et al., 2007) (20D17, 38C2, and 38D2) were positive for green fluorescent protein (GFP) expression only when Nanog was activated. 256H18, which was established by introducing only Oct3/4, Sox2, and Klf4, expressed DsRed (Nakagawa et al., 2008) constitutively. The pMx-GFP retrovirus was introduced into this clone as a silencing indicator. The control D3 ES cells were constitutively positive for GFP. All four of the iPS clones formed ES-like colonies over more than 15 passages (Fig. 1A). The FACS analysis revealed that all of the clones expressed SSEA1, E-cadherin, and CD31 (Fig. 1B), thus demonstrating the phenotypic similarity between iPS and ES cells.

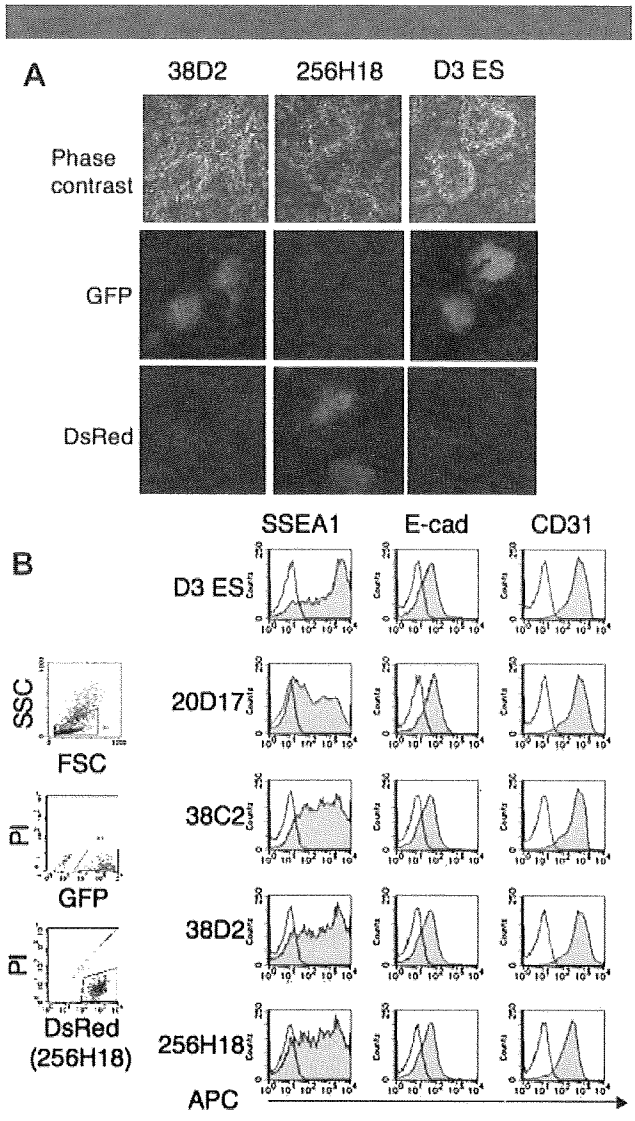


Fig. 1. Formation of ES-like colonies from iPS cells. A: Phase contrast (top row) and fluorescence (middle row: GFP, bottom row: DsRed) micrographs of Nanog-iPS cells (38D2), three-factor (without Myc) iPS (256H18) cells, and D3 ES cells maintained on SNL feeder cells. The D3 ES cells were derived from GFP$^+$ mice. Nanog-iPS cells express GFP only in the undifferentiated state. The three-factor iPS cells were derived from DsRed$^+$ mice, with additional infection by the pMx-GFP virus as a silencing marker. B: FACS analysis showing the phenotypic similarity of iPS and ES cells. The left parts show the gates for eliminating dead cells and contaminated feeders. GFP$^+$PI$^-$ cells (R2) and DSRed$^+$PI$^-$ cells (R4) were gated as ES- and iPS-derived viable cells, respectively. SSEA1, E-cadherin, and CD31 were positive in all strains (shaded bars). Open bars show staining with isotype control antibodies. Representative results from one of three independent experiments performed are presented.

To analyze the hematopoietic differentiation potential of iPS cells, we adapted the OP9 coculture system originally reported by Nakano et al. (1994, 1996). We cocultured iPS cells with OP9 stromal cells for 5 days and transferred the entire culture onto fresh OP9 layers in the presence of mSCF, mIL3, hTPO, and hEPO. Small, round cell colonies first appeared 2 days later (on day 7; Fig. 2A). These colonies gradually grew in both size and number, and a few exhibited areas with a cobblestone-like appearance. Floating cells also appeared on day 7 and thereafter. May–Giemsa staining of the floating cells on day 15 revealed enucleated red blood cells, macrophages, granulocytes, and megakaryocytes (Fig. 2B). The presence of granulocytes and megakaryocytes was confirmed by MPO and acetylcholine esterase (Maherali et al., 2007) staining, respectively. FACS analysis on day 15 confirmed the existence of various types of blood cells, including erythoid and myeloid lineage cells, but not of lymphoid lineage cells (Fig. 2C). These above results demonstrate that iPS cells, like ES cells, can produce hematopoietic cells of various lineages in vitro.

Efficient production of hematopoietic cells from sorted Flk-1$^+$ cells

To thoroughly investigate iPS cell-derived hematopoietic development, we analyzed the expression of Flk-1, a marker of hemoangiogenic progenitors. Although the proportion of Flk-1$^+$ cells in the iPS clones on day 5 varied between 20 ± 2% and 48 ± 9% (Fig. 3A), the temporal patterns of expression were similar in iPS and ES cells. No Flk-1$^+$ cells were detected at

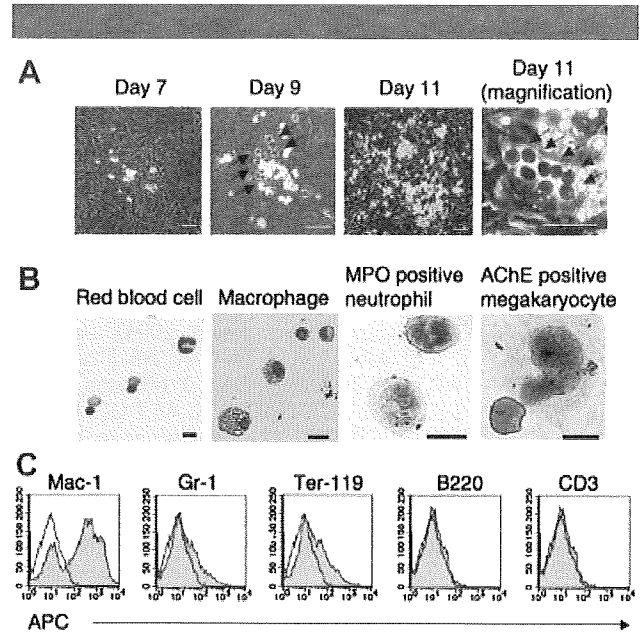


Fig. 2. Hematopoietic cells develop from iPS cells on OP9 feeders. Data from clone 38D2 are shown as representative of iPS-derived hematopoiesis. A: Small colonies first appeared on day 7 (2 days after Flk1$^+$ sorting) and then grew larger. Dark, round hematopoietic progenitors (indicated by arrows) appeared on days 9 and 11, lying beneath the OP9 layer and presenting cobblestone-like areas. Scale bars, 200 μm (left three parts) and 100 μm (rightmost part). B: Floating cells on day 15 included various lineages of hematopoietic cells; enucleated red blood cells, macrophages, MPO$^+$ neutrophils, and AChE$^+$ megakaryocytes were observed. Scale bars, 50 μm. C: Expression of lineage-specific antigens. Floating cells on day 15 were stained with antibodies against macrophages (Mac-1), granulocytes (Gr-1), erythrocytes (Ter-119), B cells (B220), and T cells (CD3). Expression of each antigen (shaded bars) was analyzed using FACS. Open bars show staining with isotype control antibodies.

