BD), anti-hCD45RA (-PE-Cy7 from BD or -FITC from Beckman Coulter), anti-ICOS (-FITC from e-Bioscience), anti-HLA-DR (-PE from BD biosciences), anti-CD31 (-APC from e-Bioscience), anti-hCD127 (-PE from Beckman Coulter and -PE-Cy5 from e-bioscience), and 7-AAD (Dako). Intracellular detection of FoxP3 with anti-hFoxP3 (PE or Alexa Fluor 647, clone 236A/E7 [e-Bioscience] or clone 259D [BD biosciences]) and of KI-67 antigen with KI-67 antibody (FITC or PE from BD) was performed on fixed and permeabilized cells via Cytofix/Cytoperm (e-Bioscience). For detection of intracellular cytokine production, $CD4^+$ T cells were stimulated with 20 ng/ml PMA and 1 μM Ionomycin in the presence of Golgi-Stop (BD Biosciences) for 5 hr and then stained with anti-hFoxP3-PE, KI-67-FITC, anti-IL2-APC (BD Biosciences), anti-IFN-γ-APC (BD), or anti-IL-17-Alexa Fluor 647 (e-Bioscience) after fixation and permeabilization. Data acquired by FACSCalibur (Becton Dickinson) were analyzed with WinMDI 2.9 software (http://facs.scripps.edu/software.html). Statistical comparisons were performed with the nonparametric Mann-Whitney U test.

Cell Culture and Suppression Assay

RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Sigma, St. Louis, MO) was used for T cell culture. Cells were labeled with 1 μM CFSE (Dojindo and Invitrogen). In suppression assays, unless otherwise indicated, 1×10^4 CFSE-labeled responder $CD25^-CD45RA^+CD4^+$ T cells were cocultured with 1×10^4 unlabeled cells assessed for their suppressive capacity and 1×10^5 irradiated autologous accessory cells and were stimulated with 0.5 μg/mL plate-bound anti-CD3 (OKT3 mAb) in 96-well round-bottom plate in supplemented RPMI medium. Proliferation of CFSE-labeled cells was assessed by flow cytometry after 84–90 hr of culture. Percent suppression was calculated by dividing the number of proliferating CFSE-diluting responder cells in the presence of suppressor cells at a 1 to 1 ratio by the number of proliferating responder cells when cultured alone, and multiplied by 100.

FOXP3 Gene DNA Methylation

The genomic DNA from purified human $CD4^+$ T cell subsets was extracted by the Blood & Tissue Genomic DNA Extraction System (Viogene). Genomic DNA from purified cells was bisulfite converted by the EpiTect Bisulfite Kit (QIAGEN) according to the manufacturer's instructions. DNA was then subjected to PCR with primers for amplification of specific targets in bisulfite-treated DNA. The PCR products obtained were cloned into the pGEM-T Easy vector (Promega) and 20 individual clones from each sample were cycle sequenced by the BigDye Terminator kit (ver. 3.1; Applied Biosystems) and the ABI automated DNA sequencer (Applied Biosystems). Primers used: Fxpro-met_F1, 5′-TTTTT GTGGTGAGGGGAAGAAATTATATT-3′; Fxpro-met_R2, 5′-TACCATCTCCTC CAATAAAACCCACATC-3′; Fxint-met_F8, 5′-TTTGGGTTAAGTTTGTTGTAG GATAGGGTAGTTAG-3′; Fxint-met_R7, 5′-AAATCTACATCTAAACCCTATTAT CACAACCCC-3′.

Single Cell Sorting, RT-PCR, and Vβ5 Sequence Analysis

PBLs were stained with anti-human CD4-FITC, anti-human CD25-PC7 (BD Biosciences), and anti-human BV5.1, BV5.2, BV5.3-PPE (Beckman Coulter). Single cells were sorted with the FACS Vantage (Becton Dickinson) into 96-well PCR plates (Abgene, Epsom). Single-cell RT-PCR conditions were as previously described (Miyara et al., 2006). In the first PCR round, BV5ext (5′-GATCAAAACGAGAGGACAGC-3′) and BC (5′-CGGGCTGCTCCTTGAG GGGCTGCG-3′) were used. Reactions were subjected after 5 min at 94°C to 8 cycles (94°C for 30 s, 60°C for 40 s, 72°C for 50 s), 32 cycles (94°C for 30 s, 55°C for 40 s, 72°C for 50 s), and a final elongation at 72°C for 5 min. In a second PCR round, nested primers BV5 (5′-AGCTCTGAGCTGAATGT GAACGCC-3′) and BC-int (5′-GCGGGTCYGTGCTGACCC-3′) were used. PCR was performed as in the first step.

Products were subjected to automated sequencing (ABI 3100, Applied Biosystems).

Specific questions regarding this repertoire analysis should be sent to guy.gorochov@upmc.fr.

Microarray and Real-Time PCR

RNA was extracted from FACS-sorted $CD4^+$ T cells according to their amounts of CD25 and CD45RA and analyzed by Affymetrix Human Genome U133 Plus 2.0 Arrays.

Real-time PCR was performed with a SYBR green assay on the LightCycler 480 system (Roche). Total RNA extracted from FACS-sorted T cells was reverse transcribed according to the manufacturer's instructions (RNeasy Micro kit, QIAGEN). In each reaction, hypoxanthine phosphoribosyltransferase-1 (HPRT-1) was amplified as a housekeeping gene to calculate a standard curve and to correct for variations in target sample quantities. Relative copy numbers were calculated for each sample from the standard curve after normalization to HPRT-1 by the instrument software. Primers used: FOXP3_F, 5′- CAGCACATTCCCAGAGTTCC-3′; FOXP3_R,5′-TGAGCGTGGCGTAGGT GAAAG-3′; RORA_F,5′- TCACCAACGGCGAGACTTC-3′; RORA_R,5′-GGCA AACTCCACCACATACTG-3′; RORC_F,5′-CGCTCCAACATCTTCTCC-3′; RO RC_R,5′-CTAACCAGCACCACTTCC-3′; AHR_F,5′-AACAGATGAGGAAGGAA CAGAGC-3′; AHR_R,5′-GAGTGGATGTGGTAGCAGAGTC-3′; AHRR_F,5′- AA GGCTGCTGTTGGAGTC-3′; AHRR_R,5′- TGGATGTAGTCATAAATGTTCTG G-3′; HPRT-1_F,5′-GCTGAGGATTTGGAAAGGGTG-3′; HPRT-1_R,5′-TGAG CACACAGAGGGCTACAATG-3′.

ACCESSION NUMBERS

Microarray data are available from the National Center for Biotechnology Information Gene Expression Omnibus (GEO) under accession number GSE15659.

SUPPLEMENTAL DATA

Supplemental Data include ten figures and two tables and can be found with this article online at http://www.cell.com/immunity/supplemental/S1074-7613(09)00202-7.

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The effects of cardioactive drugs on cardiomyocytes derived from human induced pluripotent stem cells

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ABSTRACT

Developing effective drug therapies for arrhythmic diseases is hampered by the fact that the same drug can work well in some individuals but not in others. Human induced pluripotent stem (iPS) cells have been vetted as useful tools for drug screening. However, cardioactive drugs have not been shown to have the same effects on iPS cell-derived human cardiomyocytes as on embryonic stem (ES) cell-derived cardiomyocytes or human cardiomyocytes in a clinical setting. Here we show that current cardioactive drugs affect the beating frequency and contractility of iPS cell-derived cardiomyocytes in much the same way as they do ES cell-derived cardiomyocytes, and the results were compatible with empirical results in the clinic. Thus, human iPS cells could become an attractive tool to investigate the effects of cardioactive drugs at the individual level and to screen for individually tailored drugs against cardiac arrhythmic diseases.

Introduction

The long-QT syndrome (LQTS) is characterized by an abnormal prolongation of the QT-interval on the ECG and an increased risk of sudden death, due to ventricular fibrillation known as Torsade de Pointes (TdP) [1]. In a previous study [2], four patients died suddenly (1.3% per year) during an average follow-up period of 26 months per patient and among 196 idiopathic LQTS patients, 27 experienced one or more syncopal episodes (8.6% per year). Molecular genetic studies have revealed several forms of congenital LQTS caused by mutations in genes coding for potassium, sodium and calcium channels or membrane adapters [3–6]. Preliminary clinical studies have since suggested the feasibility of performing genotype-specific therapy with therapeutic agents that abbreviate the QT-interval [7]. But it is difficult to select the correct drug because within the same LQTS subtype, the same drug can sometimes have different effects depending on the patient.

Furthermore, the diagnosis of LQTS subtypes is difficult. Genetic testing can only identify 50–75% of probands [8]. So an epinephrine challenge is needed in some patients to diagnose LQTS in a clinical setting [9]. However, this test sometimes induces TdP, so it must be done under careful patient surveillance.

The generation of iPS cells from human fibroblast using a combination of 4 transcription factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*) has opened remarkable new avenues for not only basic research but also regenerative medicine, understanding of disease mechanisms, drug screening, and toxicology [10]. A recent study reported the generation of disease-specific iPS cell lines from patients with a variety of diseases [11]. If patient-specific iPS cells could be commonly generated and employed in a clinical setting, they could become a useful tool for selecting the best drug for individual LQTS patients.

But there has been no report that cardiomyocytes derived from human iPS cells respond to drugs in the same way as human cardiomyocytes. It is important to investigate whether cardiomyocytes derived from human iPS cells react to drugs in the same way as human cardiomyocytes, if patient-specific iPS cells are to be used in a clinical setting for drug screening. Previous studies have hinted that some drugs produce the same effects in cardiomyocytes as in cardiomyocytes derived from ES cells [12–14]. In this study, we investigated whether cardiomyocytes derived human iPS cells responded to drugs in the same way as in cardiomyocytes derived from human ES cells with respect to beating frequency and contractility, and we compared these results with previously described clinical empirical results [15].

