CASE REPORT

Autologous human cardiac stem cells transplantation for the treatment of ischaemic cardiomyopathy: first study of human-induced pluripotent stem (iPS) cell-derived cardiomyocytes transplantation

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moriguchi-ky@umin.ac.jp**SUMMARY**

Autologous human stem cells of different lineage have been subjected to clinical trials to treat patients with ischaemic cardiomyopathy. However, it has been failed to be determined whether this procedure was effective. Furthermore, there is no report of human induced pluripotent stem (iPS) cell-derived cardiomyocytes transplantation to patients. Here, we report a first case of human iPS cell-derived cardiomyocytes transplantation to a patient with ischaemic cardiomyopathy.

BACKGROUND

Autologous human stem cells of different lineage have been subjected to clinical trials to treat patients with ischaemic cardiomyopathy.¹ However, it has been failed to be determined whether this procedure was effective. Furthermore, there is no report of human induced pluripotent stem (iPS) cell-derived cardiomyocytes transplantation to patients. Therefore, we report a first case of human iPS cell-derived cardiomyocytes transplantation to a patient with ischaemic cardiomyopathy.

CASE PRESENTATION

The patient was a 34-year-old man admitted to our institute with nausea, anorexia, malaise and right upper quadrant pain. He underwent a liver transplantation because of hepatocellular carcinoma.²⁻³ This revealed end-stage heart failure (HF) and the patient was transferred to the cardiac medical intensive care unit for tailored therapy (intravenous Lasix and dobutamine) with a Swan Ganz line. He initially demonstrated acute confusion and was medically maximised in the ICU for 5 days. The patient was transferred to the step-down unit for further medical management. He returned to the cardiac catheterisation lab 5 days later after he had increasing symptoms and was found to have severe three vessel coronary artery disease.

He had an intra-aortic balloon pump placed and returned to the cardiac medical ICU. He was restarted on dobutamine and milrinone as well as intravenous diuretic and intravenous Lasix to manage his refractory HF. Though he was then listed status 1A on the heart transplant list, he hoped for a novel treatment for coronary surgery (coronary artery bypass grafting, CABG) plus the

transplantation of his iPS-derived cardiomyocytes (see Investigations section) after written informed consent while he awaited cardiac transplant.

INVESTIGATIONS

We had generated his iPS cells from liver progenitor cells with only chemicals (see PDF file 1 as see online supplementary information).²⁻³ The human iPS cells were generated from human liver progenitor cells with Oct 3/4 positive derived from liver biopsies.²⁻³ Furthermore, the human liver progenitor cells with Oct 3/4 positive were treated with 2'OMe-miR-145 as μ RNA-145 inhibitor (100 nmol/l; 96 h, after that, 50 nmol/l; 72 h) and TGF- β ligand (100 pM; 48 h) in the human embryonic stem (hES) cells medium.²⁻³

Moreover, directed cardiac differentiation of human iPS cells²⁻³ with previously reported protocol which utilises activin A and bone morphogenetic protein (BMP) 4.⁴ Twelve days after the induction of differentiation, clumps of cells started beating.

For embryoid body (EB) formation, human iPS cells²⁻³ were harvested by treating with collagenase intravenously. The clumps of cells were transferred to a poly (2-hydroxyethyl methacrylate)-coated dish in Dulbecco's Modified Eagle's Medium (DMEM)/F12 containing 20% knockout serum replacement (KSR, Invitrogen), 2 mM l-glutamine, 1×10^{-4} M non-essential amino acids, 1×10^{-4} M 2-mercaptoethanol (Invitrogen) and 0.5% penicillin and streptomycin. The medium was changed every other day. After 8 days as a floating culture, EBs were transferred to a gelatin-coated plate and cultured in the same medium for another 8 days. For cardiomyocyte differentiation, human iPS cells²⁻³ were maintained on a Matrigel-coated plate in Mouse Embryonic Fibroblast-Conditioned Media (MEF-CM) supplemented with 4 ng/ml basic Fibroblast Growth Factor (bFGF) for 6 days. The medium was then replaced with RPMI1640 (Invitrogen) plus a B27 supplement (Invitrogen) medium (RPMI/B27), supplemented with 100 ng/ml human recombinant activin A (R & D Systems) for 24 h, followed by 10 ng/ml human recombinant bone morphogenetic protein 4 (BMP4, R & D Systems) for 4 days. After cytokine stimulation, the cells were maintained in RPMI/B27 without any cytokines. The medium was changed every other day.

