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Hematopoietic Microchimerism in Sheep After In Utero Transplantation of Cultured Cynomolgus Embryonic Stem Cells

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Background. Although directed differentiation of human embryonic stem (ES) cells would enable a ready supply of cells and tissues required for transplantation therapy, the methodology is limited. We have developed a novel method for hematopoietic development from primate ES cells. We first cultured cynomolgus monkey ES cells in vitro and transplanted the cells in vivo into fetal sheep liver, generating sheep with cynomolgus hematopoiesis.

Methods. Cynomolgus ES cells were induced to mesodermal cells on murine stromal OP9 cells with multiple cytokines for 6 days. The cells (average 4.8×10^7 cells) were transplanted into fetal sheep in the liver (n=4) after the first trimester (day 55–73, full term 147 days). The animals were delivered at full term, and two of them were intraperitoneally administered with human stem-cell factor (SCF).

Results. Cynomolgus hematopoietic progenitor cells were detected in bone marrow at a level of 1% to 2% in all four sheep up to 17 months posttransplant. No teratoma was found in the lambs. After SCF administration, the fractions of cynomolgus hematopoiesis increased by several-fold (up to 13%). Cynomolgus cells were also detected in the circulation, albeit at low levels (<0.1%).

Conclusions. Long-term hematopoietic microchimerism from primate ES cells was observed after in vitro differentiation to mesodermal cells, followed by in vivo introduction into the fetal liver microenvironment. The mechanism of such directed differentiation of ES cells remains to be elucidated, but this procedure should allow further investigation.

Keywords: Primate embryonic stem cells, In utero transplantation, Hematopoietic microchimerism, Sheep.

(*Transplantation* 2005;79: 32–37)

A major barrier for most tissue or cellular transplantation therapies is the shortage of donors. Because human embryonic stem (ES) cell lines have dual abilities to proliferate indefinitely and differentiate into multiple tissue cells (*1, 2*), directed differentiation of human ES cells into functionally defied tissue types is a goal in providing an inexhaustible and potentially customized supply of transplantable cells or tissues. Clearly directed differentiation of ES cells is still in its infancy, and the methodology is quite limited. Many researchers have studied in vitro specific differentiation programs through manipulation of the cytokine milieu, cellular microenvironment, and conditional activation of specific gene expression (*3–5*). On the other hand, we and other groups have shown a line of evidence that undifferentiated ES cells respond to local cues after transplantation and differentiate into site-specific cells in rodent and nonhuman primate allogeneic transplantation models (*6, 7*). These studies have highlighted the importance of the in vivo local microenvironment for directed differentiation of ES cells. ES cells can be induced to differentiate into specific cells if exposed to the proper microenvironment. In this study, we have tried to use the in vivo fetal sheep liver microenvironment for hematopoietic development from primate ES cells.

Sheep in utero transplantation has been used as an assay system for human hematopoiesis (*8*). The fetal sheep is immunologically tolerant of allogeneic skin grafts or xenogeneic human hematopoietic cells before 75 days of gestation, which allows avoidance of the immunologic barriers present in postnatal models (*8–10*). In this model, long-term human/sheep hematopoietic chimeras have been established after the transplantation of human hematopoietic stem cells into the fetal sheep at a preimmune stage (*8*). It has also been reported that human mesenchymal stem cells engraft and show site-specific differentiation after in utero transplantation in sheep (*11*).

We have used nonhuman primate (cynomolgus monkey) ES cells (*12*) because this is the most faithful model for human ES cells for generating hematopoietic chimera in sheep. We first cultured cynomolgus ES cells in vitro to differentiate into mesodermal cells and introduced the cells into fetal sheep liver after the first trimester. Fetal liver is a hematopoietic organ at this stage of fetuses. We then examined the in vivo fate of transplanted cell progeny long term.

MATERIALS AND METHODS

Cell Preparation

Cynomolgus macaque ES cells (CMK6) were maintained on a feeder layer of mitomycin C (Kyowa, Tokyo, Japan) treated mouse (BALB/c, Charles River Japan,

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Kanagawa, Japan) embryonic fibroblasts, as described previously (*12*). The mouse bone-marrow stromal-cell line OP9 was maintained in α-minimum essential medium (Gibco, Rockville, MD) supplemented with 20% fetal calf serum, as previously described (*13*).

For the in vitro differentiation (induction of hematopoietic differentiation), ES cells were seeded onto mitomycin–C-treated confluent OP9 cell layers in culture dishes in Iscove's modified Dulbecco's medium (Gibco) supplemented with 8% horse serum (Gibco), 8% fetal calf serum, 5×10^{-6} M hydrocortisone (Sigma, St. Lois, MO), and multiple cytokines including 20 ng/mL recombinant human (rh) bone morphogenetic protein-4 (BMP-4; R&D, Minneapolis, MN), 20 ng/mL rh stem-cell factor (SCF, Amgen, Thousand Oaks, CA), 20 ng/mL rh interleukin-3 (Research Diagnostics, Flanders, NJ), 20 ng/mL rh interleukin-6 (Ajinomoto, Osaka, Japan), 20 ng/mL rh vascular endothelial growth factor (VEGF, R&D), 20 ng/mL rh granulocyte colony-stimulating factor (Chugai, Tokyo, Japan), 10 ng/mL rh Flt-3 ligand (Research Diagnostics), and 2 IU/mL rh erythropoietin (Chugai). During differentiation, media were changed every 2 to 3 days. After 6 days of culture, cells were dissociated with 0.25% trypsin (Gibco), collected with a cell scraper, washed with Hanks' balanced salt solution (HBSS, Gibco), resuspended in 0.4 mL of 0.1% bovine serum albumin/HBSS, and used for transplantation. Human cord-blood $CD34^{+}$ cells used in the present study were obtained at Jichi Medical School Hospital with informed consent.

Transplant Procedures

Pregnant Suffolk ewes (Japan Lamb, Hiroshima, Japan) were bred at the Utsunomiya University Farm. Fetal sheep at 55 to 79 days of gestation (full term 147 days) were used. Before transplantation, ewes were sedated with ketamine (10 mg/kg intramuscularly) and received a 0.5% to 1.0% halothane-oxygen mixture by inhalation by way of an endotracheal tube. The uterus was exposed through a midline laparotomy incision. Donor cells were injected into the fetus in the liver through the uterine wall using a 25-gauge needle under ultrasound guidance. After closure of the abdominal wall, penicillin and streptomycin were administered. The fetus was allowed to come to term. After birth, some lambs were intraperitoneally administered rhSCF at a dosage of 60 μg/kg once a day for 18 or 5 consecutive days.