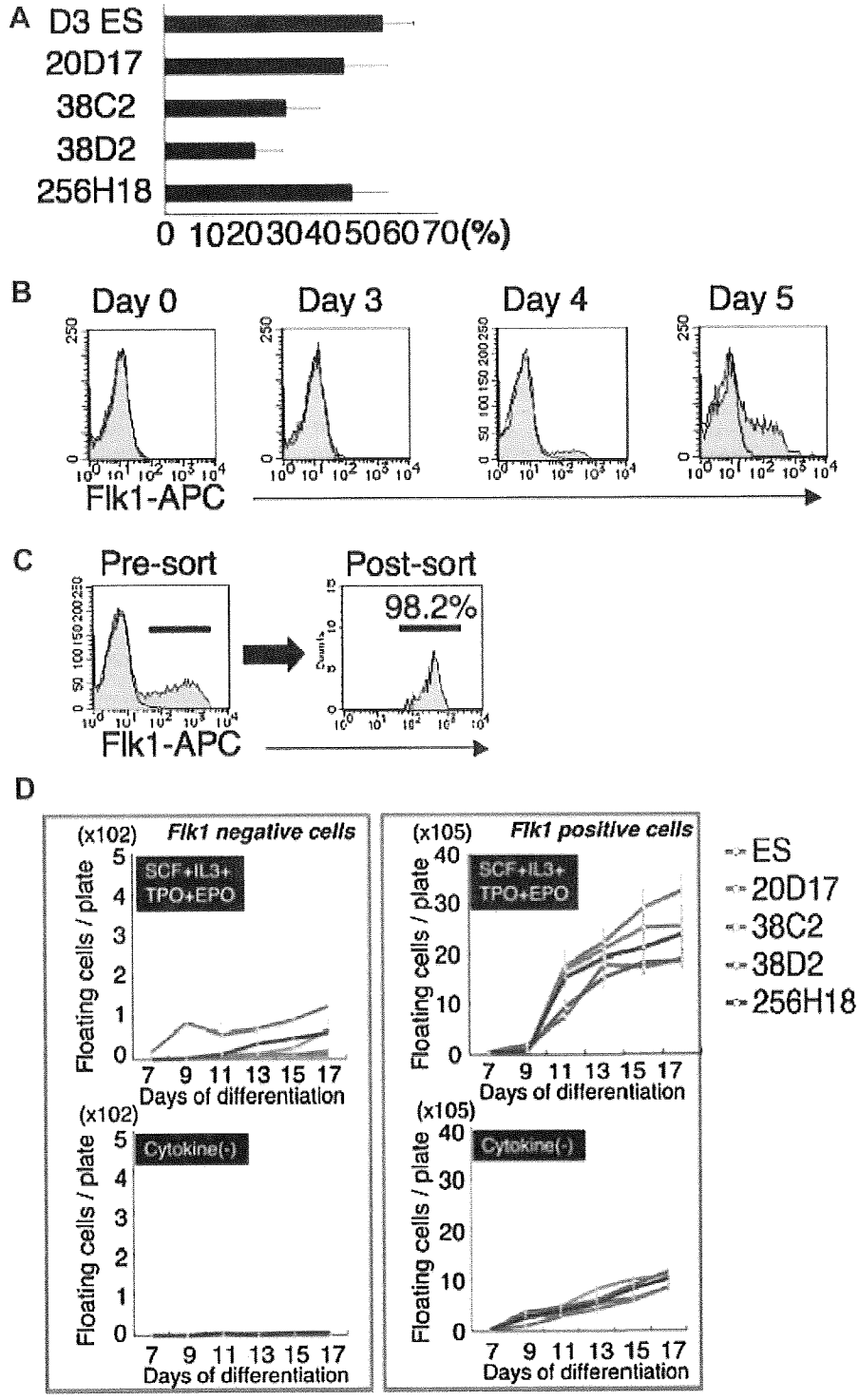


Fig. 3. Efficient production of hematopoietic cells from Flk-1$^+$ populations. A: The amounts of Flk-1$^+$ cells generated from ES and iPS cells at day 5 of differentiation were analyzed by FACS after eliminating OP9 stromal cells as described in Materials and Methods Section. Data are shown as a percentage in the total ES- and iPS-derived viable cells. B: Sequential FACS analysis reveals the emergence of Flk-1$^+$ population after day 4 of differentiation (shaded bars). Open bars show staining with isotype control antibodies. C: Purification of Flk-1$^+$ fractions by FACS on day 5. Reanalysis of the sorted cells confirmed the purity as 93.0–98.2%. D: Sequential analysis of the number of floating cells from ES and iPS cells after sorting with Flk-1 antibody. Sorted Flk-1$^+$ and Flk-1$^-$ cells were cultured in the presence or absence of SCF, IL-3, TPO, and EPO. In (A) and (D), data are presented as mean ± SE of three independent duplicate experiments. In (B) and (C), representative data from clone 38D2 are shown.

the outset, but they appeared on day 4 of culture and increased in number until day 5 (Fig. 3B).

We next sorted the Flk-1$^+$ cells on day 5 and cocultured them with fresh OP9 cells. Reanalysis of the sorted Flk-1$^+$ cells by FACS showed that their purity ranged from 93.0% to 98.2% (Fig. 3C). Regardless of the percentage of Flk1$^+$ cells before sorting, all of the iPS cell lines and ES cells could produce similar yields of hematopoietic cells predominantly from Flk-1$^+$ fractions, and exogenous cytokines increased the hematopoietic efficacy fourfold (Fig. 3D).

Primitive and definitive hematopoietic development of iPS cells

In the developing mouse embryo, primitive hematopoiesis originates in the extra-embryonic yolk sac on day 7.5 of gestation (Moore and Metcalf, 1970). Thereafter, definitive hematopoiesis emerges as a second wave in the aorta-gonad-mesonephros (AGM) region and replaces primitive hematopoiesis (Muller et al., 1994; Medvinsky and Dzierzak, 1996; Matsuoka et al., 2001). Primitive and definitive erythrocytes are morphologically distinguishable, and show distinct patterns of hemoglobin gene expression: the former are larger, nucleated cells that express not only embryonic ε-globin and ζ-globin but also adult α-globin, whereas the latter are smaller, enucleated cells expressing only adult α-globin and β-globin (Doetschman et al., 1985; Leder et al., 1992; Nakano et al., 1996; Xu et al., 2001).

To investigate whether primitive and definitive erythropoiesis can occur in iPS cells, we initially examined floating hematopoietic cells (Fig. 4A). May–Giemsa staining revealed that on day 7 the cells were large and nucleated, resembling primitive erythrocytes, whereas on day 15 they were smaller, enucleating or enucleated, and similar to definitive erythrocytes. Immunostaining revealed that day 7 cells were strongly positive for embryonic hemoglobin but negative for β-major hemoglobin, while day 15 cells expressed β-major hemoglobin strongly, with little or no expression of embryonic hemoglobin.

We also examined globin gene expression in the floating cells by sequential RT-PCR (Fig. 4B). The expressions of ε-globin and ζ-globin were strongest on day 7, decreased thereafter until day 11, and were undetectable on days 13–17. In contrast, α-globin and β-major globin expression were observed from days 9 through 17. These expression patterns were similar in iPS and ES cells, suggesting that iPS cells in vitro, like ES cells, can undergo primitive followed by definitive erythropoiesis.

Hematopoietic stem/progenitor cells develop from iPS-derived Flk-1$^+$ cells

To verify the formation of hematopoietic stem/progenitor cells in our culture system, we initially examined the expressions of c-kit, Sca1, CD34, and CD45, which are expressed by early hematopoietic progenitors (van de Rijn et al., 1989; Motro et al., 1991; Ling and Neben, 1997). FACS analysis revealed that undifferentiated iPS and ES cells expressed c-kit and Sca1, but not CD34 or CD45. Subsequent examination revealed the transient downregulation of Sca1 and c-kit on day 3, with Sca1 expression increasing again on day 5. On day 13 (8 days after cell sorting), we detected a new cell population that was positive for c-kit, Sca1, CD34, and CD45 (Fig. 5A,B).

We next investigated whether clonogenic hematopoietic cells were also produced in our culture system. Methylcellulose colony-forming assays showed that CFU-Mix, BFU-E, CFU-GM, and CFU-G colonies, as described in Materials and Methods Section, developed from the iPS-derived cells (Fig. 5C), and their numbers were not much lower than those formed by ES-derived cells (Fig. 5D). CFU-Mix and BFU-E colonies predominated the Flk-1$^+$ cells on days 7 and 9, but then

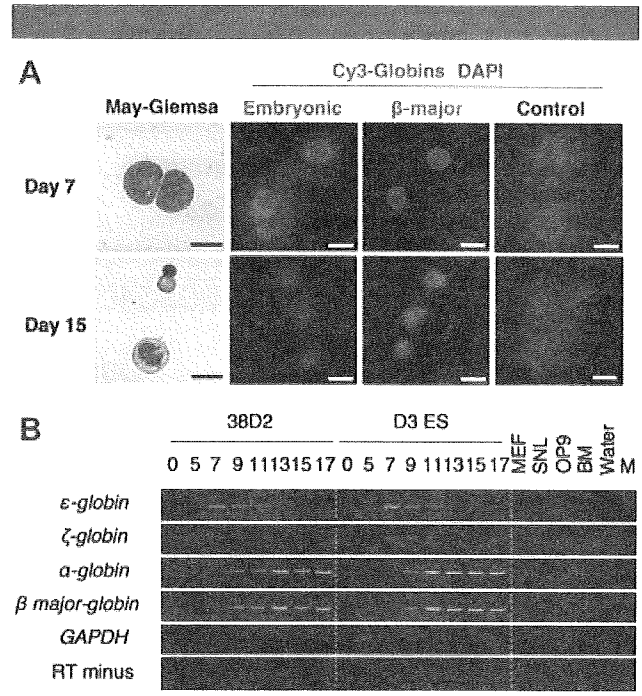


Fig. 4. Primitive and definitive erythrocytes formed from iPS cells. A: iPS-derived floating hematopoietic cells on day 7 (upper row) and day 15 (lower row) are shown. Erythrocytes on day 7 were larger and nucleated, and strongly positive for embryonic hemoglobin, but negative for β-major globin. Red blood cells on day 15 were smaller and enucleating or enucleated, and positive for β-major globin. B: Sequential RT-PCR analysis of globin gene expressions. RNA was isolated from all cells during the initiation culture (days 0 and 5), and from floating cells during the hematopoietic culture (day 7 and thereafter). GAPDH was used as a loading control. MEF, murine embryonic fibroblasts; SNL, SNL feeder cells; OP9, OP9 feeder cells; BM, adult murine bone marrow; M, 200-bp size marker. Representative results are shown from one of three independent experiments performed on clone 38D2.

decreased; the majority of cells after day 11 were from the CFU-GM and CFU-G colonies. These results suggest that iPS cells can generate multipotent hematopoietic progenitors almost as efficiently as ES cells.