Materials and methods

Human iPS and human ES cell culture. We used human ES cell line, KhES1, and human iPS cell lines 201B7. Human iPS cells and human ES cells were maintained on mitomycin-C (Kyowa Hakkoh)

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treated mouse embryonic fibroblasts (MEFs) or SNLs on cell culture dishes. In brief, both human iPS and human ES cells were maintained in DMEM/F12 culture medium (SIGMA) supplemented with 20% knock-out serum replacement (Gibco), 0.1 mmol/L nonessential amino acids (Gibco), 4 mmol/L L-glutamine, 0.8 μmol/L basic fibroblast growth factor (bFGF) (Invitrogen).

Embryoid body formation and cardiac differentiation. Colonies were detached from cell culture dishes by incubating them with PBS containing 0.25% trypsin (Gibco) and 1 mg/mL collagenase I (Worthington) at 37 °C for 3–4 min. The cells were then placed in petri dishes (Sterilin) in suspension cultures for 7 days with maintenance medium supplemented with 5 ng/ml bFGF. Embryoid bodies (EBs) were then plated on 0.1% gelatin-coated 6-well culture plates (BD Biosciences) and cultured in cardiac differentiation medium, consisting of alpha MEM (Gibco) supplemented with 0.5 μmol/L 2ME and 10% FCS (Hyclone) (changed once every 7 days). Contractile colonies appeared 15–25 days after plating on gelatin-coated dishes (Fig. 1A).

Reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated using TRIzol Reagent (Invitrogen) from undifferentiated iPS cells, EBs derived from human iPS cells, the contracting areas of differentiated human iPS cells, and human right ventricular tissue (obtained by a tetralogy of Fallot patient that had received a right flow ventricular tract ventriculotomy). Total RNA was used for oligo (dT) 12–18-primed reverse transcription using the Super Script II First-Strand Synthesis System (Invitrogen). RT-PCR was carried out using Ex Taq (TAKARA BIO). PCR conditions included denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 55–65 °C for 1 min for 25–35 cycles, with a final extension at 72 °C for 7 min. Primers used are listed in Table 3.

Immunohistochemistry. Contractile colonies were partitioned into small particles using collagenase I (Worthington) for 2 h at 37 °C. The cells were then washed and plated on 6-well culture plates coated with 0.1% gelatin for 2 or 3 days to allow attachment. Cells were fixed in 4% paraformaldehyde for 15 min at 4 °C. Then the cells were incubated with primary antibodies, such as polyclonal anti-cardiac Troponin I (IgG, 1:50 dilution; Santa Cruz Biotechnology), polyclonal anti-MLC2v (IgG, 1:50 dilution; Santa Cruz Biotechnology), or polyclonal anti-ANP (IgG, 1:250 dilution; Chemicon) in 2% skim milk with 0.1% Triton X-100 overnight at 4 °C. Secondary antibodies were cyanine 3 (Cy3)-conjugated donkey anti-rat IgG (1:200 dilution; Jackson Immunoresearch), Cy3-conjugated donkey anti-rabbit IgG (1:200 dilution; Jackson Immunoresearch), and Cy3-conjugated donkey anti-goat IgG (1:200 dilution; Jackson Immunoresearch). Nuclei were counter-stained with Hoechst 33342 (Molecular probes).

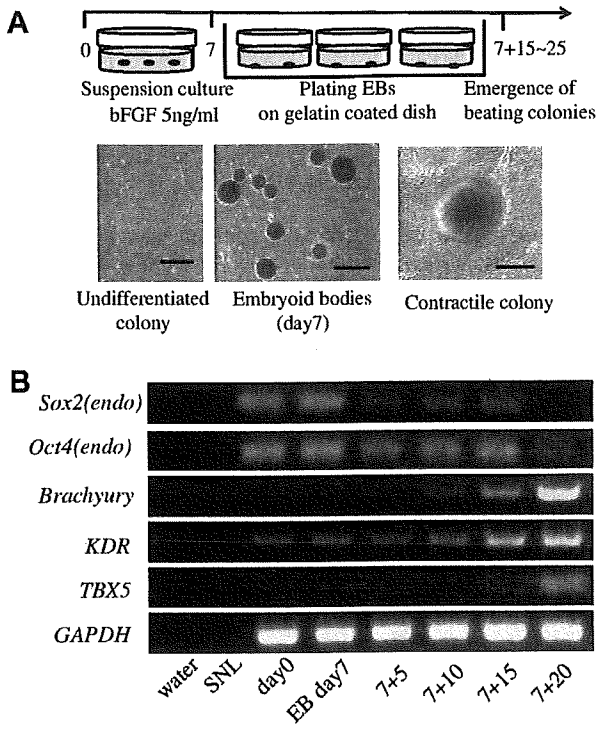

Fig. 1. An outline of the protocol used for the differentiation of human iPS cells and human ES cells. Scale bars = 200 μm (A). Time course analysis of immature gene expression, mesodermal markers, and cardiac progenitor markers during differentiation (B).

Electrophysiological examination. Microelectrode arrays analysis was performed to investigate the electrophysiological potential of cardiomyocytes derived from human iPS cells using the MED 64 system (Alpha MED Sciences) [16–18]. Micro-dissected contracting areas were plated on MED-probe dishes (Alpha MED Sciences) followed by incubation for 3–7 days to allow attachment. The potentials of the contractile colonies derived from these cells were then recorded.

Drug loading test. Differentiation medium was replaced with alpha MEM containing 10 mmol/L HEPES buffer (Nacalai tesque), 7 mol/L NaCl, and 0.5 μmol/L 2ME, which was adjusted to pH 7.4 with NaOH. After 10 min incubation at 37 °C, the frequency and contractility of the contractile colonies were measured in a movie recorded by a VB 7000 (KEYENCE) camera under drug-free medium conditions as well as under drug conditions with three different drug concentrations. Beating colonies were selected when the beating rate was 40/min to 60/min under drug-free medium conditions. Colonies whose contractile motion was distended were excluded. Loading drugs were as follows; isoproterenol (SIGMA), adrenaline (Dai-ichi Sankyo), propranolol (SIGMA), procainamide (Dai-ichi Sankyo), mexiletine (Boehringer Ingelheim), flecainide (Eisai), verapamil (SIGMA), and amiodarone (Sanofi-aventis).

Analysis of beating rate and contractility. Beating rates were counted based on the video recordings. Recently, some papers reported that video-edge detecting systems are useful for calculating the contractility of contractile colonies [12]. We imitated this method and calculated the contractility of colonies. In brief, we extracted the still images of systolic phase and diastolic phase from the recorded video images. The major axis of each phase was measured and the contractile index was defined as $a - b/a$ (a: length of diastolic phase, b: the length of systolic phase) (Fig. 4A).

Statistics. Data are presented as means ± SEM. Statistical significance was determined by the unpaired t-test for two samples and one-way ANOVA followed by the Scheffe test for more than three samples. P values <0.05 were considered to be statistically significant.

Results

Time course analysis of gene expression during cardiac differentiation

First, we examined the time course of gene expression during cardiac differentiation of human iPS cells by RT-PCR to compare it with that of normal embryogenesis (Fig. 1B). Undifferentiated human iPS cells strongly expressed endogenous *Oct4* and *Sox2*, which are undifferentiated cell markers, but did not express the mesodermal marker *Brachyury* or the cardiac progenitor marker *TBX5* (Fig. 1B). *KDR* was weakly expressed. These results show that undifferentiated human iPS cells have similar properties to undifferentiated human ES cells [19]. Endogenous *Oct4* and *Sox2* expression gradually decreased during culture in differentiation medium. The

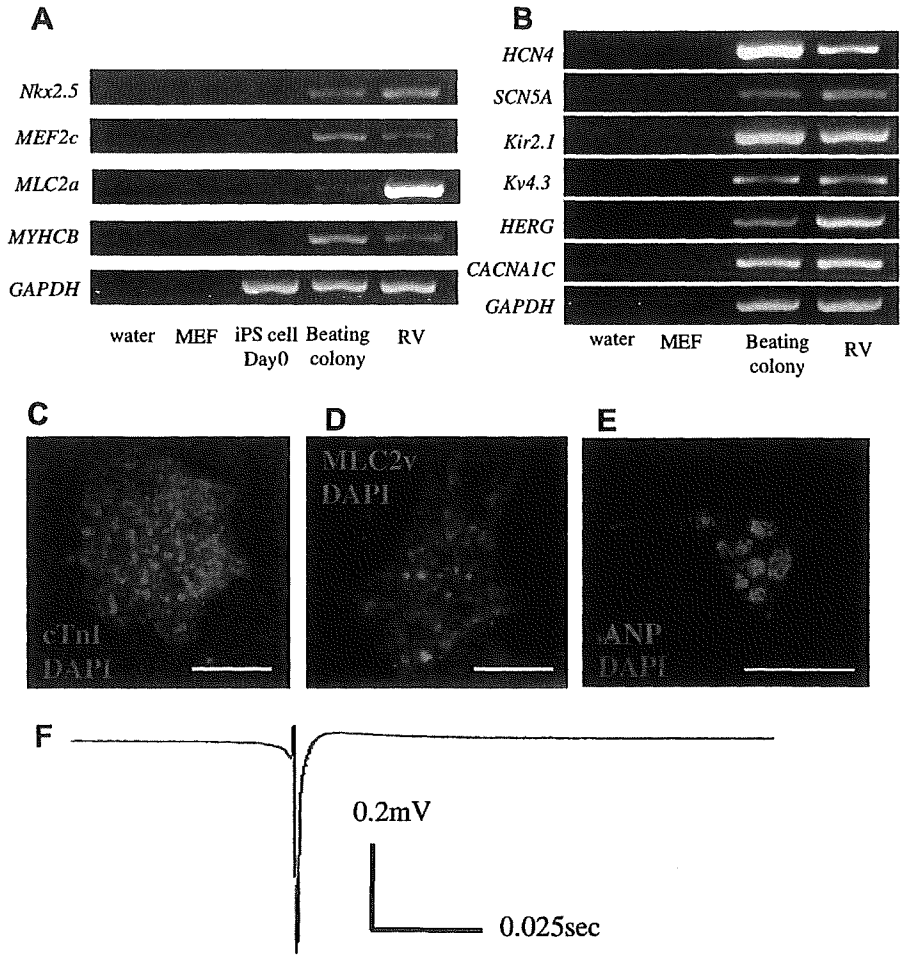

Fig. 2. Gene expression analyses of cardiac markers by RT-PCR (A). Gene expression analysis of ion channel related genes (B). Immunohistochemistry of contractile colonies. Colonies were stained with cTnI (C), MLC2v (D), or ANP (E). Scale bars = 100 μm. Field potentials of contractile colonies measured by the MED 64 system (F).