Hematopoietic Progenitor Assay

To assess cynomolgus hematopoiesis in sheep, clonogenic hematopoietic colonies were produced by growing bone-marrow cells in methylcellulose with defined rh cytokines (Methocult GF+ and MegaCult-C, Stem Cell Technologies, Vancouver, Canada). Bone-marrow cells were aspirated from the iliac bone. From harvested bone-marrow cells, a leukocyte cell fraction was obtained after red–blood-cell lysis with ACK buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA; Wako, Osaka, Japan). Cells were plated in triplicate at 1 to 5×10^{4} cells per 35-mm plate. After 14 days, individual colonies were plucked into 50 μL of distilled water and digested with 20 μg/mL proteinase K (Takara, Shiga, Japan) at 55°C for 1 hour, followed by 99°C for 10 minutes. Each sample (5 μL) was used for polymerase chain reaction (PCR) amplification to detect cynomolgus-specific β2-microglobulin gene sequences. Nested PCR was performed. The outer primer set was 5'-GTC TGG ATT TCA TCC ATC TG-3' and 5'- GGC TGT GAC AAA GTC ACA TGG-3', and the inner primer set was 5'-GTC TGG ATT TCA TCC ATC TG-3' and 5'-GGT GAA TTC AGT GTA GTA CAA G-3'. Amplification conditions for both outer and inner PCR were 25 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Amplified products (135 bp) were resolved on 2% agarose gel and visualized by ethidium bromide staining.

PCR Southern Blotting

Cellular DNA was extracted from peripheral blood and bone-marrow cells after birth and subjected to the cynomolgus-specific β2-microglobulin PCR as described above. The PCR products were resolved on 2% agarose gel and transferred to Hybond-N+ (Amersham, Cleveland, OH). The membrane was hybridized with a radiolabeled cynomolgus-specific β2-microglobulin probe generated by PCR using the following primers: 5'-GTC TGG ATT TCA TCC ATC TG-3' and 5'-GGT GAA TTC AGT GTA GTA CAA G-3'. Radiolabeling of a probe was performed using a DNA labeling kit (Amersham).

RESULTS

In Vitro Culture and In Utero Transplantation

Cynomolgus ES cells were maintained on mouse embryonic fibroblast feeder cells. They form colonies on feeder cells (Fig. 1a). For induction of hematopoietic differentiation, undifferentiated ES cells were placed on murine stromal OP9 cells (*13*) in the presence of multiple cytokines including human BMP-4 and VEGF (*14*). The cell number increased by 10-fold at day 6, and cobblestone-like cells emerged at this time point (Fig. 1b). These cells were negative or only weakly positive for CD34 (hematopoietic) and CD31 (endothelial) markers as assessed by flow cytometry and had little clonogenic hematopoietic colony forming ability in culture (data not shown). These cells likely represent a differentiated population of mesodermal cells. We then transplanted the day-6 ES derivatives (average 4.8×10^{7} cells, Table 1) into fetal sheep (n=4) by way of direct injection into the liver under ultrasound guidance after the first trimester (Fig. 1c).

Hematopoietic Microchimerism after Birth

The animals were delivered at full term. Marrow cells were harvested from the iliac bone and plated in methylcellulose. Clonogenic hematopoietic colonies (colony-forming units [CFU]) were thus produced to examine hematopoietic chimerism in the sheep (Fig. 2). Sheep bone-marrow cells generated colonies of clear hematopoietic morphology in this assay. Cynomolgus and sheep cells were equivalent in their colony-formation ability in this culture, and each colony was derived from a single cynomolgus or sheep hematopoietic progenitor cell. Therefore, the ratio of cynomolgus to sheep colony number is considered a chimeric fraction. To distinguish cynomolgus versus sheep colonies, we tried to immunostain colonies with antihuman class I, and more directly hematopoietic-specific antihuman CD45, but they reacted to sheep counterparts or generated a considerable nonspecific staining. We then conducted PCR for monkey-specific β2-microglobu-

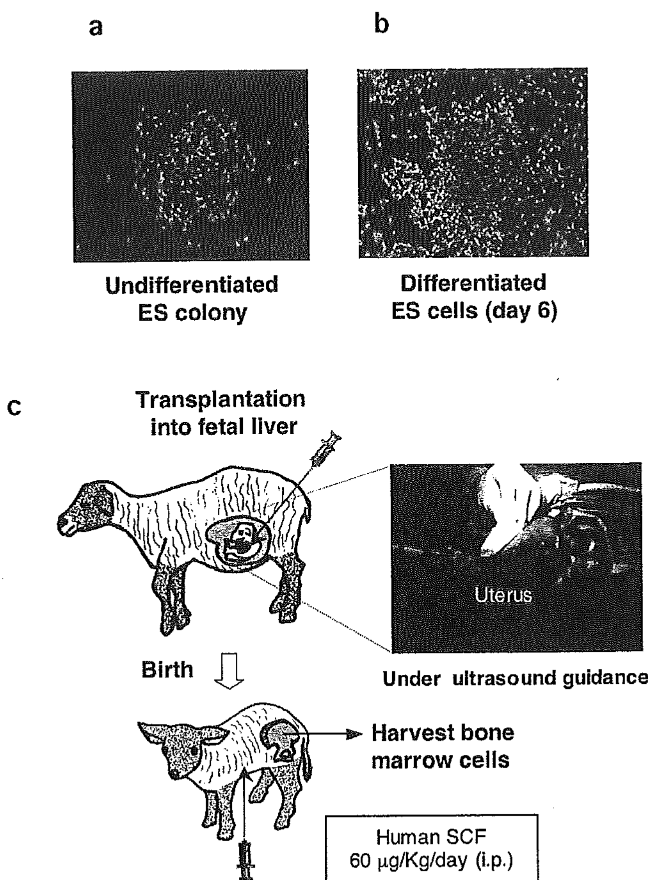

FIGURE 1. In utero transplantation of day 6 cynomolgus embryonic stem (ES)-derived cells. (a) Undifferentiated cynomolgus ES cells were maintained on mouse embryonic fibroblast feeder cells. (b) For hematopoietic induction, undifferentiated ES cells were placed on murine stromal OP9 cells in the presence of multiple cytokines. Cobblestone-like cells emerged at day 6. (c) The day 6 ES derivatives were transplanted directly into the fetal liver after the first trimester. After birth, marrow cells were harvested from the iliac bone and examined for cynomolgus versus sheep hematopoiesis. Some lambs were intraperitoneally (i.p.) administered with human stem-cell factor (SCF) to specifically stimulate cynomolgus hematopoiesis.

lin DNA sequences on DNA isolated from each colony (Fig. 2) (colony PCR). Cynomolgus CFU were clearly detected at a level of 1% to 2% in the bone marrow of all four sheep (Table 1). We repeated colony PCR and confirmed that data were reproducible. We detected both granulocytic and erythroid cynomolgus CFU. Cynomolgus megakaryocytic colonies were not detected, but it was no wonder considering the low frequency of cynomolgus megakaryocytic colony formation in vitro (*15*). The longest follow-up was 17 months after transplantation (14 months after birth), and cynomolgus CFU were still detectable. No lamb developed a teratoma.

