Transplantation of cardiac-committed mouse embryonic stem cells to infarcted sheep myocardium: a preclinical study

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Summary

Background **Heart failure develops after myocardial infarction and is a major cause of morbidity and mortality. The ability to direct differentiation of embryonic stem cells (ESC) towards a cardiomyogenic phenotype makes them an attractive therapeutic option for cardiac repair, but species-specific and individual-specific immunological imprinting remains a hurdle. Our aim was to ascertain whether the purported immune privilege of ESC allows for their cross-species engraftment in a clinically relevant large-animal model.**

Methods **We studied engraftment and differentiation of cardiac-committed mouse ESC in 18 sheep in which a myocardial infarction had been induced; nine controls received medium and nine sheep (five of which were immunosuppressed) received ESC. The gain in myocardial function was measured by echocardiography 1 month after cell transplantation.**

Findings **Cardiac-committed murine ESC engrafted in infarcted myocardium of immunosuppressed and immunocompetent sheep, and differentiated into mature cardiomyocytes that expressed connexins. Colonisation of the scar area by ESC was accompanied by a functional benefit of the damaged myocardium. Left-ventricular ejection fraction deteriorated in the control group by a median of 9·9% (range –20 to 0·3) relative to baseline (p=0·011) whereas in the treated group it improved by 6·6% (–5·7 to 50·8; comparison between groups p=0·002).**

Interpretation **These findings obtained in a clinically relevant large-animal model of heart failure strengthen the potential therapeutic use of ESC to regenerate the severely dysfunctional myocardium and bring additional evidence for an immune privilege of these cells.**

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Introduction

Congestive heart failure is prevalent in developed countries and is primarily caused by ischaemic events. It is characterised by a loss of myocytes associated with interstitial fibrosis that weakens the contractile power of the myocardium.[1] In response to an increasing mechanical load, cardiomyocytes undergo hypertrophy to transiently compensate for the loss of contractile tissue. When this adaptation is overwhelmed, heart failure results since contractile reserve is decreased below a critical threshold. Furthermore, mammalian cardiomyocytes are terminally differentiated and have a poor mitotic capacity,[2] thereby limiting myocardial self-regeneration.

The potential therapeutic benefit of stem-cell transplantation to limit the progression of heart failure has been established in small-animal models of myocardial infarction. So far, only stem cells isolated from the bone marrow have been assessed in clinical trials.[3,4] However, the developmental plasticity and the potential therapeutic benefit of these cells has been challenged, and their ability to transdifferentiate into cardiomyocytes refuted.[5–8] Furthermore, transplantation of unselected bone-marrow stem cells could result in myocardial calcification.[9] Although a subpopulation of stem cells from bone marrow has been reported to give rise to cardiomyocytes, the extent of the differentiation process was poor.[10] This finding suggests that these cells might be used to improve neoangiogenesis. An alternate, potentially non-allogeneic source of cells, is therefore needed to regenerate damaged myocardium in patients with severe heart failure.

To achieve regeneration of scanned post-infarction myocardium, the high plasticity and the likely tolerance to hypoxia of pluripotent embryonic stem cells (ESC) are attractive features. Self-renewing ESC are derived as clonogenic cell lines from the inner cell mass of blastocysts. Although they spontaneously give rise to different progenies representative of the three embryonic layers—the endoderm, mesoderm, and ectoderm[11]—the fate of ESC can be directed by addition of appropriate growth factors to specific cell lineages,[12] including the cardiac phenotype. Furthermore, the commitment process is translated into a differentiation programme when cells are engrafted into diseased organs of small animals provided that they find a favourable humoral environment.[13,14] Human ESC lines do not readily differentiate toward a cardiac lineage, calling for the use of a xenogeneic source of ESC to test the feasibility and efficacy of this mode of cell therapy in a preclinical large-animal model of heart failure.

Our aim was to ascertain whether the immune privilege of ESC allows for their engraftment across species-specific and individual-specific barriers through transplantation of cardiac-committed and genetically-labelled murine ESC into infarcted sheep myocardium.[15]

Methods

Procedures

We generated murine stem-cell clones that expressed reporter genes so that we could track them once transplanted into infarcted sheep myocardium. We engineered CGR8 cell clones to express β-galactosidase under the control of the Nkx2.5 promoter[16] and enhanced yellow fluorescent protein (EYFP) under the control of the cardiac-specific α-actin promoter, subcloned upstream of EYFP with XhoI and HindIII restriction sites of the promoterless pEYFP vector (Clonetech, St Quentin, Yvelines, France). We linearised the actin EYFP construct with XhoI, and NkxβGal with BglII. We electroporated both constructs into CGR8 stem cells, and screened colonies for the construct by PCR after 10 days of treatment with G418 sterile solution (Invitrogen, Cergy-Pontoise, France).