Concomitant development of endothelial cells from iPS-derived Flk-1$^+$ cells

We next evaluated the development of endothelial lineages in our system. At 5 days after sorting, sheet-like colonies appeared that took up Dil-acetylated low-density lipoprotein (Dil-Ac-LDL) and were positive for anti-endothelial nitric oxide synthase (eNOS), CD31, and VE-cadherin (Fig. 6A). As shown in Figure 6B, iPS-derived Flk-1$^+$ cells produced many more VE-cadherin$^+$ colonies than Flk-1$^-$ cells ($P < 0.05$).

We also analyzed the expression of the following genes that are associated with the development of hematopoietic and endothelial lineages (Fig. 6C): required for excision 1 (*Rex1*; undifferentiated cells), *Brachyury* (primitive streak and mesoderm), *Flk-1* (mesoderm), GATA-binding protein 2 (*GATA2*; hematopoietic and endothelial), *SCL* (hematopoietic), *Myb* (hematopoietic), *GATA1* (hematopoietic), and tyrosine kinase with Ig-like and endothelial growth factor-like domains 1 (*Tie1*; endothelial). Both ES and iPS cells expressed *Rex1* strongly in the undifferentiated state. *Rex1* expression gradually decreased during differentiation, and *Brachyury*, *Flk-1*, and *SCL* expressions initially appeared on day 3. Quantitative real-time PCR analyses confirmed that *Brachyury* expression increased to

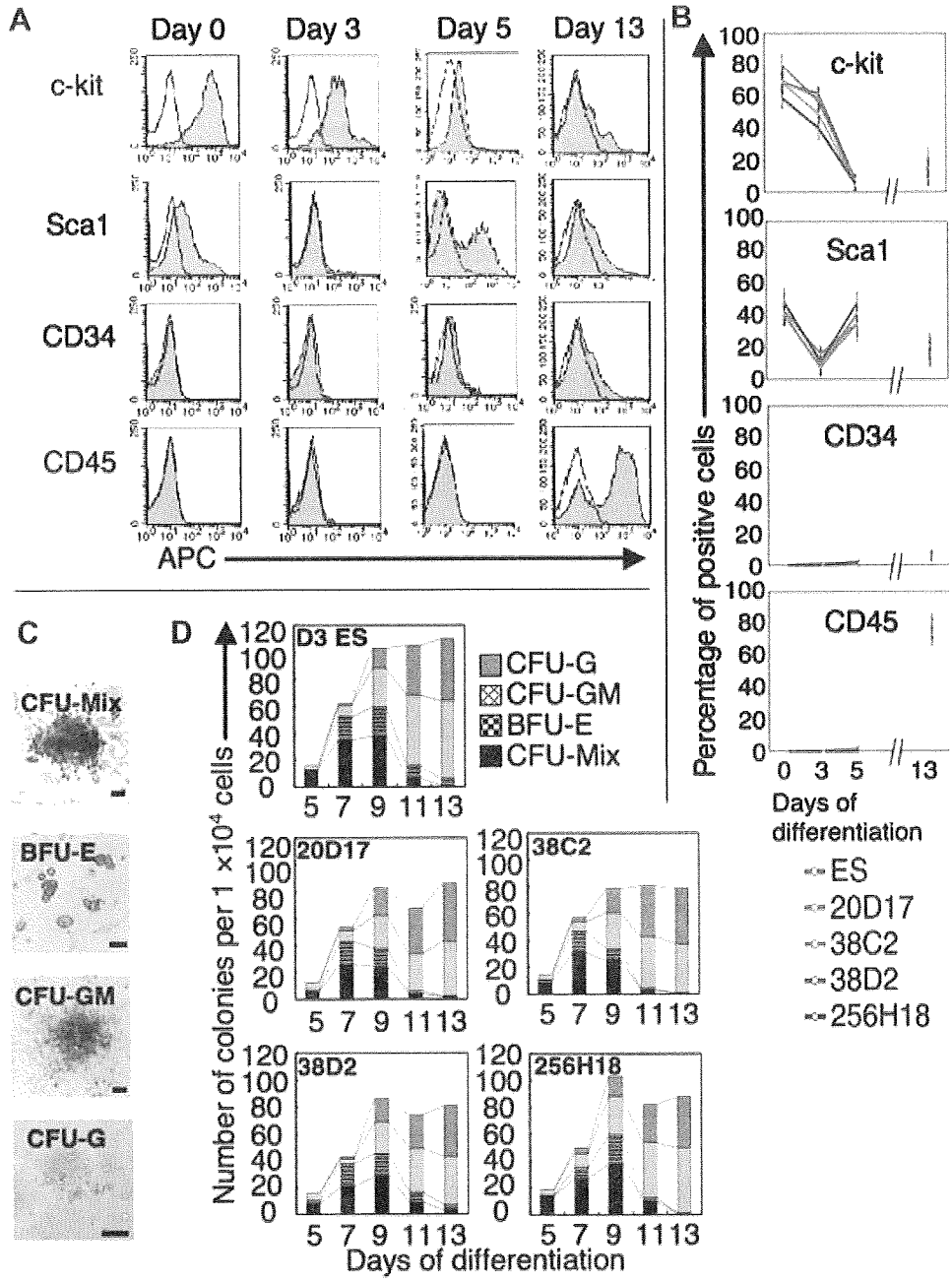


Fig. 5. Hematopoietic stem/progenitor cells emerge from Flk-1$^+$ cells. Sequential FACS analysis of c-kit, Sca1, CD34, and CD45 in ES and iPS-derived cells during differentiation. Whole culture were harvested on indicated days and analyzed by FACS as described in Materials and Methods Section. A: Representative data from clone 38D2 are shown. Histograms show the isotype control staining profile (open bars) versus the specific antibody staining profiles (shaded bars). B: Percentages of each antigen positive cells generated from ES and iPS cells are presented as mean ± SE of three independent duplicate experiments. C: The iPS cells formed various colony types on MTC-containing medium. Data from clone 38D2 are shown as representative. Scale bars, 200 μm. D: Numbers of each colony type derived from ES and iPS cells. Data represent mean of three independent triplicate experiments.

a maximum on day 3, followed by the upregulation of *Flk-1* and *SCL* (Fig. 6D). *Brachyury* expression continued until day 7, whereas that of *Flk-1* and *SCL* could be detected until day 9. *GATA2*, *Myb*, *GATA1*, and *Tie1* expressions were initially detected on day 5, and persisted thereafter. Taken together, these results demonstrate that, in our system, hematopoietic and/or endothelial differentiation of iPS cells occurs in a similar manner to that observed during embryogenesis.

Common hemoangiogenic progenitors are present in iPS-derived Flk-1$^+$ populations

Previous work has demonstrated that common hemoangiogenic progenitors are present in Flk-1$^+$ cells during ES-cell differentiation (Nishikawa et al., 1998). To investigate whether iPS-derived Flk-1$^+$ cells possess the same differentiation potential, we performed a single-cell deposition