Selective Expansion of Cynomolgus Hematopoiesis

To selectively stimulate cynomolgus compared with endogenous hematopoiesis in the sheep, we administered two animals with human SCF, a cytokine that does not cross-react to stimulate sheep hematopoietic progenitors (*16*). A time-course profile of the two animals (No. 141 and No. 182) is shown in Figure 3. In both sheep, in response to the human SCF administration for 18 days, the fractions of cynomolgus CFU increased several-fold (up to 13.2% at day 174 and 4.7% at day 112 posttransplantation, respectively). After cessation of SCF administration, the fractions fell to the original levels. Resumption of human SCF at the same dose but for 5 days produced a similar elevation in the chimeric fractions (up to 4.4% at day 364 and 8.8% at day 187 posttransplantation, respectively). No adverse effects associated with human SCF administration were observed. We did not examine antibody responses to human SCF in the sheep, but this was unlikely to occur because second trials of human SCF administration did work (i.e., increased the chimeric fractions) in both sheep.

Cynomolgus cells were also detected in the circulation, although the fraction was very low (<0.1%), even after human SCF administration as assessed by PCR Southern blotting (Fig. 4). The low levels of cynomolgus cells hampered the lineage analysis by flow cytometry. To detect cynomolgus T lymphocytes in the sheep, we collected the peripheral blood and tried to selectively expand cynomolgus T lymphocytes in the culture with human interleukin-2, antimonkey CD3 (FN-18), and antihuman CD28 (Kolt-2, cross-reacting to monkey CD28) (*17*). However, sheep lymphocytes were also stimulated to expand, and we failed to detect cynomolgus T lymphocytes.

Comparative Study of Repopulating Ability

Next, we transplanted human cord-blood hematopoietic stem cells ($CD34^+$ cells, average 1.8×10^6 cells) into fetal sheep (n=4) instead of day-6 cynomolgus ES derivatives. We used a $CD34^+$ fraction because it was widely used both in sheep in utero transplantation (*18, 19*) and in clinical transplantation of hematopoietic stem cells (*20*), although some investigators used a human $CD34^-$ fraction for sheep in utero transplantation and obtained hematopoietic chimera as well or even better in serial transplantation experiments (*21*). For the present, however, we considered that the $CD34^+$ fraction was an appropriate standard with which to compare the repopulating ability of day-6 cynomolgus ES-derived cells as well. The chimeric fraction in bone-marrow CFU after in utero transplantation of $CD34^+$ cells was 1% to 9% (average 3.7%). Fractions of donor-derived cells in the peripheral blood were also very low (<0.1%) after the transplantation of $CD34^+$ cells. The average cell numbers necessary to achieve 1% chimerism in CFU after birth were estimated to be 4.3×10^7 and 6.0×10^5 for day-6 cynomolgus ES-derived cells and human cord-blood $CD34^+$ cells, respectively.

DISCUSSION

We have generated long-term hematopoietic microchimerism in sheep derived from ES cells. Achieving hematopoietic reconstitution from ES cells has been an enormous challenge. The difficulty is attributable to the developmental immaturity of ES-derived cells, which most closely resemble primitive embryonic yolk-sac hematopoietic progenitors (*22*). The processes governing embryonic versus adult blood formation are distinct, and mouse ES cells do not contribute

TABLE 1. In utero transplantation and donor-cell engraftment in sheep

		In utero transplantation		Donor cell-derived CFU in bone marrow* (months posttransplant)		
Transplanted cells	Animals (sex)	Transplanted cell number per fetus	Gestational day at transplantation (Full term 147 days)	After birth	After human SCF administration	Average cell number necessary for 1% chimerism
Day 6 ES-derived cells	No. 57 (male)	5.0×10^7	67	1.1% (1/91) at 3.5 months	ND	4.3×10^7
	No. 55 (female)	5.0×10^7	55	1.1% (1/91) at 5 months	ND	
	No. 141 (male)	7.8×10^7	73	1.1% (1/91) at 3 months	13.2% (12/91) at 6 months	
	No. 182 (male)	1.4×10^7	66	1.6% (1/63) at 3 months	8.8% (8/91) at 6 months	
Human cord blood CD34$^+$ cells	No. 71-1 (male)	2.0×10^6	69	8.8% (8/91) at 1 months	ND	6.0×10^5
	No. 71-2 (female)	2.0×10^6	69	4.4% (4/91) at 3.5 months	ND	
	No. 99-1 (male)	1.5×10^6	79	1.1% (1/91) at 1 months	ND	
	No. 99-2 (female)	1.5×10^6	79	4.4% (4/91) at 2 months	ND	

* Percent cynomolgus CFU was calculated by dividing the number of CFU positive for the cynomolgus-specific β2-microglobulin gene sequence by the total number of CFU analyzed.

CFU, colony-forming unit; ND, not done; ES, embryonic stem.

to hematopoietic reconstitution in irradiated mice, unlike stem cells isolated from adult bone marrow (*23, 24*). An effective approach to this obstacle has recently been reported. Genetically engineering mouse ES cells to express a specific transcription factor (HoxB4) or signaling molecule (STAT5) during a specific developmental window has resulted in hematopoietic reconstitution from ES cells in irradiated mice (*5, 25*). However, the requirement for artificial over-expression of those genes is undesirable for clinical applications. Our method to develop hematopoietic engraftment from primate ES cells does not require genetic manipulation, and it is a combination of two steps: in vitro differentiation into mesodermal cells followed by in vivo development into hematopoietic cells in the proper microenvironment of fetal sheep liver.

To examine whether transplanted day-6 ES-derived cells engraft in other tissues, we did one more in utero transplantation experiment using same cultured cynomolgus ES cells (mesodermal cells at day 6) and delivered a fetus at 1 month after transplantation. Although cynomolgus CFU were detected in fetal liver and cord blood, cynomolgus cells were not detectable in any other tissues by a sensitive PCR after the fetal blood was completely washed out (data not shown). Therefore, our method appears to direct the fate of primate ES cells to the hematopoietic lineage.

We assumed that the initial in vitro culture of ES cells into mesodermal cells is crucial for successful engraftment in the fetal liver. In fact, when we transplanted undifferentiated (day 0) cynomolgus ES cells into fetal sheep (n=2), we failed to detect cynomolgus CFU or other nonhematopoietic cells in any fetal tissue at 1 month posttransplantation as assessed by a sensitive PCR (data not shown). Therefore, undifferentiated ES cells do not appear to engraft in fetal sheep, unlike adult mesenchymal or hematopoietic stem cells (*8, 11*), but day-6 ES derivatives (mesodermal cells) can engraft and are susceptible to hematopoietic specification in the fetal-liver microenvironment. For the successful initial in vitro culture of ES cells, there are some points to be noted. First, we used stromal OP9 cells as a feeder. Second, we included BMP-4 and VEGF in the culture medium. The coculture with OP9 and inclusion of BMP-4 and VEGF promote hematopoietic differentiation of ES cells (*13, 14, 26–28*). Finally, we cultured cells in vitro for a relatively short period (6 days) to avoid over-maturation of cells (*14*). The xenogeneic fetal liver is able to provide such cultured ES cells with an adequate microenvironment for support of hematopoietic development (*29*). Factors present in the fetal liver responsible for the development remain to be elucidated.