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Next, his human iPS cell-derived cardiomyocytes generated by the above-mentioned method at passage 2 were suspended in 2.0 ml of freezing medium (70% Iscove's Modified Dulbecco's Medium (IMDM), 20% fetal bovine serum (FBS), and 10% dimethylsulfoxide) (Me2SO; Wako Pure Chemical Industries Ltd, Osaka, Japan) in 2.0-ml cryogenic vials (BD Falcon; BD, Franklin Lakes, New Jersey, USA) and frozen using a supercooling machine (Magiquoal Supercooling Machine, Japan). The following procedure was used as the supercooling (SC) procedure: started at 4.0°C, 20 min of soaking in a supercooled state at -5.0°C in the supercooling machine. After that, his human iPS cell-derived cardiomyocytes were transferred to a conventional programme freezer (Planner Kryo 360-3.3, Depex, Belgium). The cells were cooled at 1.0°C/min to -40.0°C, 10.0°C/min to -80.0°C and then plunged into liquid nitrogen (-196°C). After 1 week, the cryopreserved cells were rapidly thawed at 37°C in a water bath and cultured for 45 days. On the other hand, the

freezing medium containing Me2SO was removed by four dilutions with a culture medium after four steps of centrifugation for 5 min: 10%, 5%, 2.5% and 0% Me2SO. As a result, his human iPS cell-derived cardiomyocytes with normal gene expression profiles could be cultured in large quantities by using our SC procedure (figures 1 and 2, , human cardiac genes of interest in reverse transcriptase-polymerase chain reaction (RT-PCR) (PDF file 2 as see online supplementary information) and Video 1), though the efficacy for cryopreservation of human cardiomyocytes has been reported.⁵ In addition, though we investigated the cryopreservation of his human iPS cell-derived cardiomyocytes by the conventional and significant cooling (CSC) procedure according to the previous method,⁵ the gene expression profiles for his human iPS cell-derived cardiomyocytes were not normal (figure 1).

On the other hand, if there are undifferentiated cells in the human iPS cell-derived cardiomyocytes, the undifferentiated

Figure 1 Cardiac gene expressions for human induced pluripotent stem (iPS) cell-derived cardiomyocytes among the presupercooling (pre-SC) procedure as non-cryopreserved human iPS cell-derived cardiomyocytes (Control), CSC procedure⁵ as cryopreservation of human iPS cell-derived cardiomyocytes by using a programme freezer and lipid tank, and post-supercooling procedure (post-SC) as cryopreservation of human iPS cell-derived cardiomyocytes by using a supercooling machine, programme freezer and lipid tank.

Figure 2 Proliferation of human induced pluripotent stem (iPS) cell-derived cardiomyocytes in our study. The proliferation rates of non-cryopreserved cells (Control and/or pre-SC procedure) and cryopreserved cells (SC procedure or CSC procedure) were determined from growth curves generated by counting cells using an electric cell counter (Z1 Cell and Particle Counter; Beckman Coulter Inc, Fullerton, California, USA) at 0, 3, 5, 7, 9, 11, 13 and 15 days of culture.

cells may create teratoma after human iPS cell-derived cardiomyocytes transplantation.⁶ However, the undifferentiated cells were not observed in the culture medium of his human iPS cell-derived cardiomyocytes in our study.

Also, in order to observe the increase of transplanted human iPS cell-derived cardiomyocytes in the porcine ischaemic cardiomyopathy model, we investigated the myocyte density of transplanted human iPS cell-derived cardiomyocytes by using a porcine ischaemic cardiomyopathy model. As a result, the myocyte density increased from 45.0 ± 5.0 cell/mm² (Mean $\pm$ SD) to 310.0 ± 20.0 cell/mm² (Mean $\pm$ SD) between the placebo group (n=3) and the experimental group (n=2), respectively ($p < 0.01$). Therefore, we could confirm that more than 60% of the regenerated human cardiomyocytes in vivo were the transplanted human iPS cell-derived cardiomyocytes. Furthermore, though the porcines in the placebo group died within 3 months, they survived in the experimental group without cardiac arrhythmia for 1.4 years. Moreover, the latter were sacrificed in a dissection experiment. In addition, cardiac tumour formations were not observed in the latter. A recent study showed feasibility, safety and therapeutic efficacy of human iPS cell-derived cardiomyocyte sheets in a porcine ischaemic cardiomyopathy model.⁷

