trypan blue 0.4% in normal saline (Figure 1, right).

2.4. Scanning Electron Microscopy (SEM) Of Cultispher-S Entrapped With Cardiomyocytes

CultiSpher-S microcarriers containing entrapped α-MHC cardiomyocytes were washed with 0.25 M HEPES buffer (Sigma-Aldrich, Germany) and were fixed with glutaraldehyde, which was added to the suspension in a final concentration of 2% (v/v). The suspension was washed with sodium cacodylate buffer, pH 7.2, and was embedded in 2% low melting agarose (Sigma-Aldrich & Taufkirchen, Germany). The post fixation was performed by the treatment of the microcarriers suspension in three steps using 2% osmium tetroxide, 1% tannic acid and 1% uranyl acetate in water as previously described (Katsen *et al.,* 1998). Dehydration of the suspension was carried out in increasing series of ethanol, while the last dehydrating step was performed in acetone.

The CultiSpher-S microcarriers entrapped with the cells were prepared for block-face scanning electron microscopy (SEM) (Philips, USA). Briefly the microspheres entrapped with the cells were embedded in epoxy resin, cross-sectioned with ultra-microtome, coated with carbon in SCD-030 (Balzers, Lichtenstein) and examined in a field emission scanning electron microscope (FESEM) XL30 (Philips, USA) using Back Scattered electron (BSE) modes with 12 KV accelerating voltage and 10 mm working distance (Katsen *et al.,* 1998).

2.5. Confocal Laser Scanning Microscopy (CLSM)

Detection of α-MHC+ cardiomyocytes on CultiSpher-S microcarries was done by using the confocal laser scanning microscopy (Eclipse TE2000-U microscopy, D-Eclipse C1 CLSM, Nikon, Japan). The entrapped microspheres with α-MHC+ cardiomyocytes were cultured in 20% IMDM medium, and placed into a micro-dish (IBID GmbH, München, Germany). A Z-stack was performed with 5 µm step size. Images were taken at room temperature, in separated channels for red and green emission. A three dimensional image was constructed afterwards by merging single Z-stack images by software EZ-CE1 (Nikon, Japan)**.**

3. Results and Discussion

3.1. Isolation of Highly Purified A-MHC$^+$ Cardiomyocytes from Transgenic Embryonic Stem Cell Line

In this study the cardiomyocytes were generated with high purity from transgenic α-MHC+ ES cells line and the puromycin resistance and EGFP under the control of the α-MHC$^+$ promoter was detected microscopically within the EBs after treatment with puromycin. These puromycin-resistant, EGFP-positive α-MHC $^+$ cardiomyocyte populations were isolated with over 92% purity.

The transcriptomic analysis of the α-MHC$^+$ cardiomyocytes in comparison to undifferentiated transgenic ES cells and 15 day old control EBs identified transcripts differentially regulated in the cardiomyocyte population (Figure 2.a,b,c). The specific gene expression pattern of the α-MHC$^+$ cardiomyocytes reflected the biological, physiological, and functional processes that take place in mature cardiomyocytes.

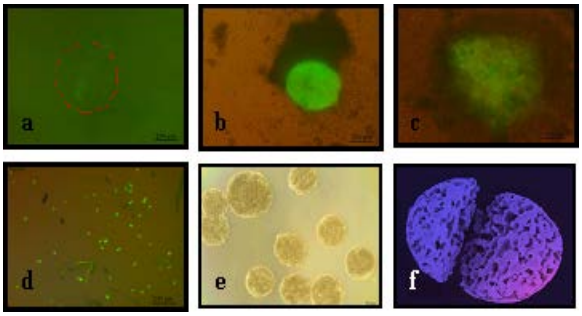

Figure 2. (a) Control group: the attachment of the cardiomyocytes aggregates without puromycin treatment. (b,c) Progressive purification of α-MHC$^+$ cardiomyocytes aggregates after treatment of the 8days EBs with puromycin for 7days. (d) Single cardiomyocytes after dissociation of the EBs with collagenase-B (e) CultiSpher-S microspheres (f) External features of CultiSpher-S. microspheres .

*3.2. In vitro Expansion of A-MHC+ Cardiomyocytes in Cultispher-S Microspheres Using Spinner Flask Bioreactor***.**

CultiSpher-S gelatine microspheres exhibited less porosity than other commercial microcarriers of (20µm in diameter) with a larger matrix component.