On the other hand, several issues remain to be further investigated. Although the chimeric fractions increased by several-fold (up to 13%) after SCF administration, the increase was transient. To stably enhance the ES-derived chimeric fraction, an enrichment of cells responsible for the engraftment will be needed before transplantation, as suggested from Table 1 (see average cell numbers necessary to achieve 1% chimerism). Another issue is a very low level of donor-derived cells in the peripheral blood. Ours is quite different from previous human-to-fetal sheep experiments that demonstrated easily detectable peripheral blood chimerism (*16, 19*). This has hampered the lineage analysis of cynomolgus cells in our sheep to obtain further evidence to support hematopoietic differentiation from ES cells. Low levels of pe-

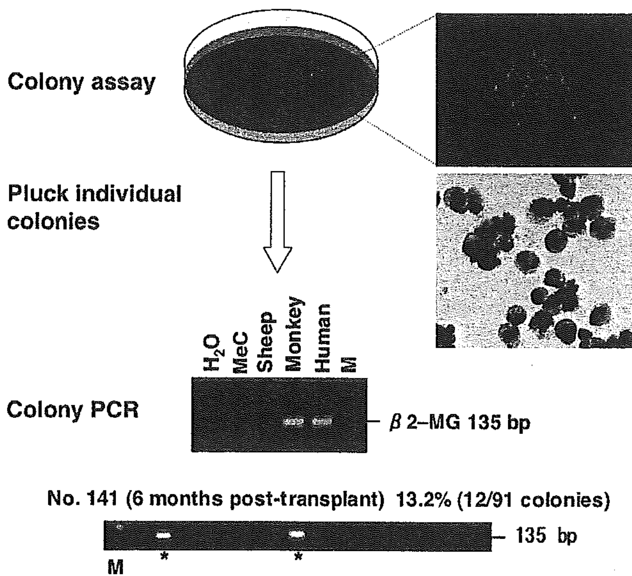

FIGURE 2. Assessment of cynomolgus hematopoiesis in sheep after birth. Bone-marrow cells were harvested from lambs and placed in methylcellulose. Hematopoietic colonies were thus formed. A cytospin specimen (stained with the Wright-Giemsa method) of plucked myeloid colonies demonstrated mature neutrophils. Each colony was derived from a single cynomolgus or sheep hematopoietic progenitor cell. To detect cynomolgus colonies, individual colonies were plucked and examined for cynomolgus-specific β2-microglobulin (β2-MG) sequences by polymerase chain reaction (PCR). PCR products were analyzed on 2% agarose gel. Plucked methylcellulose (MeC) alone (not containing colonies) and sheep colonies generated no bands by the PCR. Colony PCR was repeated at least twice. Representative colony PCR results of sheep No. 141 shown. *Bands positive for the cynomolgus-specific sequence. M, molecular weight marker.

ripheral chimerism were, however, also found after human CD34$^+$ cells were transplanted in our study. Therefore, the issue is not specific to transplanted ES cells, but is likely to be attributable to the experimental system. A possible explanation may be the different sheep species used in our study (Suffolk versus Dorset Merino). Another possible explanation is immune responses caused by relatively later gestational ages (in the second trimester, day 55–79) at transplantation in our study compared with other recent studies (day 40–45) (*19*). The immune response may have cleared xenogeneic cells from the circulation. The existence of microchimerism does not necessarily guarantee or predict tolerance in other systems (*30, 31*). "The window of opportunity" for successful tolerance induction may be earlier and narrower. To avoid sensitization, transplantation at earlier days may be more efficacious.

In conclusion, long-term hematopoietic microchimerism from primate ES cells is possible after in vitro differentiation to mesodermal cells, followed by in vivo transplantation into the fetal-liver microenvironment. We have used nonhuman primate ES cells in the current study, but if human ES cells are similarly used, human blood cells can be generated in sheep. This procedure should allow for further investigation.

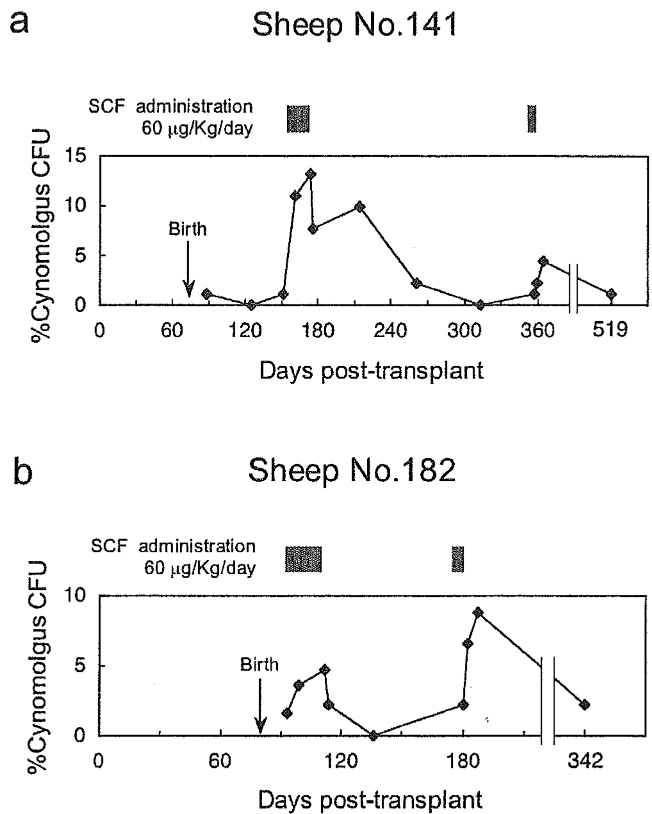

FIGURE 3. Time course of hematopoietic chimerism in the sheep receiving human SCF. (a) In sheep No. 141, human SCF was intraperitoneally administered at 60 μg/kg once a day from day 156 posttransplantation for 18 days. SCF administration was then stopped and tried again from day 352 for 5 days. (b) In sheep No. 182, human SCF was similarly administered from day 94 posttransplantation for 18 days, followed by a second administration at the same dose from day 175 for 5 days. Horizontal axis indicates days after transplantation. Vertical axis shows cynomolgus/sheep chimerism (a ratio of cynomolgus vs. sheep CFU in the bone marrow). Period of human SCF administration (*gray bars*).