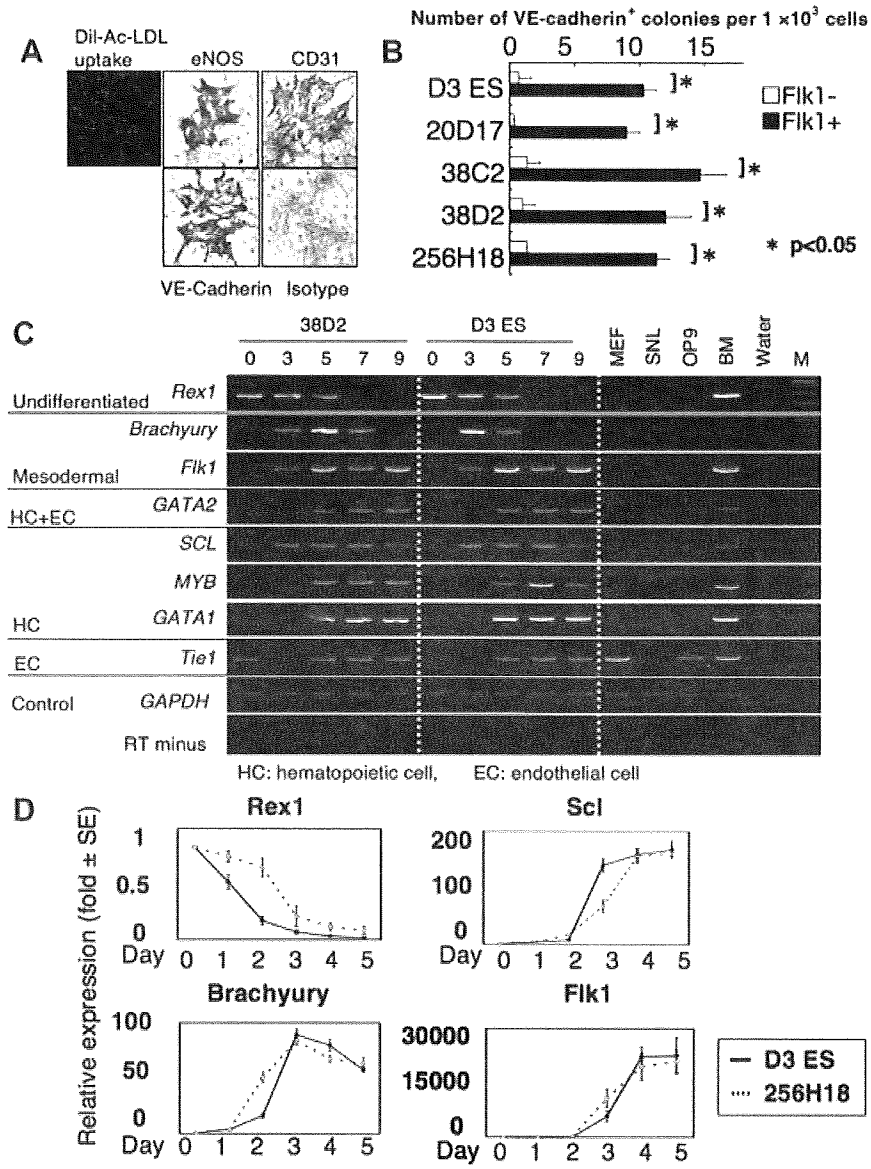


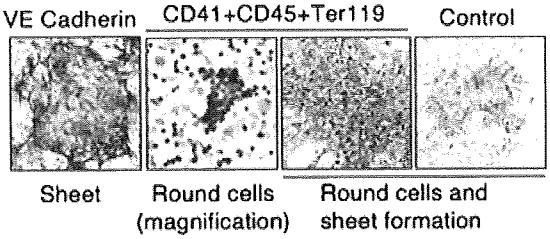
Fig. 6. Concomitant endothelial and hematopoietic development from iPS-derived Flk-1$^+$ cells. A: The sheet-like colonies took up Dil-Ac-LDL and were positive for eNOS, CD31, and VE-cadherin. Data from clone 38D2 are shown as representative. B: Number of VE-cadherin$^+$ colonies per 1 × 10^3 Flk1$^+$ or Flk1$^-$ cells derived from ES and iPS cells. Data are presented as mean ± SD of three independent experiments. C: RT-PCR using mRNA isolated from ES and iPS-derived cells during culture. HC and EC means hematopoietic and endothelial cells, respectively. GAPDH was used as a loading control. M: 200 bp size marker. Representative results from one of three independent experiments performed on clone 38D2 are shown. D: The expressions of *Rex1*, *Brachyury*, *Scl*, and *Flk-1* were evaluated by real-time quantitative RT-PCR. mRNA samples were harvested from D3 ES-derived GFP$^+$ cells or clone 256H18-derived DsRed$^+$ cells sorted by FACS on indicated days. Values were normalized to *gapdh* mRNA, and the control values were arbitrarily set to day 0 (undifferentiated ES cells). Data represent the mean ± SE of three independent duplicate experiments.

assay using the DsRed$^+$ clone 256H18. Single Flk-1$^+$ cells were deposited in four 96-well culture dishes (384 wells) containing OP9 feeder cells. Each well was observed by fluorescence microscopy 24 h after cell deposition, and wells that contained more than one DsRed$^+$ cell were excluded from further analysis. The presence of hematopoietic (Woodard et al., 2000) and endothelial (Maherali et al., 2007) colonies was confirmed not only morphologically, but also by immunostaining with a mixture of anti-CD41, CD45, and Ter119 antibodies, and anti-VE-cadherin antibodies, respectively, as previously reported (Fig. 7A) (Nishikawa et al., 1998). After 5 days of culture, the clonal outgrowth rates were 10.2% and 8.9% from 256H18 iPS and D3 ES cells, respectively. The frequencies of EC development alone, HC development alone, and HC plus EC development, respectively, were 2.7%, 5.2%, and 2.2%, respectively, from iPS cells and 2.4%, 3.5%, and 2.9%, respectively, from ES cells (Fig. 7B). Thus, the potential for mono- or bipotential progenitor development from iPS cells was almost equivalent to that from ES cells.

Discussion

Induced PS cells may serve as a novel cell source in both research and the clinic because, like ES cells, they have an

A



B

Clones	Only one cell 24 hours after sorting	After 5 days		
		HC	EC	HC+EC
DsRed iPS cells (256H18)	361 wells	10 wells (2.7%)	19 wells (5.2%)	8 wells (2.2%)
D3 ES cells	369 wells	9 wells (2.4%)	13 wells (3.5%)	11 wells (2.9%)

HC: Hematopoietic cells
EC: Endothelial cells

Fig. 7. Common hemoangiogenic progenitors are present in Flk-1$^+$ cells during iPS-cell differentiation. A: Single Flk-1$^+$ cells were cultured on OP9 layers in the wells of 96-well plates and immunostained with anti-VE-cadherin antibodies, or a cocktail of anti-CD41, anti-CD45, and anti-Ter119 antibodies, 7 days after sorting. B: Number of wells that showed EC (endothelial cells), HC (hematopoietic cells), and EC + HC development were counted as described in text.

unlimited capacity for self-renewal (Takahashi and Yamanaka, 2006; Meissner et al., 2007; Okita et al., 2007; Park et al., 2007; Takahashi et al., 2007; Yu et al., 2007; Aoi et al., 2008; Hanna et al., 2008; Nakagawa et al., 2008). The use of customized pluripotent stem cells would avoid the controversies surrounding ES cells. A recent study demonstrated that, after additional genetic manipulation and hematopoietic stem/progenitor cell expansion, autologous iPS cells could be used to treat mice with sickle-cell anemia, clearly revealing the advantage of these cells in regenerative medicine (Hanna et al., 2007).

One significant advantage of iPS cells is that cells from each patient can be used to screen drugs or to examine the effects of novel procedures against various diseases. Many diseases have a complex genetic etiology that affects the development, differentiation, and maturation of different tissues and organs, and require an experimental model system to faithfully reproduce their altered developmental processes (Lensch and Daley, 2006). In light of the heterogeneity of disease phenotypes and drug toxicity, it is desirable to establish defined sources of cells for drug discovery and research. In this area, immortalized cell lines and tissue-specific stem/progenitor cells that have been used for such studies are now being replaced by pluripotent stem cells.

The present study demonstrates that murine iPS cells can recapitulate early hematopoietic development in vitro. We confirmed the step-wise development of primitive and definitive hematopoietic cells, as well as endothelial cells, from Flk-1$^+$ hemoangiogenic progenitors, together with the upregulation of genes related to both lineages. Both lineages could be generated from individual Flk-1$^+$ cells, strongly suggesting the existence of common progenitor "hemangioblasts," as was previously reported for ES cells and embryos (Flamme et al., 1995; Risau, 1995; Risau and Flamme, 1995; Choi et al., 1998; Huber et al., 2004).

Step-wise development of primitive and definitive hematopoiesis from iPS-derived intermediate mesodermal progenitors

During embryogenesis, primitive hematopoiesis emerges in the yolk sac on 7.5 d.p.c. Following this process, definitive hematopoiesis, which is the major hematopoietic process throughout life, originates on 8.5 d.p.c. in the AGM region (Muller et al., 1994; Medvinsky and Dzierzak, 1996; Matsuoka et al., 2001). When the site of hematopoiesis shifts to the fetal liver on 10.5 d.p.c. and finally to the bone marrow, the number of blood cells massively increases; erythroid cell lineages are the major products in the fetal liver, and myeloid lineages appear at later stages. Here we demonstrated that Flk-1$^+$ mesodermal cells derived from iPS cells can lead to both primitive and definitive hematopoiesis.

Interestingly, the time courses of the hematopoietic differentiation of iPS and ES cell lines in our experiments were almost precisely synchronized with those seen in embryonic development. Hematopoietic colonies on the OP9 layer were first observed on day 7 of differentiation, and the number of cells produced increased explosively from day 11. Immunostaining and RT-PCR indicated a shift from primitive to definitive hematopoiesis as differentiation progressed over time. Moreover, the results of the MTC colony-forming assay also suggested that hematopoietic differentiation in our system reflects that occurring in embryogenesis: CFU-Mix and BFU-E colonies were mainly observed until day 9, while CFU-GM and CFU-G colonies became dominant on day 11 and thereafter.