expression of the mesodermal marker *Brachyury* increased from day 10 after EB formation. The expression of *KDR* also gradually increased from day 10 after EB formation. These patterns of mesodermal marker expression are compatible with those of human ES cells as previously described [19]. The cardiac progenitor marker *TBX5* was expressed from day 20 after EB formation, which is compatible with the gene expression patterns seen during cardiac formation in embryogenesis and human ES cell differentiation as previously described [19]. The result additionally suggests that human iPS cells differentiated into the mesodermal lineage and then differentiated into contractile colonies via cardiac progenitor cells.

Cardiac differentiation of human iPS cells via EBs

Next, we examined the contractile colonies consisting of cardiac-specific cells. Contractile colonies were observed from 15 to 25 days after EB formation both in human iPS and human ES cell populations. This result demonstrates that our differentiation methods could generate contractile colonies from both human iPS cells and human ES cells. Next, we investigated whether these contractile colonies were human cardiomyocytes. For this purpose, we carried out RT-PCR and examined for the expression of cardiac cell markers. RT-PCR showed that contractile colonies expressed cardiac markers *Nkx2.5*, *MEF2c*, *MLC2a*, and *MYHCB* (Fig. 2A). Moreover, we carried out immunohistochemical analysis to confirm that the contractile colonies were human cardiomyocytes. Contractile colonies were stained by the cardiac cell marker, cTnI, the ventricular cell marker, MLC2v, and the atrial cell marker, ANP. The colonies were also stained by cTnI, and some of them were stained by MLC2v or ANP (Fig. 2C–E). These results of immunohistochemical analysis confirmed that the contractile colonies were indeed human cardiomyocytes.

Electrical analysis of contractile colonies

To investigate whether the contractile colonies that expressed cardiac markers were electrically functional cardiac colonies, we measured their electrical potentials by microelectrode array analysis using the MED 64 system (Alpha MED Sciences) [16–18]. The field potentials of the contractile colonies were comparable to those of cardiomyocytes derived from human ES cells as previously reported (Fig. 2F) [16–18]. Moreover, RT-PCR showed that these cells expressed the If channel (*HCN4*), the L-type calcium channel (*CACNA1C*), the sodium channel (*SCN5A*), the inward rectifier (*Kir2.1*), the transient outward channel (*Kv4.3*), and the delayed rectifier IKr (*HERG*) (Fig. 2B).

Effects of drugs on the beating frequency of cardiomyocytes derived from human iPS cells

We next investigated whether the cardiomyocytes derived from iPS cells reacted with cardioactive drugs in the same manner as

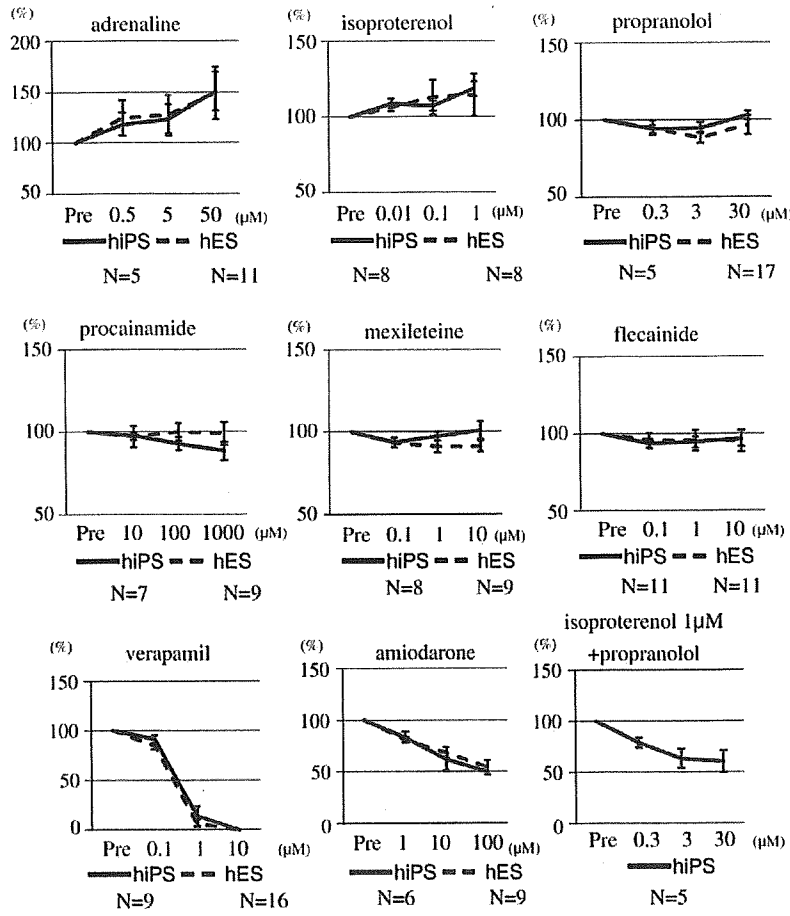

Fig. 3. The effects of cardioactive drugs on the beating rates of contractile colonies derived from human iPS cells and human ES cells. Adrenaline, isoproterenol, verapamil, amiodarone, and isoproterenol + propranolol had statistically significant effects between pre-drug loading and the maximum concentration of the drug used in cardiomyocytes derived from human iPS cells ($P < 0.05$). There were no statistically significant differences between the concentrations of drugs that elicited effects in human iPS cells and those that elicited effects in human ES cells.

cardiomyocytes derived from human ES cells by performing drug loading tests. First we compared the beating frequencies of these two cell populations. A total of eight drugs were tested (see Table 1 for the list of drugs and their concentrations). The β stimulants, adrenaline and isoproterenol increased beating frequency in a dose dependent manner. The β blocker, propranolol, and the Na channel blockers, procainamide, mexiletine, and flecainide had no effect on beating frequency. The Ca channel blocker verapamil decreased beating frequency in a dose dependent manner, and all contractile colonies ceased to contract when 1×10^{-5} M verapamil was loaded. Amiodarone, which mainly acts as a K channel blocker, decreased beating frequency in a dose dependent manner. We carried out β blocker loading in the presence of 1×10^{-6} M isoproterenol in order to mimic conditions *in vivo* [20]. Under this condition, the beating frequency decreased in a dose dependent manner. There were no statistical differences between the drug concentrations required to elicit the effects in human iPS cells and those required to elicit the effects in cardiomyocytes derived from human ES cells (Fig. 3). Previous reports showed that some drugs had similar effects on the beating frequency of cardiomyocytes derived from ES cells and on *bone-fide* human cardiomyocytes, suggesting that human iPS cells and cardiomyocytes respond similarly to these drugs as well [12–14]. Table 2 shows a comparison of the effects of drug loading on human iPS cells and the effects of these drugs in a clinical setting [15]. As the effects are broadly similar and occur within the same range of drug concentrations, we conclude that cardiomyocytes derived from human iPS cells are a good model for testing the effects of drugs on the beating frequency of human cardiomyocytes. The results are also compatible with previously reported clinical empirical results [15].

The effects of drugs on the contractility of cardiomyocytes derived from human iPS cells

Next, we investigated the effects of drugs on the contractility of human iPS cells and cardiomyocytes derived from human ES cells. The results showed that adrenaline and isoproterenol increased contractility in a dose dependent manner. Propranolol, mexiletine, or amiodarone had no effect on contractility. Verapamil decreased contractility in a dose dependent manner, and all contractile colonies ceased to contract when 1×10^{-5} M verapamil was loaded. Procainamide and flecainide also decreased the beating frequency in a dose dependent manner. We also carried out β blocker loading in the presence of 1×10^{-6} M isoproterenol with cardiomyocytes derived from human iPS cells, which showed that contractility again decreased in a dose dependent manner under these conditions. There were no statistical differences between the drug concentrations required to elicit the effects in human iPS cells and those required to elicit the effects in cardiomyocytes derived from human ES cells (Fig. 4B). Previous reports have shown that some