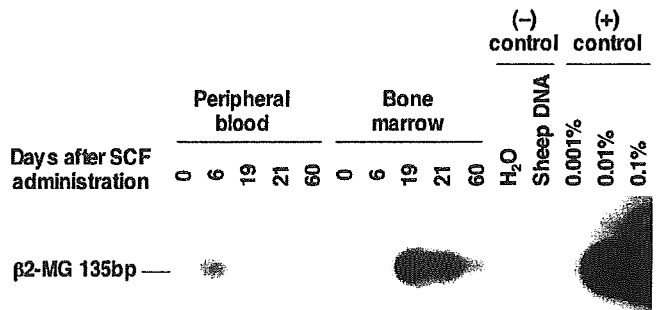

FIGURE 4. Detection of cynomolgus cells in the circulation. DNA was extracted from whole peripheral blood or bone-marrow nucleated cells after birth and subjected to cynomolgus-specific β2-microglobulin (β2-MG) PCR, and Southern blot analysis. Data before and after SCF administration shown. Positive controls show 0.001, 0.01, and 0.1% chimerism (cynomolgus to sheep). Cynomolgus cells were detectable after SCF administration, albeit at low levels (<0.1%).

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RESEARCH ARTICLE

Efficient and stable Sendai virus-mediated gene transfer into primate embryonic stem cells with pluripotency preserved

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Efficient gene transfer and regulated transgene expression in primate embryonic stem (ES) cells are highly desirable for future applications of the cells. In the present study, we have examined using the nonintegrating Sendai virus (SeV) vector to introduce the green fluorescent protein (GFP) gene into non-human primate cynomolgus ES cells. The GFP gene was vigorously and stably expressed in the cynomolgus ES cells for a year. The cells were able to form fluorescent teratomas when transplanted into immunodeficient mice. They were also able to differentiate into fluorescent embryoid bodies, neurons, and mature blood cells. In addition, the GFP expression levels were reduced dose-dependently by the addition of an anti-RNA virus drug, ribavirin, to the culture. Thus, SeV vector will be a useful tool for efficient gene transfer into primate ES cells and the method of using antiviral drugs should allow further investigation for regulated SeV-mediated gene expression.
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Keywords: *primate embryonic stem cell; Sendai virus vector; gene transfer; green fluorescent protein; pluripotency; ribavirin*

Introduction

Since human embryonic stem (ES) cell lines have the ability to both proliferate indefinitely and differentiate into multiple tissue cells,[1,2] they are expected to have clinical applications as well as to serve as models for basic research and drug development. Although efficient and stable gene transfer into primate ES cells would be useful for such purposes, it has been difficult and only lentiviral vectors have been successful in achieving it.[3–5] We have previously developed Sendai virus (SeV) vectors that replicate in the form of negative-sense single-stranded RNA in the cytoplasm of infected cells and do not go through a DNA phase.[6] SeV vectors can efficiently introduce foreign genes without toxicity into airway epithelial cells,[7] vascular tissue,[8] skeletal muscle,[9] synovial cells,[10] retinal tissue,[11] and hematopoietic progenitor cells.[12] Here we report that the SeV-mediated gene transfer into primate ES cells is very efficient and stable even after the terminal differentiation of the cells. In addition, we show that SeV-mediated transgene expression levels can be reduced by the addition of a ribonucleoside analog, ribavirin, to the culture. Ribavirin is a mutagen and inhibitor of viral RNA polymerase.[13,14] It shows antiviral activity against a variety of RNA viruses and is used to treat infections of hepatitis C virus in combination with interferon-α[15,16] and of lassa fever virus.[17] The method of using antiviral drugs might offer a novel approach for regulated SeV-mediated gene expression in primate ES cells.

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Results

SeV-mediated gene transfer into ES cells

In this study, we have used an SeV vector, which is capable of self-replication but incapable of transmitting to other cells.[6] The vector does not encode the fusion (F) protein (Figure 1a), which is essential for viral entry into cells. It can be propagated only in a packaging cell line expressing the F protein. The green fluorescent protein (GFP) gene was introduced after the leader sequence of the vector genome. Cynomolgus ES cells[18] were exposed to the SeV vector for 24 h. Flow cytometric analysis at 2 days after infection showed that 15, 38, and 61% of cells fluoresced at 2, 10, and 50 transducing units (TU) per cell, respectively (Figure 1b). The gene transfer efficiency of about 60% is comparable to or even better than that for lentiviral vectors.[3] We confirmed that the undifferentiated cell fractions remained unchanged after the infection with SeV vector, as assessed by the expression of undifferentiated markers, alkaline phosphatase and SSEA-4 (data not shown). The GFP expression after infection was stable at least for a month. On the other hand, the GFP gene transfer to cynomolgus ES cells with adenovirus- and adeno-associated virus (AAV)-based vectors resulted in much lower expression levels (<20% by flow cytometry) and the levels declined to zero within a week after infection (Figure 1c).

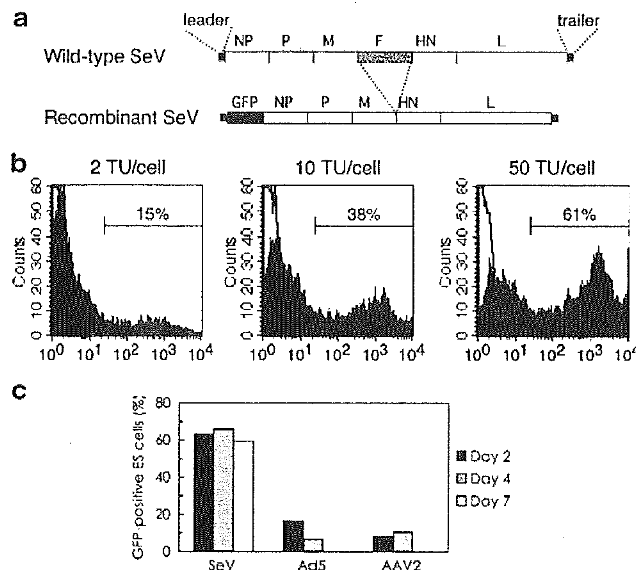

Figure 1 High-level transgene expression in cynomolgus ES cells after infection with SeV vector. (a) Schematic diagrams of the wild-type SeV genome and recombinant F-defective SeV carrying the GFP gene (SeV vector in this study). The SeV genome is 15 384 nucleotides long and its genes (NP, P, M, F, HN, and L) are in order from 3′ to 5′ in the negative-strand RNA. In the SeV vector, the entire fusion (F) gene was removed and the GFP gene was introduced at a unique NotI site between the leader sequence and NP gene. (b) The GFP expression by the SeV vector in cynomolgus ES cells. Cynomolgus ES cells were infected with the SeV vector at 2, 10, and 50 TU/cell. The flow cytometric profiles at day-2 postinfection are shown in gray. The white areas indicate uninfected ES cells. The fractions of GFP-positive cells are indicated. (c) The GFP expression levels in cynomolgus ES cells infected with the SeV (50 TU/cell), adenovirus serotype 5 (Ad5, 3.4×10^2 g.c./cell), and AAV serotype 2 (AAV2, 2.4×10^4 g.c./cell) vectors. The fractions of GFP-positive cells were examined by flow cytometry at 2, 4, and 7 days postinfection.