Identifying and inducing hematopoietic stem cells (HSCs) in vitro is of great biological interest. Previous studies have suggested that the cobblestone area forming cells (CAFCs) observed in the OP9 system are indicative of the existence of primitive hematopoietic progenitors (Suwabe et al., 1998); CD34, c-kit, and Sca1 are among the characteristic markers of HSCs or very immature progenitors. In our study, we observed CAFCs derived from iPS cells, and FACS analyses revealed that many of the iPS-derived hematopoietic cells expressed the progenitor markers mentioned above. Taken together, these findings suggest that iPS cells can produce very immature hematopoietic progenitors in vitro. In the future, further study will be necessary to investigate whether iPS cells can generate true HSCs that demonstrate long-term multilineage marrow reconstitution in lethally irradiated mice without any additional gene manipulation.

Concomitant differentiation of iPS cells into hematopoietic and endothelial lineages

Several in vivo and in vitro studies have demonstrated a close association between hematopoietic and endothelial differentiation. Previous experiments have shown that murine and primate ES cells differentiate into hematopoietic cells via common Flk1$^+$ hemoangiogenic progenitors (Nishikawa et al., 1998; Umeda et al., 2006). Using RT-PCR and the single-cell deposition assay, our study demonstrated that iPS cells, like ES cells, can generate hematopietic and endothelial cells concomitantly, as is observed in embryogenesis.

The RT-PCR data demonstrated that the expression of the early mesodermal marker *Brachyury* was followed by that of *flk-1* and *scl*, both of which are crucial for the development of common progenitors (Nishikawa et al., 1998; Chung et al., 2002). The expression of genes associated with both lineages began thereafter. These results suggest that the orchestrated process from mesoderm development to the specification of either lineage during embryogenesis is also recapitulated in the iPS-cell model.

The single-deposition assay demonstrated that iPS cells possess an equivalent capacity to ES cells to develop bipotent progenitors at the single cell level. However, the frequency of progenitor development was unexpectedly low. One possible reason for this is the low clonal growth rate in our single-cell culture condition. Another possibility is that the sorted Flk-1$^+$ cells included, besides hemangioblasts, progenitors that contribute to other mesodermal lineages. Recent studies on murine ES and iPS cells demonstrated the development of cardiac muscles, vascular smooth muscles, and pericytes from Flk-1$^+$ fractions (Yamashita et al., 2000; Iida et al., 2005; Baba et al., 2007b; Narazaki et al., 2008). We also observed the formation of contractile colonies from Flk-1$^+$ fractions (data not shown). This may be one alternative reason for the observed low frequency of differentiation of either lineage. Further studies will enhance our understanding of the developmental biology of iPS cells.

Hematopoietic potential of iPS-derived Flk-1$^+$ progenitors is equivalent, regardless of the clone

In our experiments, the efficacy of Flk-1$^+$ cell induction varied between the clones, although the timing of their differentiation was the same. This may be potentially due to contamination by the SNL feeder cells. As these feeder cells were not eliminated at the start of differentiation, they would have remained throughout the assay and might have inhibited differentiation. However, to address these problems, it will be necessary to study the biological characteristics of iPS cells further, including their epigenetic behavior during differentiation. The most interesting and encouraging finding in our study was that the sorted Flk-1$^+$ cells derived from all analyzed iPS and ES clones were similar in their ability to generate hematopoietic cells.

In conclusion, our results demonstrate that iPS cells can develop into hematopoietic cells in vitro via hemoangiogenic progenitors, the so-called "hemangioblasts." Furthermore, iPS cells traverse the primitive and definitive hematopoietic stages in a manner similar to that observed during embryogenesis. Although future investigations at the biological and molecular levels are highly desirable, our study suggests that iPS cells hold great promise in medicine, and may aid in attaining the long sought goal of patient-specific stem cells.

Acknowledgments

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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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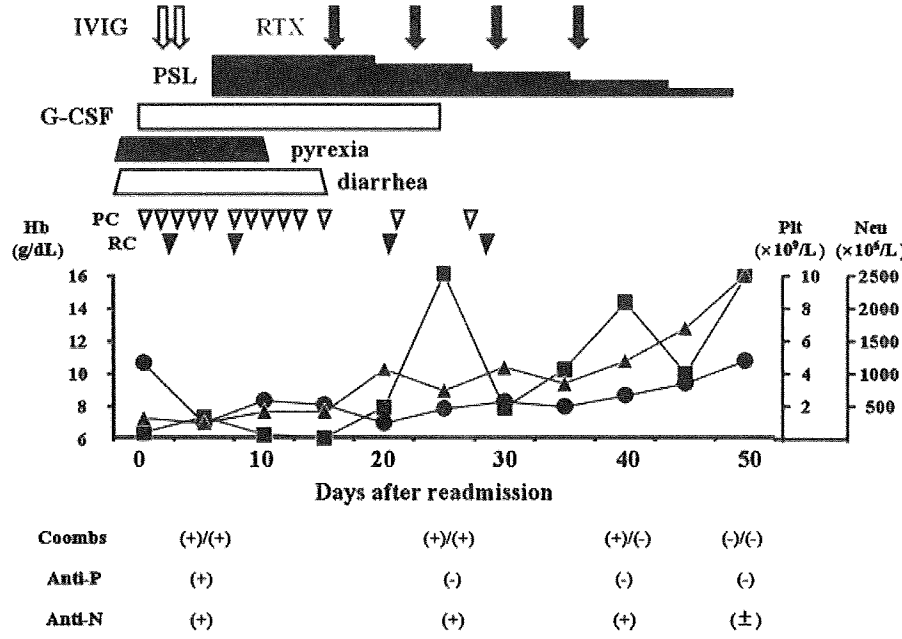

Fig. 1. The clinical course after readmission. IVIG, intravenous immunoglobulin; RTX, Rituximab; PSL, prednisolone; G-CSF, granulocyte colony-stimulating factor; RC, packed red blood cell concentrate; PC, packed platelet concentrate; Plt, platelet counts; Neu, neutrophil counts; Coombs, direct/indirect 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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PEDIATRIC ALLERGY AND IMMUNOLOGY

Allergic status of schoolchildren with food allergy to eggs, milk or wheat in infancy

Kusunoki T, Morimoto T, Nishikomori R, Heike T, Fujii T, Nakahata T. Allergic status of schoolchildren with food allergy to eggs, milk or wheat in infancy.
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Although children allergic to eggs, milk or wheat in infancy tend to become tolerant by school age, the allergic status of these children at school age has not been well evaluated. To investigate the allergic status of schoolchildren who avoided eggs, milk or wheat because of an immediate-type allergic reaction at < 1-yr-old (food avoiders in infancy), we conducted a large-scale questionnaire-based survey of schoolchildren. A questionnaire on allergic diseases was distributed to the parents of 14,669 schoolchildren aged 7 to 15 yr in 30 schools in Kyoto, Japan. Of these, 13,215 responded (response rate, 90.1%). The rate of 7-yr-old children who were food avoiders in infancy was 5.4%. This rate decreased as the current age of the children increased, down to 3% in 15-yr-old children, indicating that food allergy in infancy tended to become more prevalent over the past 8 yr. Although more than 80% became tolerant to these foods by school age, the prevalence of bronchial asthma, atopic dermatitis, allergic rhinitis and allergic conjunctivitis were significantly higher in this group. Moreover, avoidance of other foods (buckwheat, shellfish, fruits and others) at school age was seen at much higher frequencies than in non-food avoiders in infancy (adjusted odds ratio, 7.7; confidence interval, 5.9–10.2). This risk did not differ significantly between those who did and did not develop tolerance to eggs, milk and wheat by 3 yr old. In conclusion, food avoiders in infancy appear to have a higher risk of not only other allergic diseases ('atopic march') but also allergy to other foods ('food allergen march') at school age, indicating the need for continuous attention to food allergy.

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Key words: allergy; atopic march; bronchial asthma; atopic dermatitis; allergic rhinitis; allergic conjunctivitis; epidemiology; food allergy; food avoidance; schoolchildren

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An increasing prevalence and concern about food allergy, especially among children, has been reported in developed countries (1–4). In the United States, food allergy usually starts in infancy as an immediate-type allergy to eggs, milk, peanuts or soy (5). In Japan as well, three major foods (eggs, milk and wheat) have been shown to be the most frequent allergens for immediate-type allergic reactions in infancy (6). Although children allergic to these food allergens in infancy have a tendency to become tolerant by school age (7), patients allergic to other foods, such as peanuts, tree nuts, fish, shellfish and especially buckwheat in Japan (8), are much more likely to maintain their clinical reactivity during or even after school age.