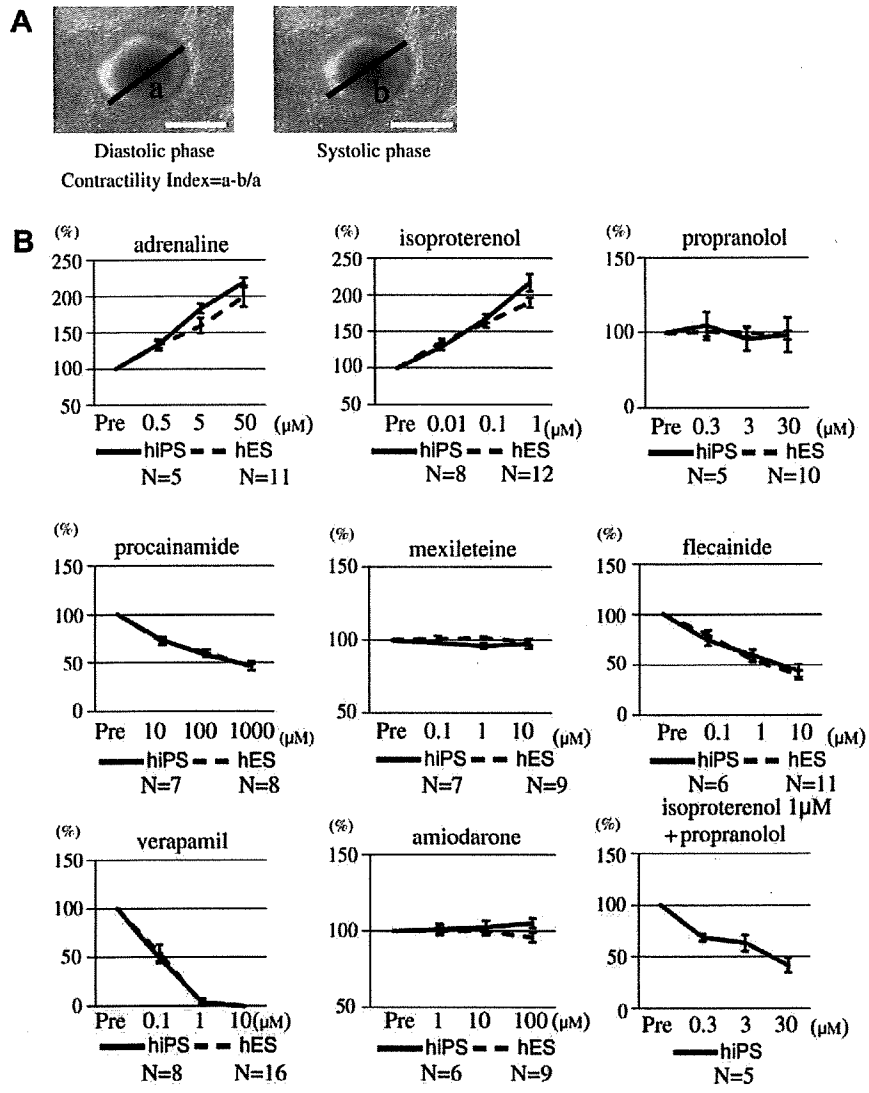

Fig. 4. Calculation of the contractility index. Right panel; diastolic phase, left panel; systolic phase. Scale bars = 200 μm (A). The effects of cardioactive drugs on the contractility of contractile colonies derived from human iPS cells and human ES cells. Adrenaline, isoproterenol, procainamide, flecainide, verapamil, and isoproterenol + propranolol had statistically significant effects on human iPS cells between pre-drug loading and the maximum concentration of the drug used in cardiomyocytes derived from human iPS cells ($P < 0.05$). There were no statistically significant differences between the concentrations of drugs that elicited effects in human iPS cells and those that elicited effects in human ES cells (B).

drugs had similar effects on the beating frequency of cardiomyocytes derived from ES cells and on *bone-fide* human cardiomyocytes, suggesting that human iPS cells and cardiomyocytes respond similarly to these drugs as well [12]. The results were compatible with clinical empirical results [15]. So we conclude that cardiomyocytes derived from human iPS cells respond similarly to drugs that affect contractility in human cardiomyocytes.

Table 1
Drugs and concentrations.

Class	Drugs	Concentration (M)
Na channel blocker	Procainamide	1×10^{-5}–1×10^{-3}
	Mexiletine	1×10^{-7}–1×10^{-5}
	Flecainide	1×10^{-7}–1×10^{-5}
β blocker	Propranolol	3×10^{-7}–3×10^{-5}
K channel blocker	Amiodarone	1×10^{-6}–1×10^{-4}
Ca channel blocker	Verapamil	1×10^{-7}–1×10^{-5}
α, β stimulant	Adrenaline	5×10^{-7}–5×10^{-5}
β stimulant	Isoproterenol	1×10^{-8}–1×10^{-6}

Table 2
Comparison with clinical empirical result.

Drugs	Result		Clinical efficacy	
	Contractility	Beating frequency	Contractility	Beating frequency
Procainamide	↓	→	↓	→
Mexiletine	→	→	→	→
Flecainide	↓	→	↓	→
Propranolol	↓	↓	↓	↓
Amiodarone	→	↓	→	↓
Verapamil	↓	↓	↓	↓
Isoproterenol	↑	↑	↑	↑
Adrenaline	↑	↑	↑	↑

Table 3
Primers for RT-PCR.

Genes	Direction	Sequence
Oct4 (endo)	Forward	GACAGGGGGAGGGGAGGAGCTAGG
	Reverse	CTTCCCTCCAACCAGTTGCCCCAAAC
Sox2 (endo)	Forward	GGGAAATGGGAGGGGTGCAAAAGAGG
	Reverse	TTGCGTGAGTGTGGATGGGATTGGTG
C-KIT	Forward	ATTCCCAGCCCATGAGTCCTTGA
	Reverse	ACACGTGGAACACCAACATCCT
Brachyury	Forward	AAGGTGGATCTTCAGGTAGC
	Reverse	CATCTCATTGGTGAGCTCC
KDR	Forward	AAAACCTTTGTTGCTTTTGG
	Reverse	GAAATGGGATTGGTAAGGATG
Nkx2.5	Forward	GCGATTATGCAGCGTGCAATGAGT
	Reverse	AACATAAATACGGGTGGGTGCGTG
TBX5	Forward	AAATGAAACCCAGCATAGGAGCTGGC
	Reverse	ACACTCAGCCTCACATCTTACCCT
MEF2c	Forward	TTTAACACCGCCAGCGCTCTTCACCTTG
	Reverse	TCGTGGCGCGTGTGTTGTGGGTATCTCG
MLC2a	Forward	ACATCATCACCCACGGAGAAGAGA
	Reverse	ATTGGAACATGGCCTCTGGATGGA
MYHCB	Forward	CTGGAGGCCGAGCAGAAGCGCAACG
	Reverse	GTCCGCCCGCTCCTCTGCCTCATCC
HCN4	Forward	GGTGTCCATCAACAACATGG
	Reverse	TGTACTGCTCCACCTGCTTG
SCN5A	Forward	CCTAATCATCTTCCGCATCC
	Reverse	TGTTCATCTCTCTGTCCTCATC
Kir2.1	Forward	GACCTGGAGACGGACGAC
	Reverse	AGCCTGGAGTCTGTCAAAGTC
Kv4.3	Forward	GCCAGTCCCTGTGATTGTTT
	Reverse	CTCCATGCAGTTCTGCTCAA
HERG	Forward	TCCAGCGGCTGTACTCGGGC
	Reverse	TGGACCAGAAGTGGTCGGAGAACTC
CACNA1C	Forward	AACATCAACAACGCCAACAA
	Reverse	AGGGCAGGACTGTCTTCTGA
GAPDH	Forward	CACCAGGGCGCTTTTAACTCTG
	Reverse	ATGGTTCACACCCATGCGAAC

Discussion

In this report, we differentiated human iPS cells into cardiomyocytes, and compared the effects of drugs on cardiomyocytes derived from these cells and on cardiomyocytes derived from human ES cells, as well as with empirical results obtained in a clinical setting. The time course analysis of gene expression during cardiac differentiation was compatible to that seen during cardiogenesis of normal embryogenesis, and the results of the drug loading tests showed that cardiomyocytes derived from human iPS cells responded to drugs in much the same way as cardiomyocytes derived from human ES cells. The results were also compatible to empirical results obtained in a clinical setting.

Human iPS cells can be generated from somatic cell by introducing transcriptional factors. This technology is expected to generate patient-specific iPS cells suitable for the study of disease mechanisms, drug screening, and toxicology studies. This technology is easier to implement for the generation of patient-specific pluripotent cells than current technology which relies on nuclear transplantation technology to generate patient-specific pluripotent cells from ES cells. If cardiomyocytes derived from iPS cells could be shown to respond to drugs in the same way as human derived cardiomyocytes, then this technology would also constitute a major advance because it would allow the use of patient-specific iPS cell for the screening of patient-specific drugs against arrhythmic diseases, especially for lethal arrhythmic diseases such as LQTS where it is often very difficult to select for the best drug.

As the generation of cardiomyocytes from human iPS cells relies on the introduction of exogenous genes, we addressed the troublesome issue of whether cardiomyocytes derived from human iPS cells would respond to drugs in the same way as normal human cardiomyocytes. We considered the beating frequency and contractility to be very important indicators, because heart pump function is defined by beating frequency and contractility. So we investigated the effects of drugs on these two indicators, and found that drugs affect the beating frequency and contractility of cardiomyocytes derived from human iPS cells in much the same way as they do in a clinical setting. This result suggests that cardiomyocytes derived from human iPS cells could be used for drug screening tests instead of current screening procedures in a clinical setting. Cardiomyocytes derived from ES cells also responded to drugs in the same way as cardiomyocytes derived from human iPS cells.

Thus, these results suggest that patient-specific iPS cells could be used to select for the best drug to treat arrhythmic disease at the individual level, and would have the additional advantage of allowing the massive and rapid screening of drugs at concentrations that would be normally prohibitive in patients. However, until further studies are carried out, it is probably still too early to conclude that the drug effects on human iPS cell lines and patients are identical.

In conclusion, cardiomyocytes derived from human iPS cells have tremendous potential for drug screening, which should open the possibility of using patient-specific iPS cells in a clinical setting. The best drugs could be selected safely and rapidly by using human iPS cells from individual patients.