We plucked fluorescent ES cell colonies under a fluorescent microscope once at 1 month after infection and propagated them. After this selection procedure, approximately 90% of the ES cells expressed GFP (Figure 2a and b) and the high-level expression was stable for a year as assessed by flow cytometry (Figure 2c, upper). The mean fluorescence intensity per cell was also stable (Figure 2c, lower), indicating that the replicating vector genome was almost equally delivered to each cell of all progeny. The self-replication of the SeV vector in infected cells was confirmed by RNA-PCR that amplified the viral RNA genomic sequence (Figure 3a). The GFP cDNA sequence, however, could not be detected by DNA-PCR in the infected cells (Figure 3b), indicating that no DNA phase was involved in the GFP expression.

Pluripotency of infected ES cells

The SeV-infected, fluorescent cynomolgus ES cells were able to form fluorescent tumors when transplanted into immunodeficient mice (Figure 4a–c). The fluorescence was observed uniformly by fluorescent microcopy (Figure 4d and e). The tumors consisted of all three embryonic germ layer cells (Figure 4f–i). Thus, the SeV-infected ES cells were capable of forming teratomas and the SeV infection did not spoil the pluripotency of ES cells. The infected, fluorescent cynomolgus ES cells were also able to generate fluorescent embryoid bodies (Figure 5a and b), MAP-2-positive neurons (Figure 5c), clonogenic hematopoietic colonies (Figure 5d and e), and mature functional (NBT test-positive) neutrophils (Figure 5f and g), all of which fluoresced. In addition, the GFP expression levels were not decreased during the teratoma formation or differentiation, indicating that no 'silencing' of the transgene occurred.

Drug-inducible reduction of transgene expression

Next, we examined whether ribavirin inhibits the replication and transcription of the SeV vector resulting in a reduction of transgene expression. We first used a rhesus monkey kidney cell line (LLC-MK2) to test the effect of ribavirin on the replication and transcription of the SeV vector. LLC-MK2 is a standard control cell line for SeV infection. Ribavirin was added at various concentrations 2 days after the infection. The formation of viral particles quantified by the hemagglutination assay decreased drastically upon the addition of ribavirin (Figure 6a). The decrease was dependent on the dose of ribavirin. The GFP expression was also depressed dose-dependently (Figure 6b). Thus, ribavirin dose-dependently inhibits the replication and transcription of the SeV vector in LLC-MK2 cells. The toxicity associated with ribavirin was not observed in LLC-MK2 cells.

We then examined the effect of ribavirin on SeV-infected, fluorescent cynomolgus ES cells. The addition of ribavirin also resulted in a dose-dependent reduction of GFP expression in the cells (Figure 6c). Although the GFP expression was almost completely inhibited after a 3-day exposure with 4 mM of ribavirin, the cells could not be propagated thereafter. Ribavirin at high concentrations (>1 mM) hampered the proliferation of cynomolgus ES cells. With lower concentrations (0.5–0.75 mM) of ribavirin, the GFP expression level decreased by half. After the discontinuation of ribavirin treatment, the cells could be propagated and nearly regained the original level of GFP expression. The undifferentiated cell fractions were unchanged after the discontinuation as assessed by alkaline phosphatase and SSEA-4 staining (Figure 6d).

Discussion

There are several advantages in using SeV vectors over other vectors. (i) SeV vectors can infect nondividing, quiescent cells as well as dividing cells unlike oncoretroviral vectors.[7–11] Thus, they can be used to infect cells that are terminally differentiated as well as at various stages of differentiation, whether they are dividing or not. (ii) SeV vector-mediated gene transfer does not require a DNA phase. Thus, there is no concern about the unwanted integration of foreign sequences into the host genome unlike with oncoretroviral or lentiviral vectors. (iii) Transgene expression is stable even in dividing cells since the SeV vector replicates by itself in the cytoplasm of host cells. On the other hand, gene transfer using nonreplicating adenoviral and AAV vectors resulted in decreased levels of transgene expression in dividing cells over time, since the non-replicating transgene was

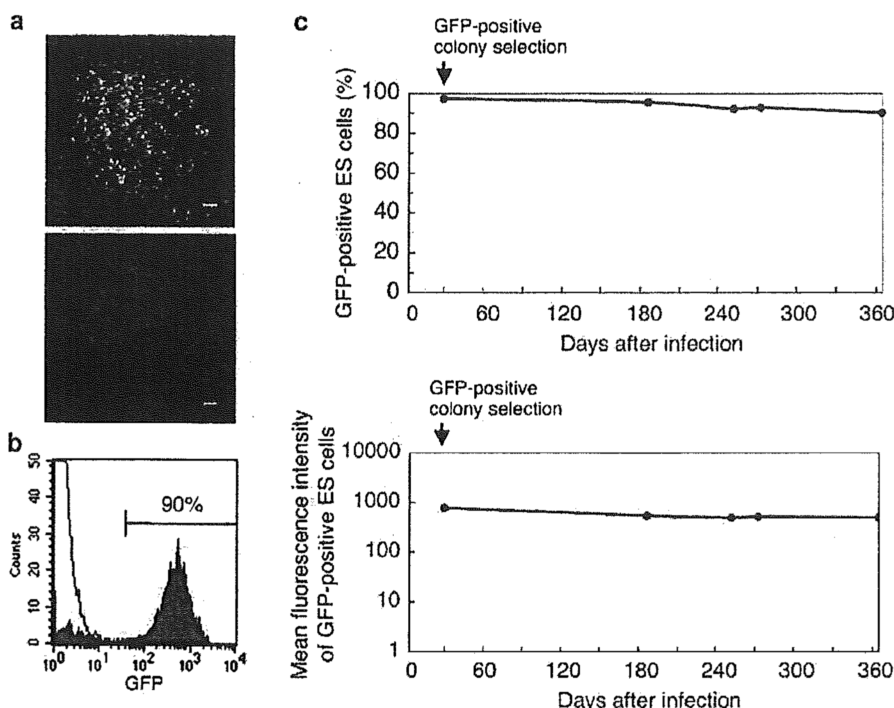

Figure 2 Stable SeV-mediated transgene expression in cynomolgus ES cells. Fluorescent ES cell colonies were plucked under a fluorescent microscope once at 1 month after infection and the cells were further propagated. (a) Phase-contrast (upper) and fluorescence (lower) images of a cynomolgus ES cell colony at day 370 after infection. Bar = 100 μm. (b) Flow cytometric analysis of SeV-infected cynomolgus ES cells at day 370 after infection (shown in green). The percentage of GFP-positive cells is indicated. Uninfected, parental cynomolgus ES cells are indicated by another line (white area). (c) The percentage of GFP-positive cells (upper) and mean fluorescence intensity per GFP-positive cell (lower) after infection with the SeV vector at 10 TU/cell are shown as a function of time (days).