Transplantation of ES cells-derived cardiomyocytes is currently limited by the low survival rate of the transplanted cells. The loss of transplanted cells might be partially due to mechanical stress and could be avoided by delivering cells embedded in carriers offering a protected environment for cells. We therefore investigated whether cardiomyocytes can be colonised on CultiSpher-S microbeads in spin cultures. EGFP$^+$ cardiomyocytes were co-incubated with the beads, with and without stirring for four days in culture and when examined by fluorescence microscopy, the entrapment was more efficient in the case of stirred cultures, and less efficient in the case of without stirring. These cells were adherent, spread and occupied the CultiSpher-S gelatin microspheres (Figure 3.A-F). From this result it's appeared that stirring conditions such as spinning is important for enhancing attachment of the cells to the microspheres. Fernandes *et al.,* (2007) reported that a stirred culture system (spinner flask) and CultiSpher-S microspheres was successfully used to scale up mouse ES cells expansion.

Medium replacement in the stirred microcarrier culture system allowed the supply of nutrients to the cells and the removal of waste products inhibiting cell growth. Notably, the cardiac cell expansion in the CultiSpher-S beads led to the maintenance of the cultures in a steady state from several days up to four weeks, indicating efficient supply of nutrients to the cells.

The macroporous microcarriers allowed cardiomyocytes to attach to both the external and internal surfaces of the matrix, thus increasing the usable surface area of the matrix and providing cells with protection from mechanical stress. For a multitude of cell types, the three-dimensional macroporous collagen surface had been previously demonstrated to contribute to increased cellular attachment, resulting in high cell density (Ohlson *et al.*, 1994; Werner *et al*., 2000). A variety of microcarriers including Cytodex 3 (Abranches *et al*., 2007; Fernandes *et al*., 2007) and CultiSpher-S microcarriers (Tielens *et al*., 2007) have already been investigated in mouse ES cells with potential applications for tissue engineering. Alternatively, polystyrene-based cationic trimethyl ammonium-coated microcarriers in spinner flasks were used to expand human fibroblasts enabling extended self-renewal and expansion while retaining full differentiation potential (Phillips *et al*., 2008).

Surface area, bead size, cell adhesion and cell spreading as well as spinning condition are all critical factors that may modulate cell attachment, and ultimately the cellular expansion potential (Koller and Papoutsakis, 1995; Hammond and Hammond, 2001). Notably, $EGFP^+$ cardiomyocytes growing on gelatine beads exhibited similar growth kinetics and beating behaviour to control $EGFP^+$ cardiomyocyte cultures (Figure 2.b,c).

In order to determine the optimum conditions for obtaining entrapment cardiomyocytes, the microbeads colonisation profiles were analysed and the average number of $EGFP^+$ cardiomyocytes that can be seeded per bead were estimated, we found that about 1400 cardiac cells were entrapped by the CultiSpher-S beads. Under these conditions, 80% of total microcarries were populated by cardiomyocytes. From this result its appeared that for optimum entrapment of cardiomyocytes, a large excess of cells to microbeads was needed (Figure 3A-F, and Table.1).

Table 1. Correlation between the number of cardiomyocytes and entrapment

	Exp 1	**Exp 2**	**Exp 3**	**Exp 4**
Number of Cells	50000	100000	300000	500000
% Entrapment	10 %	20 %	40 %	80 %

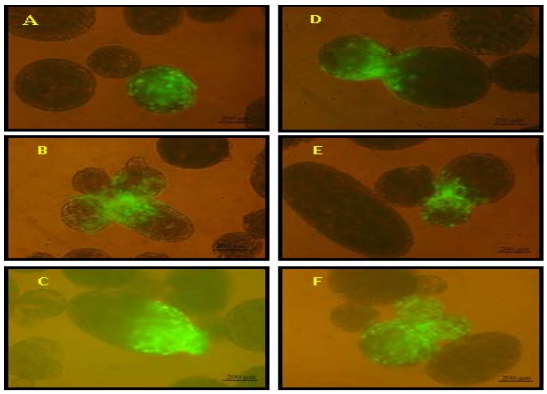

Figure 3.A-F Progressive entrapment of the 7 days old α-MHC^+ cardiomyocytes in CultiSpher-S microspheres by using the Techne spinner flask for four days at 50 rpm. Magnification 10 x, scale bar = 200 µm.