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BRIEF REPORT
Successful Treatment of Refractory Donor Lymphocyte Infusion-Induced Immune-Mediated Pancytopenia with Rituximab

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A 6-year-old male with chronic granulomatous disease, who was transplanted with bone marrow and exhibited increasing mixed chimerism, subsequently received two donor lymphocyte infusions (DLI). Two weeks after the second DLI, the patient developed acute graft-versus-host disease (GVHD) and progressive pancytopenia that was associated with autoantibody production. Conventional treatment did not improve the pancytopenia. However, administration of Rituximab (RTX) (375 mg/m^2/week for four consecutive weeks) resulted in a rapid resolution of the pancytopenia. The patient achieved full donor chimerism without GVHD symptoms. RTX can be valuable for managing immune-mediated cytopenias that arise after DLI and are refractory to conventional therapies. Pediatr Blood Cancer 2010;54:329–331. © 2009 Wiley-Liss, Inc.

Key words: allogeneic stem cell transplantation; antibodies; graft rejection; graft-versus-host disease; immune responses; Rituximab

INTRODUCTION

Donor leukocyte infusion (DLI) is used as an immunotherapy not only for preventing the reemergence of malignancies, but also for preventing graft rejection after allogeneic hematopoietic stem cell transplantation (hSCT) that results in the development of mixed increasing chimerism [1]. However, DLI treatment is also associated with substantial toxicity. For example, it has been shown that up to 41% of patients receiving DLI suffer from myelosuppression, which could lead to death from causes other than the underlying disease [2,3]. Like the cytopenias associated with graft-versus-host disease (GVHD), the cytopenias that can arise after DLI are conventionally treated by steroids, intravenous immunoglobulin (IVIG), and splenectomy. However, the prognosis of cases that are refractory to conventional treatments remains dismal as the treatment of such cases has not been established. Anti-CD20 antibody (Rituximab, RTX), a humanized murine monoclonal antibody that is often used to treat B-cell malignancies, has been shown to effectively treat various autoimmune diseases that arise after hSCT [4–6]. Here, we describe a patient with severe immune-mediated pancytopenia after DLI who responded well to RTX therapy.

CASE REPORT

A 4-month-old male was diagnosed with X-linked chronic granulomatous disease on the basis of his reduced NADPH oxidase levels (<5%) and the complete absence of gp91-phox. Despite prophylactic treatment with trimethoprim–sulfamethoxazole and itraconazole, and interferon-γ, he suffered repeatedly from severe bacterial and fungal infections, including multiple episodes of pulmonary aspergillosis. Therefore, allogeneic hSCT was planned, and the patient was transferred at 6 years of age to our hospital for bone marrow transplantation (BMT) from a genotypically HLA-matched, blood-type compatible unrelated donor. The HLA type of the donor and the patient was HLA-A 33/24, -B 58/52, -DR 1302/1502. The conditioning regimen included fludarabine 30 mg/m^2/day for 6 days from day −7 to −2, cyclophosphamide 30 mg/kg/day for 4 days from day −6 to −3, anti-T lymphocyte globulin 2.5 mg/kg/day for 4 days from day −6 to −3, and total body irradiation 300 cGy on day −1. To prevent GVHD, the patient received tacrolimus and short-methotrexate (day 1: 10 mg/m^2, day 3.6: 7 mg/m^2), as previously reported [7]. Subsequently, 4.8×10^8/kg mononucleated cells were infused without T-cell depletion. The patient's bone marrow (BM) was analyzed serially for chimerism by micro-satellite PCR, and the presence of oxidase-positive neutrophils in the peripheral blood (PB) was determined by fluorescence-activated cell sorting using a dihydrorhodamine oxidation assay. Hematopoietic engraftment occurred rapidly. The neutrophil count exceeded 0.5×10^9/L on day 10, the reticulocyte count exceeded 10‰ on day 17, and the platelet counts did not drop below 40×10^9/L during this period. However, the donor chimerism of the patient was unstable. After the dosage of tacrolimus was reduced on day 25, grade II acute GVHD of the skin developed on day 37, which was resolved by a short course of prednisolone (PSL) treatment. Subsequently, the patient achieved full donor chimerism of BM on day 61, and the oxidase-positivity of PB neutrophils was 100% on day 82. The GVHD did not worsen after treatment with PSL and tacrolimus was discontinued on days 98 and 361, respectively.

Although the patient was asymptomatic and there were no abnormal laboratory findings, the oxidase-positivity of PB neutrophils gradually decreased to 50% and 13% on days 404 and 758,

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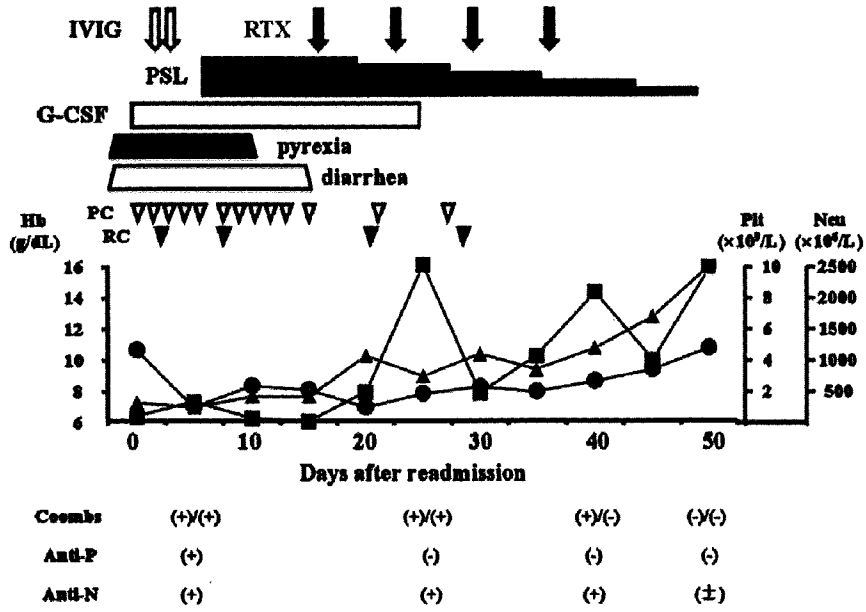

Fig. 1. The clinical course after readmission. IVIG, intravenous immunoglobulin; RTX, Rituximab; PSL, prednisolone; G-CSF, granulocyte colony-stimulating factor; RC, packed red blood cell concentrate; PC, packed platelet concentrate; Plt, platelet counts; Neu, neutrophil counts; Coombs, direct/indirect Coombs test; Anti-P, anti-platelet antibody; Anti-N, anti-neutrophil antibody. Closed circles, triangles, and squares indicate Hb levels, platelet counts (Plt), and neutrophil counts (Neu), respectively.

respectively. In an attempt to induce his return to full donor chimerism, the patient was given frozen 1.0×10^7 and 5.0×10^7 PB lymphocytes/kg on days 805 and 850, respectively, which had been harvested from the same donor who had provided the BM. Before this second DLI, the patient had not undergone any notable events such as contracting an infectious disease, medication changes or vaccinations. The clinical course after the second DLI is shown in Figure 1. Two weeks after it was delivered, the patient developed a skin rash, diarrhea, fever, elevated serum liver enzyme value, and thrombocytopenia. The patient was diagnosed clinically as having GVHD. Since restarting the patient on tacrolimus did not improve his symptoms, he was readmitted to our hospital on day 44 after the second DLI.

On readmission, the physical examination revealed no abnormal symptoms except for a persistent high fever. The results of the laboratory investigations are shown in Table I. Antibody screening tests revealed strong positivity in the direct and indirect Coombs test, and the presence of anti-platelet antibodies and anti-neutrophil antibodies specific for HNA-1a and 1b. However, other antibody screening tests were negative. There was no fungal infection or recurrence of CMV and EBV. An examination of the BM on day 896 after the BMT revealed a hypocellular marrow, but no

TABLE I. Laboratory Data on Readmission

	Value	Unit	Normal range		Value	Unit	Normal range		Value	Normal range
WBC	3.1	10^9/L	3.6–9.8	AST	38	IU/L	13–33	CMVpp65	(−)	(−)
Neu	0.93	10^9/L	1.6–6.0	ALT	36	IU/L	8–42	EBV-DNAPCR	(−)	(−)
Lymph	2.2	10^9/L	1.1–3.9	LDH	273	IU/L	129–241	Aspergillus-Ag	(−)	(−)
Hb	10.6	g/dl	11.3–13.7	ALP	473	IU/L	115–359	Candida-Ag	(−)	(−)
Reti	7.1	10^9/L	2.7–9.3	T.Bil	0.8	mg/dl	0.3–1.3	Direct Coombs Test	(+)	(−)
Plt	12	10^9/L	192–456	TP	7.3	mg/dl	6.3–8.1	Indirect Coombs Test	(+)	(−)
Haptoglobin	102.4	mg/dl	14–294	Alb	4.3	mg/dl	3.9–5.1			
CRP	4.9	mg/dl	<0.2	Soluble IL2	972	U/ml	145–519	Anti-neutrophil antibodies	(+)	(−)
β-DG	7.473	ng/ml	<11	Ferritin	7.3	ng/ml	<155	Anti-HNA-a	(+)	(−)
Endotoxin	<1.76	pg/ml	<5	Triglycerides	58	mg/dl	34–173	Anti-HNA-b	(+)	(−)
								Anti-platelet antibodies	(+)	(−)

WBC, white blood cell; Neu, neutrophil; Lymph, lymphocyte; Hb, hemoglobin; Reti, reticulocyte; Plt, platelet; CRP, C-reactive protein; β-DG, β-D-glucan; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; T.Bil, total bilirubin; TP, total protein; Alb, albumin; CMVpp65, Cytomegalovirus pp65; EBV, Epstein–Barr virus; Ag, antigen; HNA, human neutrophil antigen.

evidence of malignancy or hemophagocytosis. Chimerism studies of the BM revealed 55% of the cells were composed of donor cells. Only 17% of the PB neutrophils were oxidase-positive. The patient was first treated with IVIG (1 g/kg/day for 2 days), PSL (2 mg/kg/day daily), and granulocyte colony-stimulating factor (G-CSF). Although this initial treatment resolved the pyrexia and diarrhea, the patient's pancytopenia gradually progressed and multiple transfusions became necessary. Given his refractory autoimmune pancytopenia, he was treated with RTX (375 mg/m^2/week for four consecutive weeks). The neutrophil counts rose markedly within a few days after the first RTX infusion, which was followed by the gradual increase in Hb and platelet counts. The patient became transfusion-independent after the third RTX course, and pancytopenia did not recur when the patient stopped receiving G-CSF and PSL. The hematological values normalized 21 days after the initial RTX infusion. The autoimmune antibody levels dropped during RTX treatment and eventually disappeared almost completely. Both BM chimerism studies and analysis of the oxidase-positivity of the PB neutrophils revealed 100% donor chimerism 80 days after the initial RTX infusion. Three years after the RTX treatment, the patient was alive and free of disease and showed no signs of mixed chimerism or GVHD.