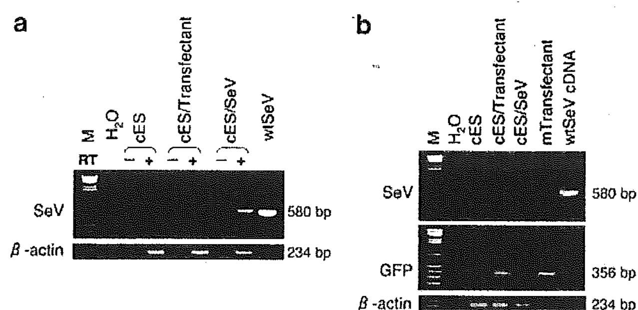

Figure 3 DNA-independent replication and transcription of SeV vector. Total cellular RNA and DNA were extracted from cynomolgus ES cells at day 284 after infection with the SeV vector. RNA-PCR (a) and DNA-PCR (b) for the SeV RNA genome or GFP sequence were conducted. The cynomolgus β-actin sequence was used as an internal control. In the RNA-PCR (a), negative results obtained without reverse transcriptase (designated RT−) confirmed that the amplified products were not derived from cellular DNA. M, 100-kb DNA ladder; cES, naive cynomolgus ES cells; cES/Transfectant, cynomolgus ES cells stably expressing the GFP gene after transfection,[33] cES/SeV, cynomolgus ES cells infected with the SeV vector; wtSeV, wild-type SeV genome; mTransfectant, a GFP-positive mouse cell line after transfection.

diluted out. (iv) The SeV vector is much less unlikely to generate wild-type virus *in vitro* or *in vivo* than oncoretroviral and lentiviral vectors, since homologous recombination between RNA genomes is very rare indeed in negative-strand RNA viruses.[19] (v) The SeV genome is not subject to cellular epigenetic modifications such as methylation, and thus it is unlikely that methylation-based silencing of transgene expression occurs.

No cytotoxic or differentiating effect on ES cells associated with the SeV infection was observed in our study. However, the wild-type SeV contains immunogenic surface proteins, hemagglutinin-neuraminidase (HN) and F proteins, which potentially induce antibody responses.[20,21] For future clinical applications, it would be desired that as many viral genes as possible are deleted from the vector backbone to permit reapplication, improve the safety, and lessen the possible toxicity of SeV vectors. To this end, we have developed a series of attenuated SeV vectors that are F gene-deleted,[6] F gene-deleted with preferable mutations,[22] M gene-deleted,[23] or have deletions of both F and M genes.[24] The modified vectors would be safer for *in vivo* use.

Ribavirin at high concentrations seems toxic to ES cells; presumably, it directly hampers viability and proliferation potential of ES cells. However, we cannot tell whether the observed toxicity is simply due to its toxicity to ES cells, as feeder cells are more highly sensitive to ribavirin than ES cells. In fact, while feeder cells died at 1 mM of ribavirin, cocultured ES cells were alive at this concentration for some time. Cynomolgus ES cells lose pluripotency and proliferation potential without feeder cells. Thus, the observed toxicity to ES cells may also be a secondary event following the injury of feeder cells. Whether the cytotoxicity is primary or secondary, it will be necessary to find modified compounds of less cytotoxicity.

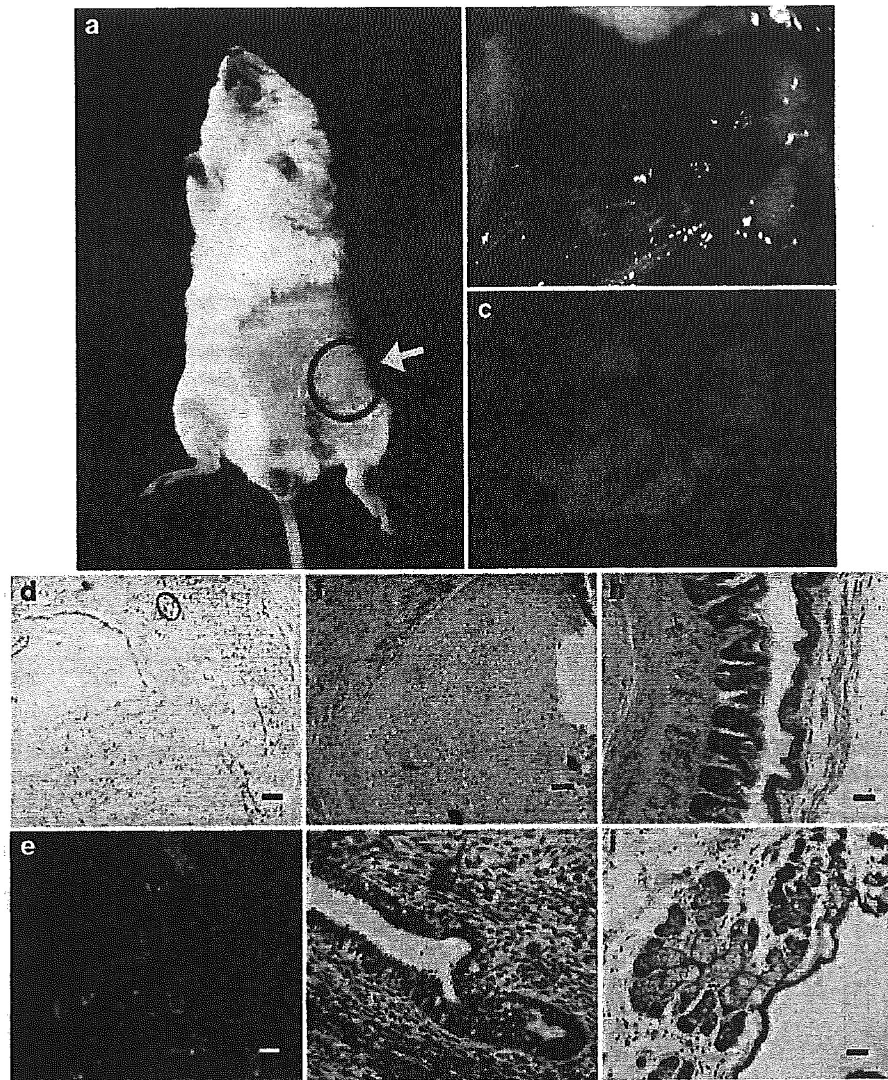

Figure 4 *Pluripotency of SeV-infected cynomlgus ES cells. Tumors formed in NOD-SCID mice after inoculation of the SeV-infected cynomolgus ES cells (a). The tumor was fluorescing ((b), bright field; (c), dark field). Fluorescence was observed uniformly in the tumor under a fluorescent microscope ((d), bright field; (e), dark field). The tumor contained all three embryonic germ layer cells; cartilage (f), ciliated columnar epithelium (g), skin (h), and sebaceous gland (i) (stained with hematoxylin and eosin). Bar = 100 μm.*