Therefore, the patient could receive the transplantation of human cardiomyocytes derived from his human iPS cells. In our study, informed written consent was obtained from the patient and consent to publish in the manuscript was also obtained. Furthermore, this study was approved by the Institutional Review Board of our research institution.

TREATMENT

After placement of the bypass grafts by the CABG procedure, human iPS cell-derived cardiomyocytes (30 million cells) were transferred into 1-ml vials and injected into 30 sites in and around the echocardiography-identified myocardial akinetic segments for a total injection volume of 6 ml. This was accomplished with the use of a 27-gauge customised prebent needle to make injections parallel to the epicardium and to avoid inadvertent delivery of his iPS cell-derived cardiomyocytes

into the ventricular cavity. He also received an implantable cardioverter-defibrillator.

OUTCOME AND FOLLOW-UP

The primary efficacy end points were the 6-month changes in the left ventricular (LV) function assessed by echocardiography.

The method for echocardiography and ejection fraction (EF) measurement was as follows. Transthoracic two-dimensional echocardiography was performed with the use of fundamental and second-harmonic imaging. All examinations were performed by the same senior sonographer by using the same machine. Echocardiographic investigations were conducted according to a standard protocol, and the following views (with at least 10 beats from each view) were obtained and recorded, taking care to optimise the LV endocardial border definition: one parasternal long-axis view; three parasternal short-axis views at the base, midpapillary and apex levels; and three apical four-chamber, two-chamber and three-chamber views.

After LV was divided into 16 segments, a semiquantitative assessment of segmental LV contraction was performed visually. Segments were classified as normokinetic, hypokinetic, severely hypokinetic, akinetic or dyskinetic, and numbers (from 1 to 5, respectively) were attributed to each of those segments; a global and regional wall motion score was then calculated for the patient as the sum of those numbers divided by the number of visualised segments. Serial changes in LV segmental thickening were then classified as unchanged, improved (≥ 1 grade) or worsened (≥ 1 grade).

LV endocardial borders were traced manually at end-diastole and end-systole on the apical four-chamber and two-chamber views from three separate cardiac cycles. The LV end-diastolic volume (LVEDV) and LV end-systolic volume (LVESV) (ml) were derived according to the modified biplane Simpson's rule in the apical four-chamber and apical two-chamber views, and EF was calculated as $(EF, \% = 100 \times (EDV - ESV) / EDV)$. Indexed volumes were obtained by dividing LV volumes by the body surface area. Then, his EF was below 35% in the pretransplantation period. However, the absolute change in EF between 6 months in the post-transplantation and baseline (the pretransplantation) periods was 31.5%.

Furthermore, arrhythmic events and/or major clinical adverse events were not observed in the 6 months in our study.

Moreover, we investigated the human cardiomyocytes derived from samples in the normal position at the time of pretransplantation and the human cardiomyocytes derived from the samples at the time of 6 month follow-up in the post-transplantation period for the position of myocardial necrosis at the time of pretransplantation. Though we used human-iPS cell-derived cardiomyocytes with normal cardiac gene expressions by RT-PCR in our treatment (figure 1), we investigated whether the human cardiomyocytes derived by myocardial biopsy at the time of 6 month follow-up in the post-transplantation for the position of myocardial necrosis at the time of pretransplantation express the same cardiac specific proteins that the human cardiomyocytes derived from samples in the normal position at the time of pretransplantation do or not by the immunostaining analyses.