To commit ESC toward a cardiac lineage, we cultured the cells for 36 h in 7·5% fetal calf serum, leukaemia inhibitory factor 1000 U/L, and 2·5 μg/L bone morphogenetic protein 2 (BMP2) before transplantation.

We induced myocardial infarction in sheep, that weighed 45–50 kg, by intracoronary embolisation under fluoroscopic control.[15] After selective opacification, we released coils (Cook, Bloomington, IN, USA) into the mid portion of the left circumflex coronary artery to achieve a thrombolysis in myocardial infarction (TIMI) flow of 0–1, and, under echocardiographic monitoring, to check for the occurrence of an akinetic area. 2 weeks later, we approached the heart of every animal through a left thoracotomy and, after individualisation by sequential numbers, randomised the sheep in blocks of two to receive either 30×10^6 cardiac-committed murine ESC injected at 25 sites ($1\cdot2\times10^6$ cells per site) along the borders and in the centre of the infarcted area, or an equivalent volume of serum-free culture medium (Dulbecco's modified eagle medium [DMEM]). We delineated the sites of injection with small stitches. Some animals were immunosuppressed with ciclosporin 500 mg/kg daily from day 5 before transplantation. We identified this regimen from results of pilot studies, and it achieved average cyclosporine serum concentrations of 390 ng/L.

After euthanasia, 1 month after injection of ESC, we removed and froze the hearts of the sheep. We cut cryosections parallel to the prevailing fibre direction on superfrost slides and stored them at –80°C. Thawed sections were permeabilised with 1% TRITON X-100, and blocked with phosphate buffer saline supplemented with 3% bovine serum albumen before adding the primary and then the alexa-conjugated secondary antibody. The primary antibodies include specific antibodies against β-myosin heavy chain (β-MHC),[17] Connexin 43, and β-galactosidase (Sigma, L'isle, d'Abeau, France), against Nkx2.5 (Santa Cruz, CA, USA), and against mouse Ki67 (Dako, Traffes, France). We used a confocal microscope (ZEISS LSM 510 META, Jena, Germany) equipped with an Arg/Xe (488 nm) or He (534 nm) laser, dichroic mirrors, and narrow range filters, using the META polychromatic 32-channel detector (515–530 nm for detection of the alexa 488-conjugated antibody and or 595–615 nm for detection of the secondary alexa 546-conjugated antibody) to get 2048×2048 pixels images. We quantified the extent of β-MHC positive ESC-derived cardiomyocytes in the scar area with a thresholding method (Metamorph software, Universal imaging, Molecular Devices, St Gregoire, France).

We extracted RNA from cryosections and reversed-transcribed it with murine moloney leukaemia virus reverse transcriptase. We ran PCR on cDNA with a taq polymerase (Invitrogen, Cergy-Pontoise, France) as previously described.[18] We ran negative controls in the absence of reverse transcriptase.

We prepared total RNA from CGR8 stem cells with a kit (Zymo, Orange, CA, USA). After reverse transcription, we used 10 ng cDNA for real-time quantitative PCR,

Panel: PCR primers

β galactosidase
Forward: TTCACTGGCCGTCGTTTTACAACGTCGTGA
Reverse: ATGTGAG CGAGTAACAACCCGTCGGATTCT

EYFP
Forward: ACGTAAACGGCCACAAGTTC
Reverse: AAGTCGTGCTGCTTCATGTG

Tubulin
Forward: CTTCGTGGAATGGATCCCCAACAA
Reverse: GGAAAGCCTTCCGACGGAACATTG

Nkx2.5
Forward: ACATTTTACCCGGGAGCCTACGGTG
Reverse: GCTTTCCGTCGCCGCCGTGCGCGTG-

GATA4
Forward: GGTTCCCAGGCCTCTTGCAATGCGG
Reverse: AGTGGCATTGCTGGAGTTACCGCTG

Mef2c
Forward: AGATACCCACAACACACCACGCGCC
Reverse: ATCCTTCAGAGAGTCGCATGCGCTT

Tbx6
Forward: AGGCCCGCTACTTGTTTCTTCTGG
Reverse: TGGCTGCATAGTTGGGTGGCTCTC

Mesp1
Forward: GCGACATGCTGGCTCTTCTA
Reverse: TGGTATCACTGCCGCCTCTTCC

Mesp2
Forward: GTGCCTTTATCTGCCTCTTCTG
Reverse: AGCGGGGGTGTCTTGTCTC-

α-actin
Forward: CACTGAAGCCCCGCTGAACG
Reverse: TCGCCAGAATCCAGAACAATGC