Atopic march usually refers to the tendency of a child with eczema in infancy to develop asthma and allergic rhinitis as he or she becomes older (9, 10). Recent epidemiological studies have shown that the same tendency exists for children with food allergy in infancy. A study of 1749 infants has shown that those with IgE-mediated milk allergy had a significantly increased risk for persistent milk allergy, development of other food allergies, asthma and rhinoconjunctivitis

(11). Also, another study has shown that in 118 children with milk allergy, the children with IgE-positive milk allergy were more likely to have other allergic diseases and sensitization to any allergen by school age (12). Similar increases in respiratory allergic symptoms and aero-allergen sensitization have been shown in infants with egg allergy (13). However, there so far has been a paucity of data regarding risk of other food allergies in school-age children with food allergy in infancy. Thus, it would be clinically important to see whether those who had been allergic to eggs, milk or wheat in infancy had a greater risk for allergy to other food antigens as they grow older, even if they outgrow their initial food allergy.

For that purpose, we investigated the allergic status of schoolchildren who avoided eggs, milk or wheat because of immediate-type allergic reactions at < 1-yr-old (food avoiders in infancy), through analysis of a questionnaire-based survey of more than 13,000 schoolchildren.

Subjects and methods

Epidemiological studies on the prevalence of allergic diseases in schoolchildren

In June 2006, a questionnaire dealing with allergic diseases was distributed through teachers to the parents of all 14,669 children aged 7 to 15 yr attending 30 randomly selected schools in Kyoto, Japan. Informed consent was obtained from the parents who responded to the questionnaires. We collected the questionnaires through the schools. This study was designated as the Allergic Schoolchildren in Kyoto (ASK) study and was approved by the Ethics Committee of Kyoto University Graduate School of Medicine.

Definition of 'food avoiders in infancy' and other allergic diseases

With respect to food allergy, we asked the following questions: (1) Does your child ever have allergic symptoms, such as skin symptoms like hives or respiratory symptoms like cough/wheeze, within 1 to 2 h after ingesting a particular food? (2) Does your child avoid particular foods due to these symptoms? (3) If so, what are the kinds of foods and the duration of avoidance? Those who answered 'yes' to both questions 1 and 2 were regarded as having either a past history or present illness of immediate-type food allergy, and the kinds of foods avoided were tabulated. Among them, those who avoided any of the three major food allergens (eggs, milk or wheat) from < 1 yr of age were defined as 'food avoiders in infancy'. These three foods were chosen because, among 589 subjects who avoided any foods from < 1 yr of age, 551 subjects (93.5%) avoided either eggs (n = 439), milk (n = 202) or wheat (n = 114), indicating that these are exclusively important food allergens during infancy in Japan, as shown previously (6). The food avoiders in infancy were further divided into two subgroups, depending on whether they developed early tolerance (avoidance could be terminated for all three foods by 3 yr old). The questionnaire on the prevalence of four other allergic diseases [bronchial asthma (BA), atopic dermatitis (AD), allergic rhinitis (AR) and allergic conjunctivitis (AC)] was based on and comparable to the one used by the International Collaborative Study of Asthma and Allergies in Childhood (ISAAC) (14) and was prepared and validated by the Study Group of Epidemiology of Allergic Diseases founded by the Japanese Ministry of Public Health and Welfare in 1993 (15). Definitions of these allergic diseases based on the questionnaire are described elsewhere (16).

Statistical analysis

Following the descriptive statistics, we developed univariate and multivariate logistic regression models to evaluate the effects of early food avoidance on present food avoidance and other allergic diseases. The dependent variables included bronchial asthma, atopic dermatitis, allergic rhinitis and allergic conjunctivitis, and the independent variables included age, gender and birth order. p-values < 0.05 were considered statistically significant. All statistical analyses were carried out using SAS software (Version 9.1; SAS Institute Inc., Cary, NC, USA).

Results

A total of 13,215 questionnaires were collected (response rate, 90.1%). The rate of food avoiders in infancy was 5.4% in 7-yr-old children. This rate decreased as the current age of the children increased, down to 3% in 15-yr-old children (Fig. 1). The overall rate of food avoiders in infancy was 4.2%. Sex ratio (male/female) and age distribution (y, mean ± standard deviation) of food avoiders in infancy vs. non-food avoiders in infancy were 1.35 vs. 1.02 ($p = 0.02$) and 9.3 ± 2.4 vs. 9.9 ± 2.5 ($p < 0.0001$), respectively, indicating that there were significantly

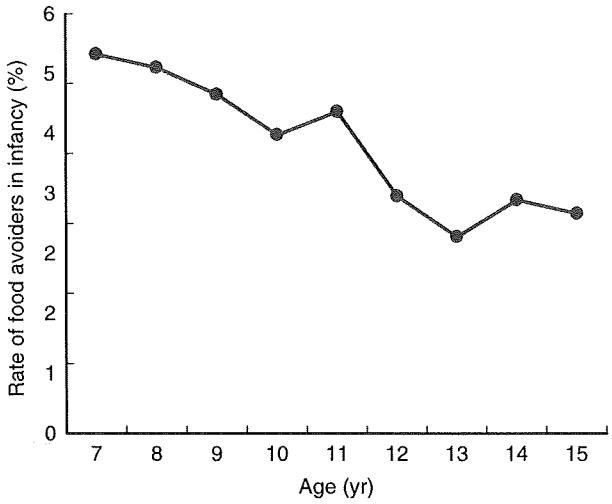

Fig. 1. Rate of food avoiders in infancy according to present age.

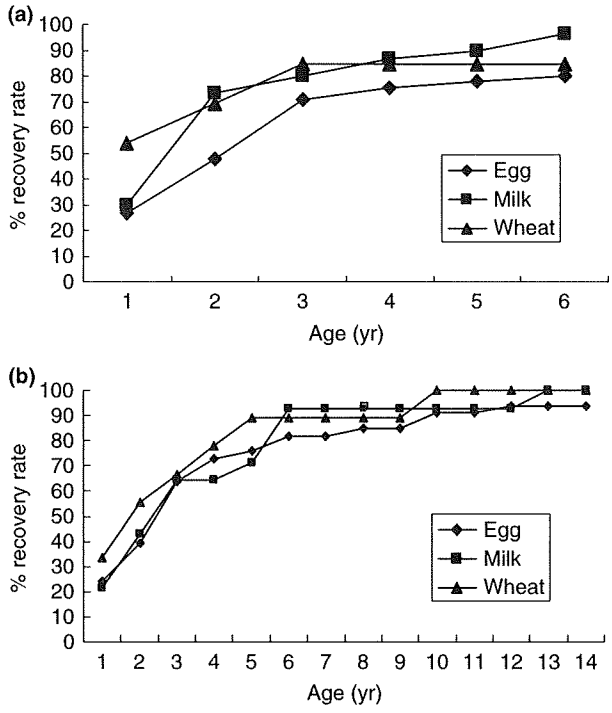

Fig. 2. Recovery from egg, milk or wheat avoidance according to age. Data with food avoiders in infancy now at (a) 7 yr olds and (b) 15 yr olds are shown.

more male and younger children among food avoiders in infancy. Analysis of the age at which the avoidance was terminated showed that more than 80% became tolerant to these foods by school age (Fig. 2). However, prevalence of BA, AD, AR and AC were significantly higher in food avoiders in infancy (Table 1) at school age, both by univariate and multivariate analysis. In these analyses, the adjusted odds ratio was highest in AD (3.18), followed by BA (2.68), AC (1.76) and AR (1.57).

Moreover, present avoidance of other foods was seen at much higher frequencies in food avoiders in infancy compared to non-food avoiders in infancy (adjusted odds ratio, 7.7; confidence interval, 5.9–10.2) at school age (Table 2). Foods other than eggs, milk and wheat were avoided in 17.0% of food avoiders in infancy, whereas only 1.9% of non-food avoiders in infancy refrained from eating these foods. These risks did not differ significantly in those with or without early tolerance (Table 3).

The frequencies of the kinds of other foods that were avoided are shown in Fig. 3. The most frequently avoided food was buckwheat, followed by shellfish and fruit in both groups. Compared to children in the United States, relatively fewer children avoided peanuts.

There was a striking difference in the age distribution at which the present avoidance of other foods was started. While 54% started avoiding those foods at the age of 4 yr or more in non-food avoiders in infancy, 52% started at < 1 yr old in food avoiders in infancy (Fig. 4).

Discussion

The rate of food avoiders in infancy decreased as the current age of the children increased, confirming a rising trend of food allergy in infancy over the past 8 yr. More than 80% of this population outgrew allergies to eggs, milk and wheat by school age, which is in accordance with most of the previous studies, but contrary to the

Table 1. Prevalence of allergic diseases in schoolchildren with or without food avoidance in infancy

	Food avoiders in infancy (n = 556)	Non-food avoiders in infancy (n = 12,659)	p-value (univariate)	p-value (multivariate)*	Adjusted OR	95% CI
BA	95 (17.1%)	569 (4.5%)	<0.0001	<0.0001	2.68	2.08–3.46
AD	106 (19.1%)	629 (5.0%)	<0.0001	<0.0001	3.18	2.50–4.04
AR	263 (47.3%)	3358 (26.5%)	<0.0001	<0.0001	1.57	1.29–1.91
AC	245 (44.1%)	3079 (24.3%)	<0.0001	<0.0001	1.76	1.45–2.15

OR, odds ratio; CI, confidence interval; BA, bronchial asthma; AD, atopic dermatitis; AR, allergic rhinitis; AC, allergic conjunctivitis.
*Adjusted for age, gender, birth order and other allergic diseases.