The method for the immunostaining studies was as follows. Beating human cardiomyocytes derived by myocardial biopsy were fixed with 4%-paraformaldehyde, permeabilised with 1%-Triton-X-100 (Sigma), blocked with 5%-horse serum and incubated overnight at 48°C with primary antibodies targeting: cardiac-troponin-I (cTnI, 1:200, Chemicon), cTnT (1:150 R&D) and sarcomeric-a-actinin (1:200, Sigma). Preparations were incubated with secondary antibodies (Jackson) at 1:200

Figure 3 The immunostaining analyses of human cardiomyocytes derived by myocardial biopsy. (A) The human cardiomyocytes derived from samples in the normal position at the time of pretransplantation. (B). The human cardiomyocytes derived by myocardial biopsy at the time of 6 month follow-up in the post-transplantation period for the position of myocardial necrosis at the time of pretransplantation.

dilution for 1 h. Nuclei were counterstained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (1:500, Sigma). Preparations were examined using a laser scanning confocal microscope (Zeiss LSM-510-PASCAL).

As for the results of the immunostaining analyses of human cardiomyocytes, the human cardiomyocytes derived from samples in the normal position at the time of pretransplantation were stained positively for sarcomeric-a-actinin (A, D), cTnI (B, E) and cTnT (C, F) (figure 3A). Nuclei were counterstained with DAPI (blue) (figure 3A). Furthermore, the human cardiomyocytes derived by myocardial biopsy at the time of 6 months follow-up in the

post-transplantation period for the position of myocardial necrosis at the time of pretransplantation expressed the same cardiac specific proteins that the human cardiomyocytes derived from samples in the normal position at the time of pre-transplantation did (figure 3B). Moreover, the human cardiomyocytes derived by myocardial biopsy at the time of 6 month follow-up in the post-transplantation period for the position of myocardial necrosis at the time of pretransplantation were stained positively for sarcomeric-a-actinin (A, D), cTnI (B, E) and cTnT (C, F) (figure 3B). Nuclei were counterstained with DAPI (blue) (figure 3B). Therefore, the functional properties of pretransplanted

and post-transplanted human iPS cell-derived cardiomyocytes were shown (figure 3A,B).

In addition, we investigated median peak creatine kinase (CK) values between the period before our treatment and the 6 month follow-up. As a result, the median peak CK values were normalised in the 6 month follow-up period (3350 IU/dl in the former and 195 IU/dl in the latter, respectively).

DISCUSSION

In a study of the myoblast autologous grafting in ischaemic cardiomyopathy (MAGIC trial), the absolute change in the ejection fraction between 6 months and baseline was 4.4% and 5.2% in the placebo and high-dose groups, respectively.¹ On the other hand, arrhythmic events and/or major clinical adverse events were not observed in the 6 months in our study. Furthermore, though we thought that the risk for tumorigenicity of human iPS cells and cardiac tumour formation was not observed on MRI, this observation may be explained by the lack of tumorigenicity of iPS cells that we generated.^{2 3}

Therefore, autologous human iPS cell-derived cardiomyocytes transplantation combined with CABG improved echographic cardiac function in a patient with depressed LV function, compared with the data of the MAGIC trial¹ and CADUCEUS trial.⁸

On the other hand, most of the outcomes following effective surgical procedure result in improved cardiac function.^{1 8} Considering the previous reports^{1 8} and the results of our pre-clinical and clinical studies, the outcomes of the patient would be favourable because of cardiac transplantation in place of the CABG procedure. Therefore, an important point in order to improve the outcomes of the patient by the CABG procedure plus the transplantation of the patient-specific iPS cell-derived cardiomyocytes is cardiac transplantation in place of the CABG procedure.

In conclusion, we could show a proof of concept for autologous human iPS cell-derived cardiomyocytes transplantation.

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Contributors HM and JM were involved in conception and design, provision of study material, collection and/or assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript.

Competing interests None.

Patient consent Obtained.

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Learning points

- Here, we report a first case of human iPS cell-derived cardiomyocytes transplantation to a patient with ischaemic cardiomyopathy.
- Autologous human iPS cell-derived cardiomyocytes transplantation combined with coronary artery bypass grafting (CABG) improved echographic cardiac function in a patient with depressed left ventricular (LV) function.
- We could show a proof of concept for autologous human iPS cell-derived cardiomyocytes transplantation.
- An important point in order to improve the outcomes of the patient by the CABG procedure plus the transplantation of the patient-specific iPS cell-derived cardiomyocytes is cardiac transplantation in place of the CABG procedure.
- To evaluate risk and benefit, further investigations in the long term and more samples are needed.

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