DISCUSSION

Cytopenias that follow allogeneic hSCT can be immune-mediated and are frequently associated with GVHD. Autoimmune hemolytic anemia (AIHA) and immune thrombocytopenia (ITP) occur frequently, but immune-mediated cytopenias, including autoimmune neutropenia (AIN), are relatively rare [4,5]. Cytopenias are also often seen in patients after DLI and are thought to be mediated by autoimmune mechanisms, as with GVHD. In our case, pancytopenia developed soon after DLI, along with acute GVHD and the emergence of autoantibodies against multilineage blood cells. Notably, the levels of these antibodies decreased in parallel with the improvement of the pancytopenia, while the blood and BM analyses suggested that other possible causes of cytopenias, such as viral infections and hemophagocytic histiocytosis, were unlikely. However, the BM examination also showed a hypocellular marrow, which suggested that the pancytopenia did not arise from antibody-mediated cell destruction alone. Our findings suggest that autoimmunity was the major cause of the severe pancytopenia exhibited by our patient.

Most patients with autoimmune cytopenias are rescued by the administration of high-dose IVIG and standard immunosuppressive agents such as steroids [8,9]. Furthermore, RTX has been demonstrated to be useful for treating the AIHA and ITP that follow GVHD, which is refractory to conventional treatment [5,6,10–13]. However, the prognosis of patients who develop autoimmune pancytopenia remains to be determined. Page et al. [4] reported two cases that developed pancytopenia after umbilical cord blood transplantation. Despite receiving immunosuppressive treatment, including RTX, one patient continued to need the therapy while the other required a second transplantation because of pancytopenia.

Despite the fact that our patient was initially treated with PSL, high-dose IVIG, and G-CSF, and showed improvements in the other symptoms of acute GVHD, his pancytopenia progressed. Given this rapid and potentially fatal progression, we chose to start a salvage therapeutic approach rather than continue such conventional treatments, which would result in a slower response. The institution of RTX resulted in the resolution of the pancytopenia and the almost complete disappearance of the autoimmune antibodies. Furthermore, the response to RTX was already obvious 1 week after the first RTX infusion, which is consistent with a study that showed that RTX induces a prompt response in a subpopulation of patients [14].

Although no definite conclusions can be drawn from a single case with a relatively short period of follow-up, this case strengthens the hypothesis that RTX can be a beneficial treatment for refractory DLI-induced immune-mediated pancytopenia. This case suggests that further clinical research examining the merits of RTX in such cases is warranted.

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Deficiency of regulatory T cells in children with autoimmune neutropenia

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Summary

$CD4^+$ 25^+ regulatory T cells (Tregs) play a role in controlling the development and progression of autoimmunity. The transcription factors Foxp3 and NFATC2 (NFAT1) play key roles in regulating the development and function of Tregs. The present study examined the involvement of Tregs in the pathophysiology of autoimmune neutropenia in children. Tregs were analysed by flow cytometry, based on the expressions of CD4, CD25, and intracellular Foxp3. The expressions of *FOXP3* and *NFATC2* mRNA in the $CD4^+$ 25^+ cells were determined by quantitative real-time polymerase chain reaction. The percentage of $CD4^+$ 25^{high} Tregs in patients with autoimmune neutropenia was significantly lower than that in age-matched healthy subjects. The intracellular expression of Foxp3 of $CD4^+$ 25^+ cells in patients similarly decreased in comparison to that in healthy subjects. The expression of *FOXP3* and *NFATC2* mRNA of $CD4^+$ 25^+ cells in patients also significantly decreased in comparison to that in healthy subjects. These results suggest that the deficiency of Tregs might thus play an important role in the immunopathophysiology of autoimmune neutropenia in children.

Keywords: infant, autoimmunity, neutropenia, regulatory T cells, Foxp3.

Regulatory T cells (Tregs) are a specialised subpopulation of T cells that are phenotypically characterised by the cell surface expression of the proteins CD4 and CD25. $CD4^+$ 25^+ Tregs, which constitute from 5 to 10% of peripheral $CD4^+$ T cells in mice and human subjects, act to suppress the activation of other immune cells and, thereby maintain immune system homeostasis (Ng *et al*, 2001; Chatila, 2005; Dejaco *et al*, 2005; Sakaguchi *et al*, 2008). The decreased frequencies or reduced effector function of Tregs is associated with several autoimmune disorders (Brusko *et al*, 1998; Dejaco *et al*, 2005; Lan *et al*, 2005). Recent studies have shown the forkhead family transcription factor Foxp3 to be critically important in the development and function of Tregs (Lan *et al*, 2005). Genetic mutations in the gene encoding Foxp3 have been identified in both humans and mice. Humans with mutations in *FOXP3* suffer from a severe and rapidly fatal autoimmune disorder known as immune regulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome (Bennett *et al*, 2001). The transcriptional factor NFATC2 (NFAT1) induces Foxp3 expression by binding to its promoter, and Foxp3 expresses its repressive effects in cooperation with NFATC2 (Mantel *et al*, 2006; Wu *et al*, 2006).

Autoimmune neutropenia (AIN) is a common form of neutropenia in childhood and it is characterised by chronic neutropenia presenting a low absolute neutrophil count (ANC) of $<1{\cdot}5 \times 10^9$/l for more than 6 months, the detection of anti-neutrophil antibodies in sera, and spontaneous resolution of neutropenia within several months to a few years (Bux *et al*, 1998; Kobayashi *et al*, 2002). To date, the precise pathophysiology of AIN remains unclear. One possible mechanism suggests the immaturity of T cell functions in infancy and early childhood (Yachie *et al*, 1981).

The present study examined the involvement of Tregs in the immunopathophysiology of patients with AIN. The results demonstrate the decreased frequency of Tregs through the low levels of Foxp3 expression as mediated by NFATC2.

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Methods

Patients and healthy subjects

Peripheral blood samples were obtained from healthy neonates (n = 14), infants (n = 22, age range: 1 month to 3 years old, mean: 8·1 months old), adults (n = 12: 26–40 years old) and AIN patients (n = 22, age range: 1 month to 3 years old, mean: 8·8 months old) after informed consent was obtained from patients' guardians, healthy subjects, and/or their guardians. Approval was obtained from the institutional board for these studies. AIN was diagnosed when the absolute neutrophil count was $<0{\cdot}5 \times 10^9$/l for more than 1 month without an underlying disease, a history of drug administration causing neutropenia, or a history of blood transfusions. Patients with severe congenital neutropenia, cyclic neutropenia, or alloimmune neutropenia were excluded. Antineutrophil antibodies were analysed by the granulocyte indirect immunofluorescence test as previously described (Kobayashi *et al*, 1989). Twenty-two patients with AIN included 12 males and 10 females that had a white blood cell count and absolute neutrophil count (mean ± SD) of $66 \pm 2{\cdot}7 \times 10^9$/l and $0{\cdot}18 \pm 0{\cdot}098 \times 10^9$/l respectively. Antibody against human neutrophil antigen (HNA)-1a antigen was detected in 10 patients, against HNA-1b in one patient, and against pan FcγRIIIb in seven patients. No antibodies were detected in the remaining four patients.

Flow cytometry

Peripheral blood mononuclear cells were isolated from peripheral blood samples by density centrifugation over Lymphoprep (1·077 g/ml; Nycomed Pharma AS, Oslo, Norway), and washed twice and resuspended in wash solution (phosphate-buffered saline containing 0·1% bovine serum albumin and 0·1% sodium azide). Cells (10^6) were simultaneously incubated with fluorescein isothiocyanate (FITC)-labelled monoclonal anti-CD4 antibody (Becton-Dickinson (BD) Biosciences, San Jose, CA, USA) and phycoerythrin (PE)-labelled monoclonal anti-CD25 antibody (BD Biosciences) for 30 min at 4°C. The cells were then washed twice and resuspended in wash solution. In cytosolic Foxp3 analysis, cells were simultaneously incubated with allophycocyanin (APC)-labelled monoclonal anti-CD4 antibody (Pharmingen, San Diego, CA, USA) and PE-labelled monoclonal anti-CD25 antibody for 30 min at 4°C. The cells were then washed and permeabilized with Cytofix/Cytoperm™ solution (Pharmingen) following the manufacturer's protocols and stained with FITC-labelled monoclonal anti-Foxp3 antibody (eBioscience, San Diego, CA, USA) for 30 min. The cells were then washed twice and resuspended in wash solution. FITC, PE or APC-conjugated mouse IgG1a was used as an isotype control. The stained cells were analysed using FACSCalibur™ (BD Biosciences) and the CELLQUEST software programme.