Table 2. Present food avoidance (other than eggs, milk or wheat) in schoolchildren with or without food avoidance in infancy

	Food avoiders in infancy (n = 556)	Non-food avoiders in infancy (n = 12,659)	p-value (univariate)	p-value (multivariate)*	Adjusted OR	95% CI
Present food avoidance (other than eggs, milk or wheat)	94 (17.0%)	243 (1.9%)	<0.0001	<0.0001	7.72	5.87–10.16

OR, odds ratio; CI, confidence interval.
*Adjusted for age, gender, birth order and other allergic diseases.

Table 3. Present food avoidance (other than eggs, milk or wheat) in food avoiders in infancy with or without early tolerance

	Early tolerance*		p-value		
	Yes (n = 362)	No (n = 194)	(univariate)	OR	95% CI
Present food avoidance (other than eggs, milk or wheat)	64 (17.7%)	30 (15.5%)	0.51	0.85	0.53–1.37

OR, odds ratio; CI, confidence interval.
*Early tolerance means that avoidance could be terminated for all three foods by 3 yr old.

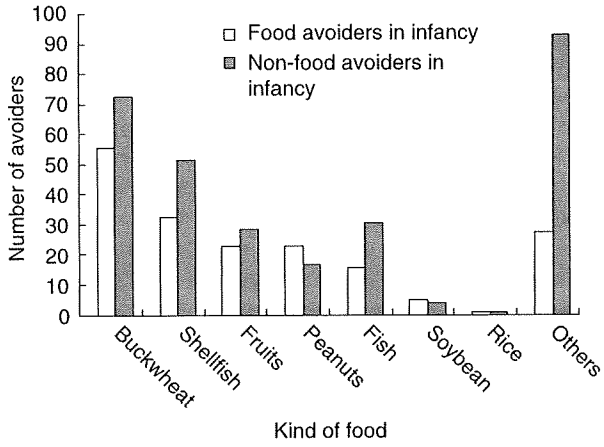

Fig. 3. Distribution of foods, other than eggs, milk or wheat, being avoided at present.

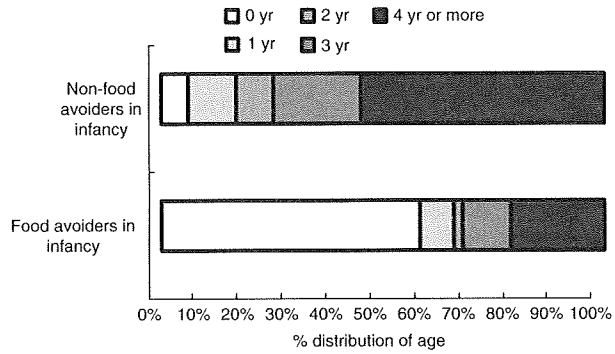

Fig. 4. Distribution of starting age for food avoidance other than eggs, milk or wheat.

recent reports by Skripak et al. (17) who showed that rates of resolution of milk allergy were only 42% and 64% by age 8 and 12 yr respectively. As their study design is a retrospective review of the clinical records of two tertiary care centres, possible population bias toward more severe cases might explain the different results. As expected from the recent literature (11–13), food avoiders in infancy appear to have a higher risk of other allergic diseases at school age. AD was most strongly linked to food avoidance in infancy, supporting the proposed relationship between food allergy and AD (18). Our data also showed that food avoiders in infancy avoid other foods at much higher frequency at school age, suggesting the existence of not only 'atopic march' but also 'food allergen march'. Moreover, the risk of present food avoidance did not differ significantly between those with and without early food tolerance, indicating that food avoiders in infancy are at risk of having food allergy from other causes at school age, whether they could achieve tolerance to eggs, milk and wheat at earlier ages (< 3 yr old) or not.

Differences in ages at which the present food avoidance started suggest that food avoiders in infancy develop symptoms of other food allergies at a much lower age. There might be some genetic predisposition to allergies to various kinds of foods in these individuals. In this respect, we previously reported that *SPINK5* polymorphism, known to cause skin barrier dysfunction, was associated not only with AD but also with food allergy (19), suggesting the existence of genetic barrier dysfunction of not only skin but also intestinal epithelium.

An alternative explanation to the association between early and present food avoidance might be that parents of food avoiders in infancy tend to avoid other foods with only subtle, 'possibly

allergic' symptoms at a much lower age because of concerns about food allergy. Actually, a population-based study of 798 6-yr-old children in the United Kingdom revealed that the rates of perception of food hypersensitivity are higher than the prevalence of sensitization to main food allergens and the prevalence of food hypersensitivity based on food challenges (4). The possible overconcern might be associated with the notion that the foods avoided at present, such as buckwheat, shellfish and fish, cause more severe anaphylactic reactions (20). Thus, appropriate medical assessment, such as measurement of allergen-specific IgE and food challenge tests, should be performed and unnecessary food avoidance, if any, be discontinued, because food avoidance has been shown to negatively affect health-related quality of life in children (21). Also, food avoidance may cause growth disturbance (22) and psychological burden (23) in children.

One limitation of the study is that the validity of food avoidance in infancy cannot be confirmed by physician diagnosis or any laboratory data because it is a large population-based questionnaire survey. However, parents were asked about the existence of food-induced allergic reactions, and those avoiding foods without any food-specific immediate-type reactions were excluded from the analysis, making the data more reliable than simply including all those who avoided foods in the analysis. In support of this, the rate of food allergy prevalence of 3% to 5% in infancy in our data is comparable with other recently reported prevalence rates (1, 24). Another limitation is possible recall bias, in which parents of food avoiders in infancy might remember more about their children's allergic diseases, although we do not think it is a significant concern because we defined the presence of each disease based on the existence of current symptoms deliberately described in the questionnaire (16), which is unlikely to be affected by the parent's memory.

In conclusion, our data clearly show the significant link between food allergy to eggs, milk or wheat in infancy and that to other foods at school age and calls for continuous attention to food avoiders in infancy up to school age, with respect not only to other allergic diseases but also to other food allergies.

Acknowledgments

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ORIGINAL ARTICLE

Changing Prevalence and Severity of Childhood Allergic Diseases in Kyoto, Japan, from 1996 to 2006

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ABSTRACT

Background: Published data regarding changes in the prevalence of childhood allergic diseases in Japan have been limited.

Methods: To observe changes in the recent trends of the childhood allergy epidemic in Japan, a population-based questionnaire survey of allergic diseases was conducted among 13,215 schoolchildren, aged 7 to 15 years, in Kyoto, Japan in 2006. The results were compared with those obtained in the 1996 survey using the same scale and methods in the same region.

Results: The prevalences of bronchial asthma (BA), atopic dermatitis (AD), allergic rhinitis (AR), and allergic conjunctivitis (AC) in 1996 and 2006 were 5.1% and 5.0% ($p = 0.58$), 4.2% and 5.6% ($p < 0.0001$), 20.3% and 27.4% ($p < 0.0001$), and 13.3% and 25.2% ($p < 0.0001$), respectively. Although the distribution of BA severity improved, the severity distribution of AD, AR, and AC all deteriorated. The lifetime prevalence (present prevalence and past history combined) of BA increased from 6.5% to 7.6% ($p < 0.0001$). The sex ratio analysis showed that the female predominance in the prevalence of AD observed in 1996 disappeared in 2006, indicating a particular rise in AD prevalence among boys.

Conclusions: Overall, the results indicate that the rising trend of allergic diseases, especially in AD, AR, and AC, continues among schoolchildren living in Kyoto, Japan. Special attention should be paid to skin and naso-ocular symptoms.