Characterization of Endogenous and Exogenous Expansion of Cardiomycytes on Cultispher-S Microspheres

The highly cross linked matrix of the CultiSpher-S beads permits the entrapment of a high cell numbers. In this study we estimate that about 1400 cardiac cells can be entrapped per microcarrier in both interior and exterior surfaces. Microscopical investigations by block face SEM and CLSM showed the attachment of cardiomyocytes in pore channels with cells not being restricted to the outer surface but also populating the inner surfaces of the gelatine beads after 7 days (Figure 4 A-F and Figure 5.A-H). The ability of ES cells-derived cardiomyocytes to attached in different sites of CultiSpher-S gelatine beads and survives for extended periods of time, this results agreement with the results of DelGuerra *et al.*, (2001), who reported that, the endogenous and exogenous colonisation of microspheres. The successful entrapment of cardiomyocytes on gelatine beads as well as migration of the cells deep into the macroporous matrix as demonstrated by SEM and CLSM is due to the macroporous structure of gelatine beads, which have closely mimicked a (required microenviromental niche) (Yang *et al.*,2007).

Identification features representative of the cardiac cytoskeleton, including myofilaments and muscle Z discs, were well defined in the cardiomyocytes populating the CultiSpher-S microcarriers. The lateral boundaries of the sarcomeres defined by the Z disc constitute the anchoring site for actin, titin, and nebulin filaments and are the primary conduits of the force generated by contraction (Guan *et al*., 1999). Crosslinked cardiac actinin and titin are organised into repeated sarcomeres along the length of the myofibril and give skeletal and cardiac muscles their striated appearance (Figure 5 E,F)

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The presence of these cellular characteristics in the cardiomyocytes cultured on the microcarriers as well as their contactile behaviour and electrophysiological properties demonstrate that the present microcarrier culture technique is suitable to maintain ESC-derived cardiomyocytes in culture.

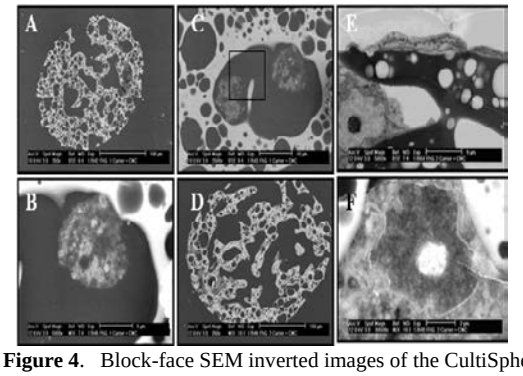

Figure 4. Block-face SEM inverted images of the CultiSpher-S microspheres colonised with α-MHC^+ cardiomyocytes. (A-D) Block-face SEM images showing the entrapment of cardiomyocytes in the pore channel of CultiSpher-S gelatine beads; B- view of area squared in A, rotation 90°. (E,F) block-face SEM images demonstrating the microcarrier - cardiomyocyte contact and typical features of cardiomyocytes.

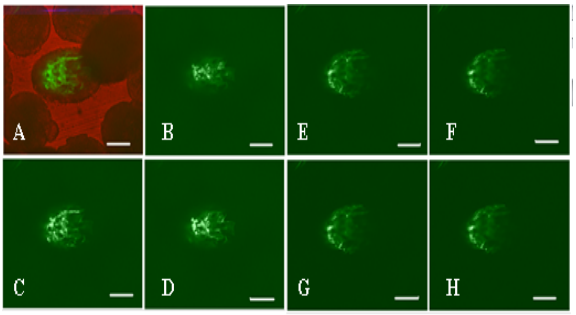

Figure 5. CLSM pictures of an entrapped CultiSpher-S microspheres with α-MHC+ cardiomyocytes (7 days old). A three dimensional image of merged Z-stack images. (A-H) Different sections of the scanned slides. Magnification 20 x. Scale bar = 100 µm.

4. Conclusion

In conclusion, α-MHC^{+} cardiomyocytes derived from ES cells attached efficiently both endogenously and exogenously to CultiSpher-S microspheres in agitated cultivation system and successfully expand *in vitro*. The cardiomyocytes entrapped within the CultiSpher-S microcarriers remain functional as demonstrated by the observation of intact action potentials and cytoskeletal features. The present study thus offers a perspective for transplantation strategies.

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