Purification of $CD4^+$ 25^+ cells

Peripheral blood samples were centrifuged over lymphoprep. The light density cells were carefully harvested and washed three times and resuspended in the above described wash solution. The cells (2×10^7/ml) were incubated with FITC-labelled monoclonal anti-CD4 antibody and PE-labelled monoclonal anti-CD25 antibody for 30 min at 4°C. FITC or PE-conjugated mouse IgG1a was used as an isotype control. The cells were washed twice and then resuspended in wash solution. The purification of $CD4^+$ 25^+ Tregs was carried out using the FACSAria™ (BD Biosciences). A low–medium forward scatter and low side scatter were used to establish sort windows. Tregs expressing both CD4 and CD25 were purified by cell sorting. The purity of $CD4^+$ $CD25^+$ cells was >95%. The purified cells were stored at −80°C until the extraction of nucleic acids and real-time polymerase chain reaction (PCR) analysis were performed.

Quantification of FOXP3 and NFATC2 mRNA by real-time PCR

$CD4^+$ $CD25^+$ cells were purified as mentioned above. Total cellular RNA was extracted using RNA extraction kit (RNeasy Mini Kit; Qiagen Sciences, MD, USA) and converted into cDNA by reverse transcriptase for real-time PCR. The PCR primers and Taq Man probe were designed as follows with the assistance of the PRIMER EXPRESS software programme (PE Biosystems, Foster City, CA, USA), based on the information of the sequence of *FOXP3* and *NFATC2* mRNA; *FOXP3*: forward primer F: 5′-GAGAAGCTGAGTGCCATGCA-3′ 751–770, reverse primer R: 5′-GCCACAGATGAAGCCTTGGT-3′ 818–799, TaqMan probe P: 5′-CCACCTGGCTGGGAAAATGGCA-3′ 774–795 and *NFATC2*: forward primer F: 5′-CGACCTGTGTCCG CAGTTT-3′ 597–615, reverse primer R: 5′-TGGTTCGAGGT GACATTATTGG-3′ 673–652, TaqMan probe P: 5′-ACATCC CTGCTCATTATTCCCCCAGAAC-3′ 620–647. The commercial reagents (TaqMan PCR reagent kit; Applied Biosystems) and the PCR conditions were used according to the manufacturer's protocol. Thereafter, 10 μl cDNA and 5 μl oligonucleotides at a final concentration of 200 nmol/l primers and 100 mmol/l TaqMan hybridisation probe were added to a 25 μl reaction mixture. The amplification conditions for quantification were an initial incubation at 50°C for 2 min and then 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. The reactions were performed with the ABI PRISM 7700 sequence detection system equipped with a 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA). The *FOXP3* and *NFATC2* values were collected together with the values obtained for *ACTB* from the same cDNA.

Statistical analysis

Statistical significance of the data between two groups was determined using Mann–Whitney's *U*-test and that among

three groups was determined using the Kruskal–Wallis test followed by a *post hoc* analysis. Any associations between continuous variables were evaluated by using the Pearson correlation. These statistical analyses were performed by using the STATVIEW software programme (version 5.0; SAS Institute, INC., Cary, NC, USA). P values < 0·05 were considered significant.

Results

The frequencies of Tregs in various normal age groups

The frequency of circulating Tregs was first examined in healthy neonates, infants and adults. Flow cytometric analysis of peripheral blood samples revealed different intensities of CD25 expression on CD4-positive cells. The $CD4^+$ 25^+ Tregs fraction was clearly divided into two populations, $CD4^+$ $CD25^{high}$ and $CD4^+$ 25^{low} cells (Fig 1). The representative staining patterns of CD4 and CD25 in the three age groups are shown in Fig 1. The percentage of CD4-positive lymphocytes expressing CD25 was significantly higher in adulthood (18·5 ± 3·5%) than neonates (11·9 ± 2·6%) and infants (10·1 ± 2·0%; P < 0·01; Table I). Similarly, a higher percentage of $CD4^+$ $CD25^{low}$ cells was noted in adulthood than neonates and infants. In contrast, the percentage of $CD4^+$ lymphocytes expressing $CD25^{high}$, known as the pure Tregs fraction, was the highest in neonates and decreased significantly with age (P < 0·01).

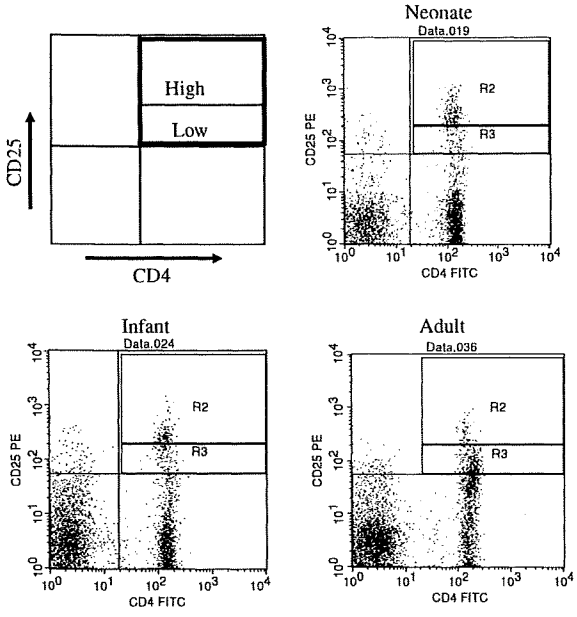

Fig 1. $CD4^+$ $CD25^+$ regulatory T cells in various age groups. Peripheral blood leucocytes from each sample were stained with anti-CD4 and anti-CD25 monoclonal antibodies. $CD4^+$ $CD25^+$ regulatory T cells were divided in two fractions, termed $CD4^+$ 25^{high} cells and $CD4^+$ 25^{low} cells (upper left). Representative dot plots are shown from the three age groups.

Table I. Percentages of $CD25^+$, $CD25^{high}$ and $CD25^{low}$ in CD4 positive cells in three age groups.

	Healthy neonates (n = 14)	Healthy infants (n = 22)	Healthy adults (n = 12)
$CD25^+$ cells	11·9 ± 2·6	10·1 ± 2·0	18·5 ± 3·5
$CD25^{high}$ cells	8·3 ± 2·7	5·6 ± 1·9	4·2 ± 1·1
$CD25^{low}$ cells	3·6 ± 1·2	4·5 ± 1·9	14·3 ± 3·6

Data represent the mean ± SD of $CD25^+$, $CD25^{high}$ and $CD25^{low}$ in CD4 positive cells. The frequency of $CD4^+$ lymphocyte expressing $CD25^{high}$ among the three age groups was statistically significant (P < 0·01).

The frequencies of $CD4^+$ 25^+ Tregs in AIN patients

Next, the frequencies of $CD4^+$ 25^+ cells in AIN patients were compared with age-matched control infants. No difference in the percentage of CD4-positive cells in mononuclear leucocytes was noted between age-matched healthy subjects and patients with AIN (data not shown). As shown in Table II, the percentage of $CD4^+$ lymphocytes expressing $CD25^{high}$ in patients with AIN (4·0 ± 1·6%) was significantly decreased in comparison to that in healthy infants (5·6 ± 1·9%, P < 0·01). No difference in the frequency of $CD4^+$ $CD25^{low}$ cells was noted between AIN patients and healthy subjects. Tregs are characterised by the intracellular expression of the transcriptional factor Foxp3. Therefore, intracellular Foxp3 expression was examined in $CD4^+$ 25^+, $CD4^+$ $CD25^{high}$ and $CD4^+$ 25^{low}. As presented in Table III, intracellular Foxp3 expression in AIN patients was significantly decreased in comparison to those in healthy subjects in all $CD4^+$ 25^+, $CD4^+$ $CD25^{high}$ and $CD4^+$ 25^{low} fractions (P < 0·05).

Table II. Percentages of $CD25^+$, $CD25^{high}$ and $CD25^{low}$ in CD4 positive cells in healthy infants and AIN patients.

	Healthy infants (n = 22)	AIN patients (n = 22)	P value
$CD25^+$ cells	10·1 ± 2·0	9·5 ± 1·9	n.s
$CD25^{high}$ cells	5·6 ± 1·9	4·0 ± 1·6	<0·01
$CD25^{low}$ cells	4·5 ± 1·9 5	5 ± 1·5	n.s

Data represent the mean ± SD of $CD25^+$, $CD25^{high}$ and $CD25^{low}$ in CD4 positive cells.

Table III. Percentages of Foxp3 positive cells in $CD4^+$ $CD25^+$ cells.

	Healthy infants (n = 22)	AIN patients (n = 22)	P value
$CD25^+$ cells	12·3 ± 5·9	5·6 ± 4·9	<0·05
$CD25^{high}$ cells	13·0 ± 8·1	7·4 ± 4·8	<0·05
$CD25^{low}$ cells	10·9 ± 16·8	5·1 ± 5·0	<0·05

Data represent the mean ± SD of cytoplasmic Foxp3 positive cells in $CD4^+$ $CD25^+$ cells.

Expressions of FOXP3 and NFATC2 mRNA

To confirm the decreased expression of Foxp3 in $CD4^+$ 25^+ cells from patients with AIN, the *FOXP3* and *NFATC2* mRNAs expression was quantified in $CD4^+$ 25^+ cells by quantitative real-time PCR. NFATC2 is known to regulate the activation of Foxp3 transcription and to form cooperative complexes with Foxp3 (Mantel *et al*, 2006; Wu *et al*, 2006). *FOXP3* mRNA expression of $CD4^+$ 25^+ cells from AIN patients was significantly decreased in comparison to that from age-matched healthy subjects (Fig 2). Furthermore, the levels of *NFATC2* mRNA expression in $CD4^+$ 25^+ cells from AIN patients were also significantly decreased in comparison to that in healthy subjects (Fig 2B). As shown in Fig 3, the decreased expression level of *FOXP3* mRNA was strongly correlated with that of *NFATC2* mRNA in $CD4^+$ 25^+ cells from both AIN patients and healthy subjects. Therefore, the decreased expression of *NFATC2* and *FOXP3* mRNA may lead to the decreased expression of intracellular Foxp3 expression in $CD4^+$ 25^+ cells from AIN patients.