KEY WORDS

allergic disease, epidemiology, prevalence, questionnaire, schoolchildren

INTRODUCTION

The prevalence of childhood allergic diseases has increased over the last few decades and has become a significant social and public health problem, especially in industrialized countries.[1] However, recent continuing trends which show an increased prevalence might be misinterpreted due to changes in diagnostic labeling, heightened awareness of the problem, and the presence of selection or information bias in previous studies.[2] Thus, in order to accurately evaluate the recent trends in the childhood allergy epidemic, it is crucial to repeatedly compare sequential data using identical, simple, validated questionnaires involving children of the same age and region sampled in the same way.[3] Phase III of the International Study of Asthma and Allergies in Childhood (ISAAC) was performed for this purpose between 1999 and 2004 (mostly 2002-03),[4] and there are abundant data comparing the results with those of the Phase I ISAAC study between 1992 and 1998 (mostly 1994-95). The data included mixed results, with some studies showing an increased prevalence,[5-7] while others showed trends that plateaued or decreased.[1,8-10] There was also a variation in the trend for prevalence depending on the kind of disease, as well as geographical differences.[2,11-16]

In Japan, there have been very few published data regarding the prevalence of childhood allergic diseases over time, with one set of data showing an in-

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creased prevalence of BA, allergic rhinitis (AR), and allergic conjunctivitis (AC) and a decreased prevalence of atopic dermatitis (AD) from 1982 to 2002,[17] while more recent data showed a decreased prevalence of AD and no significant changes in prevalence of BA, AR, and AC from 1996 to 2006.[18] ATS-DLD questionnaires were used to obtain the data, and neither severity nor past disease history were analyzed. In 1996, we conducted a large-scale population-based survey of allergic diseases among more than 50,000 schoolchildren between the ages of 7 to 15 years in Kyoto and its suburban areas[19] of which 16,176 children lived in Kyoto City, one of the largest cities in Japan with a typical urban lifestyle. The questionnaires were based on and comparable with the questionnaire used in ISAAC,[20] and not only prevalence but also the past history and severity of the diseases were evaluated. In 2006, to observe changes in the recent trends of the childhood allergy epidemic, a survey using the identical scale and questionnaire was repeated in the same region in 2006. The results of the 2 surveys were compared in order to clarify any changes in the prevalence, past history, or severity of allergic diseases between 1996 and 2006.

METHODS

EPIDEMIOLOGICAL STUDIES ON THE PREVALENCE OF ALLERGIC DISEASES IN SCHOOLCHILDREN

A questionnaire survey dealing with the prevalence of 4 allergic diseases (BA, AD, AR, and AC) was administered to the parents of schoolchildren aged 7 to 15 years. The questionnaire was based on and comparable with that used by the ISAAC[20] and was prepared and validated by the Study Group of Epidemiology of Allergic Diseases founded by the Japanese Ministry of Health and Welfare in 1993.[21] This study was designated as the Allergic Schoolchildren in Kyoto (ASK) study, and was approved by the Ethics Committee of Kyoto University Graduate School of Medicine.

Details of the questionnaire, in addition to the definitions and severity of the diseases have been previously published.[22] In brief, BA was defined as repeated episodes of wheezing with dyspnea during the preceding 2 years. AD was defined as chronic eczema having more than 3 typical symptoms of AD. AR was defined as the presence of at least 3 of the 4 chronic nasal symptoms: sneezing, rhinorrhea, scratching around the nose, and nasal obstruction. AC was defined as the presence of bilateral itchy-watery eyes. BA severity was defined as mild, moderate, or severe according to the number of absences from school due to asthmatic symptoms, i.e., 3 days or less, 4 to 6 days, or more than 7 days, respectively. AD severity was defined as mild, moderate, or severe, according to the sum of the symptom scores.[22] AR severity was defined as mild, moderate, or severe when nasal symptoms were experienced sometimes, often, or so often as to disturb quality of life, respectively. AC severity was defined as mild, moderate, or severe, when itchy eyes were experienced sometimes, always, or always in combination with sleep disturbances due to symptoms, respectively.

A past history of each disease was defined as the previous presence of each symptom but not during the preceding 2 years. Suspected Japanese cedar pollinosis (sJCP) was defined as having both AR and AC with aggravated symptoms during the spring season in which Japanese cedar pollinosis is prevalent.

In June 1996 and 2006, schoolteachers distributed the questionnaire to parents of schoolchildren aged 7 to 15 years of the same 30 randomly-selected schools in Kyoto City, which were filled out and returned to the school. A total of 16,176/17,906 questionnaires were collected (response rate, 90.3%) in 1996 and 13,215/14,669 questionnaires (response rate, 90.1%) in 2006. There was no significant difference in the age distribution, sex ratio or birth order distribution, among children in 1996 and 2006 (unpublished data).

STATISTICAL ANALYSIS

Univariate and multivariate logistic regression analysis were performed to determine the differences between data from 1996 and 2006, using SAS software (Version 9.1; SAS Institute Inc. Cary, NC, USA). *P* values less than 0.05 were considered to indicate a statistically significant difference.

RESULTS

CHANGE IN THE PREVALENCE AND SEVERITY OF BA

No significant changes were found in the current prevalence of BA, from 5.1% to 5.0% ($p = 0.58$) (Table 1A). Among those with current BA, the prevalence of severe cases was 12.4% to 10.2% ($p = 0.21$) and showed no significant change, while that of mild cases increased from 73.7% to 81.5% ($p < 0.0001$) (Table 2). Meanwhile, the prevalence of a past history of BA increased in 2006, resulting in a statistically significant increase in lifetime prevalence (current prevalence and past history combined) from 6.5% to 7.6% ($p < 0.0001$) (Table 1B).

CHANGE IN THE PREVALENCE AND SEVERITY OF AD

The current prevalence of AD increased from 4.2% to 5.6% ($p < 0.0001$) (Table 1A). Among those with current AD, a significant increase was found in severe cases, from 38.2 to 44.5% ($p = 0.02$), while mild cases decreased from 25.6% to 17.0% ($p < 0.0001$) (Table 2). Prevalence of a past history of AD also increased in 2006, resulting in an increase in lifetime prevalence from 10.1% to 13.6% ($p < 0.0001$) (Table 1B).

Table 1

(A) Current prevalence of allergic diseases among schoolchildren in Kyoto, Japan

	Year		*p*-Value
	1996 (*n* = 16,176)	2006 (*n* = 13,215)	
BA	829 (5.1%)	664 (5.0%)	0.58
AD	685 (4.2%)	735 (5.6%)	< 0.0001
AR	3279 (20.3%)	3621 (27.4%)	< 0.0001
AC	2158 (13.3%)	3324 (25.2%)	< 0.0001
sJCP	495 (3.1%)	1059 (8.0%)	< 0.0001

(B) Lifetime prevalence of allergic diseases among schoolchildren in Kyoto, Japan

	Year		*p*-Value
	1996 (*n* = 16,176)	2006 (*n* = 13,215)	
BA	1055 (6.5%)	1005 (7.6%)	< 0.0001
AD	1636 (10.1%)	1803 (13.6%)	< 0.0001
AR	3525 (21.8%)	3838 (29.0%)	< 0.0001
AC	3971 (24.5%)	3959 (30.0%)	< 0.0001

Lifetime prevalence is defined as current prevalence and past history combined.

BA, bronchial asthma; AD, atopic dermatitis; AR, allergic rhinitis; AC, allergic conjunctivitis; sJCP, suspected Japanese cedar pollinosis.

Table 2 Distribution of disease severity among children with current allergic diseases

		Year		*p*-Value
		1996	2006	
BA	mild	611 (73.7%)	541 (81.5%)	< 0.0001†
	moderate	115 (13.9%)	55 (8.3%)	
	severe	103 (12.4%)	68 (10.2%)	0.21‡
AD	mild	175 (25.6%)	125 (17.0%)	< 0.0001†
	moderate	248 (36.2%)	283 (38.5%)	
	severe	262 (38.2%)	327 (44.5%)	0.02‡
AR	mild	1632 (49.8%)	1463 (40.4%)	< 0.0001†
	moderate	1192 (36.4%)	1506 (41.6%)	
	severe	455 (13.8%)	652 (18.0%)	< 0.0001‡
AC	mild	1745 (80.8%)	2484 (74.7%)	< 0.0001†
	moderate	146 (6.8%)	156 (4.7%)	
	severe	267 (12.4%)	684 (20.6%)	< 0.0001‡

† Change in distribution of those with mild symptoms were compared.

‡ Change in distribution of those with severe symptoms were compared.

BA, bronchial asthma; AD, atopic dermatitis; AR, allergic rhinitis; AC, allergic conjunctivitis.

CHANGE IN THE PREVALENCE AND SEVERITY OF AR

The current prevalence of AR increased from 20.3% to 27.4% ($p < 0.0001$). Among those with current AR, there was an increase in severe cases from 13.8% to 18.0% ($p < 0.0001$), while mild cases decreased from 49.8% to 40.4% ($p < 0.0001$) (Table 2). The prevalence of a past history of AR showed no significant difference, resulting in an increase in lifetime prevalence from 21.8% to 29.0% ($p < 0.0001$) (Table 1B).

CHANGE IN THE PREVALENCE AND SEVERITY OF AC

The current prevalence of AC increased from 13.3% to 25.2% ($p < 0.0001$). Among those with current AC, there was an increase in severe cases from 12.4% to 20.6% ($p < 0.0001$), while mild cases decreased from 80.8% to 74.7% ($p < 0.0001$) (Table 2). Although the prevalence of a past history of AC decreased, there was an increase in lifetime prevalence from 24.5% to only 30.0% ($p < 0.0001$) (Table 1B).

CHANGE IN THE PREVALENCE OF SUSPECTED JCP (sJCP)

The current prevalence of sJCP, defined as having both presence of AR and AC symptoms plus aggravated symptoms during the spring cedar pollen season in Japan, increased from 3.1% to 8.0% ($p < 0.0001$) (Table 1).