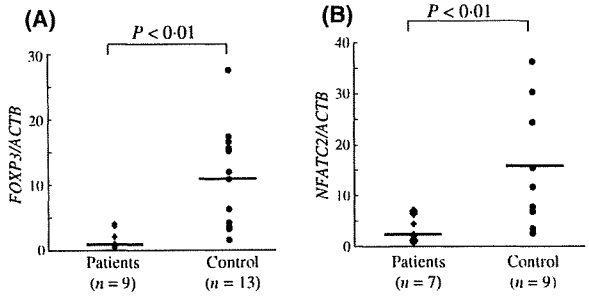

Fig 2. Expression of *FOXP3* and *NFATC2* mRNA in patients with AIN and control infants. The mRNA from $CD4^+$ CD25 cells that was purified by cell sorting was analysed for *FOXP3* (A) and *NFATC2* (B) expression by real-time PCR. The ratios of the copies of *FOXP3* or *NFATC2* to those of *ACTB* from patients and age-matched control are presented. Differences in the expression of *FOXP3* and *NFATC2* mRNA between the two groups were statistically significant ($P < 0{\cdot}01$).

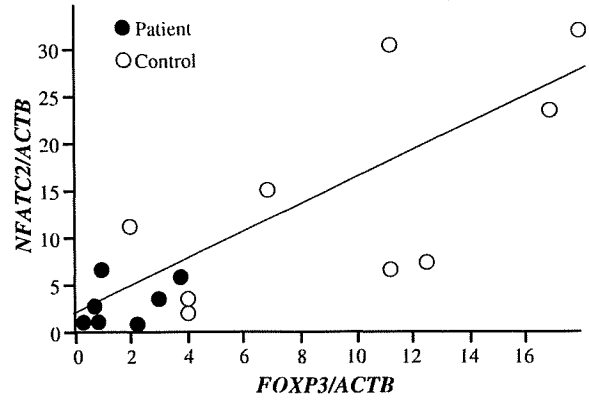

Fig 3. Relation of *FOXP3* mRNA to *NFATC2* mRNA expression in each sample. Closed symbols indicate samples of patients with AIN and open symbols indicate those of age-matched subjects. The expression of these two mRNAs was significantly correlated (Pearson correlation coefficient; $r = 0{\cdot}801$, $P < 0{\cdot}01$).

Discussion

This study attempted to elucidate the unknown standard value of Tregs in paediatric age groups and its involvement in AIN, which is known to be a representative autoimmune disease in this age group.

The results show that the percentage of $CD4^+$ $CD25^{high}$ cells was highest in neonates and decreased significantly with age. Recent studies show that most Tregs exists in the $CD4^+$ 25^{high} cell fraction, nevertheless, $CD4^+$ 25^{low} cells contain a minority of Tregs and newly activated responder cells (Holm *et al*, 2004; Roncador *et al*, 2005; Baecher-Alan & Hafler, 2006). The higher frequency of $CD4^+$ 25^{high} cells in the neonatal group is consistent with previous studies demonstrating that the frequencies of Treg were higher in cord blood than adult peripheral blood (Wing *et al*, 2002; Takahata *et al*, 2004). This finding may imply a tendency toward immunological tolerance during the neonatal period, which is particularly important for prevention against allo-reactive reactions between the fetus and pregnant women. On the other hand, the lower frequency of $CD4^+$ 25^{high} cells in the adult age group may be concerned with the relatively high proportion of autoimmune diseases in adult.

As Tregs suppress auto immunological responses, the decreased frequencies and reduced effector function of Tregs play a role in the activation of self reactive T cells. The involvement of Tregs has been investigated in several autoimmune diseases, mainly in adults (Dejaco *et al*, 2005; Lan *et al*, 2005; Mqadmi *et al*, 2005; Baecher-Alan & Hafler, 2006; Anderton & Liblau, 2008; Wei *et al*, 2008). Patients with multiple sclerosis (Viglietta *et al*, 2004; Dejaco *et al*, 2005; Anderton & Liblau, 2008) or myasthenia gravis (Balandina *et al*, 2005) showed the decreased activity of Tregs in suppressing proliferation and cytokine production of $CD4^+$ 25^- cells is observed in comparison to healthy controls, whereas there were no differences in the frequencies of Tregs. On the other hand, patients with aplastic anaemia (Solomou *et al*, 2007), juvenile idiopathic arthritis (Wei *et al*, 2008) or Kawasaki disease (Furuno *et al*, 2004), had significantly lower frequencies of Tregs and Foxp3 expression in comparison to the normal control. The current data demonstrate that in AIN patients, the frequency of $CD4^+$ 25^{high} Tregs significantly decreased in comparison to that in age-matched normal infants whereas, there were no differences in the frequencies of $CD4^+$ 25^+ Tregs and $CD4^+$ 25^{low} Tregs between the two groups. Similarly, Foxp3 protein expression in each population was significantly decreased in patients in comparison to controls. Furthermore, the *FOXP3* and *NFATC2* mRNA expression in $CD4^+$ 25^+ Tregs also significantly decreased. These data demonstrate that decreased frequencies of Tregs

and attenuated expression of Foxp3 in Tregs play a pivotal role in the pathogenesis of AIN in infancy.

The mechanisms of these abnormalities found in patients with AIN are unknown. Given that Foxp3 is known as the master switch molecule in the generation and function of Tregs (Mantel *et al*, 2006), it is plausible that the decreased frequencies of Tregs in patients with AIN might be the consequence of the attenuated expression of Foxp3. Recently a functional polymorphism in the promoter/enhancer region of *FOXP3* (Bassuny *et al*, 2003) or a decreased expression of NFATC2, which binds to the promoter of *FOXP3* gene (Solomou *et al*, 2007) was reported to be associated with other autoimmune diseases. In the current findings, *NFATC2* in addition to *FOXP3* mRNA expression were attenuated in patients, thus suggesting their possible role in the pathogenesis in AIN. Further study will be needed to elucidate the implications of such factors in patients with AIN.

In conclusion, these studies strongly suggest that the deficiency of Tregs might play an important role in the immunopathophysiology of AIN in childhood.

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Short-Term Culture of Umbilical Cord Blood-Derived CD34 Cells Enhances Engraftment into NOD/SCID Mice Through Increased CXCR4 Expression

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Human umbilical cord blood (CB) has been used successfully in stem cell transplantation. A subpopulation of $CD34^+$ cells expresses chemokine receptor CXCR4 on the cell surface that is critical for bone marrow engraftment in human hematopoietic stem cells. Here, we demonstrate the effect of short-term culture on CXCR4 expression on umbilical CB-derived $CD34^+$ cells and subsequent engraftment capability in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. Surface CXCR4 expression on $CD34^+$ cells increased after incubating the cells in medium alone for 2 h; this effect was blocked by the addition of AMD3100. No difference in CXCR4 mRNA expression was noted after incubating $CD34^+$ cells in culture for 2 h, although these cells showed significantly increased transmigrational activity toward SDF-1 and homing activity in NOD/SCID mice. Furthermore, cultured human $CD34^+$ cells showed improved engraftment into the bone marrow of NOD/SCID mice compared to noncultured or AMD3100-treated $CD34^+$ cells. These observations suggest that increased cell surface expression of CXCR4 on $CD34^+$ cells improved the engraftment of human umbilical CB cells into bone marrow through enhanced homing activity.

Introduction

HUMAN CORD BLOOD (CB), collected from the postpartum placenta and umbilical cord, is a rich source of hematopoietic stem cells (HSCs) and provides an attractive alternative to bone marrow or mobilized peripheral blood transplantation. However, a major disadvantage of CB transplantation is the relatively low number of HSCs in each CB unit that severely limits its usefulness in clinical transplantation [1]. Therefore, the development of ex vivo culture systems to expand CB HSC numbers is important to stem cell research and clinical application. Previous studies showed that the transplantation of HSCs into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice was shown to be a reliable model for the detection of regenerative human HSCs [2,3].

CXCR4 is the seven-transmembrane receptor of SDF-1 and is widely expressed in a variety of hematopoietic cell types, neuronal cells, and immature $CD34^+$ progenitor cells. The chemokine receptor, CXCR4, and its ligand, stromal cell-derived factor-1 (SDF-1, also known as CXCL12), play a central role in the migration, proliferation, differentiation, and survival of both murine and human hematopoietic stem/progenitor cells [4–7]. The SDF-1–CXCR4 axis has been proposed to be essential for the homing and repopulation of HSCs transplanted into immunodeficient NOD/SCID mice [8,9]. Recently, Kollet et al. [10] demonstrated that $CD34^+/CXCR4^-$ cells expressed intracellular CXCR4, the cell surface expression of which was stimulated by cytokines. The overexpression of CXCR4 on $CD34^+$ cells via gene transfer improved human stem cell motility, retention, and multilineage repopulation [11].

In the present study, we examined the effect of short-term culture on CXCR4 expression and engraftment in CB-derived $CD34^+$ cells transplanted into NOD/SCID mice. Our results demonstrated that short-term culture increased cell surface CXCR4 expression in $CD34^+$ cells and enhanced the retention of these human cells in mouse bone marrow through enhanced homing activity.

Materials and Methods

Isolation of $CD34^+$ cells

Umbilical CB was obtained from normal full-term deliveries, after first obtaining informed consent from all participants and the approval of the Chugoku-Shikoku Regional Cord Blood Bank (Hiroshima, Japan). Mononuclear cells

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