Materials and methods

Cell culture

Cynomolgus ES cells (CMK6) were maintained on a feeder layer of mitomycin C (Kyowa, Tokyo, Japan)-treated mouse (BALB/c) embryonic fibroblasts as described previously.[18] The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 15% ES cell-qualified fetal calf serum (FCS; Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma, St Louis, MO, USA), 2 mM glutamine (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Irvine Scientific, Santa Ana, CA, USA). The ES cell colonies were routinely passaged every 3–4 days after dissociation with a combined approach of 0.25% trypsin (Invitrogen) digestion and mechanical cutting. Alkaline phosphatase staining was conducted with an Alkaline Phosphatase Chromogen Kit (Biomeda, Foster City, CA, USA). Embryoid bodies were produced by culturing ES cell aggregates in Petri dishes. LLC-MK2 cells (1×10^6) were grown in six-well plates and cultured in Eagle's minimal essential medium (Invitrogen) supplemented with 10% FCS.

Vectors

The F-defective SeV vector carrying the GFP gene was constructed as previously described.[6] The vector titer was 1.8×10^9 TU/ml determined by counting fluorescent cells after the infection of LLC-MK2 cells. Gene transfer was conducted by adding various concentrations of the SeV vector solution to culture media. After 24 h of incubation, the cells were washed twice with phosphate-buffered saline (PBS) and fresh medium was added. In some experiments, ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; Sigma) was added at various concentrations to the culture media after infection. The

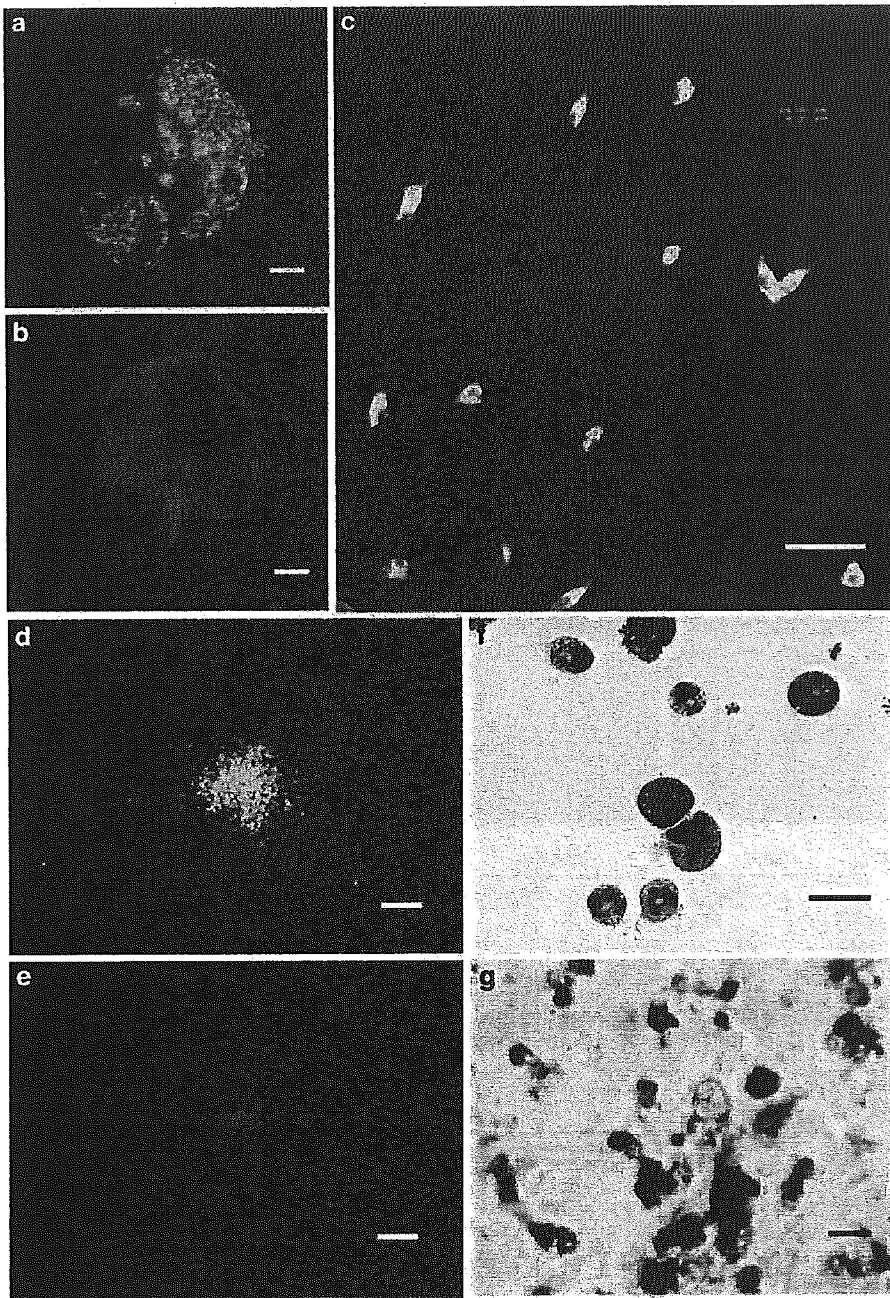

Figure 5 Stable transgene expression during differentiation. A day-20 cystic embryoid body was observed under a fluorescent phase-contrast microscope, confirming that the embryoid body was fluorescing ((a), bright field; (b), dark field). After infection with the SeV vector, fluorescent cynomolgus ES cells differentiated into neural cells. Double immunostaining with anti-GFP (green) and ant-MAP-2 (red) confirmed that differentiated neural cells expressed GFP (c). Yellow cells indicate GFP-expressing neurons. SeV-infected, fluorescent cynomolgus ES cells also differentiated into fluorescent hematopoietic cells. A clonogenic hematopoietic colony was fluorescing ((d) bright field; (e), dark field). A cytospin specimen of hematopoietic colony cells (Wright–Giemsa staining) showed that the cells were mature granulocytes (f). The infected ES cell-derived, fluorescent neutrophils were positive for NBT (stained in black (g)). Bar = 100 μm (a, b, g); 50 μm (c, f); 500 μm (d, e).

viral particles in infected cells were quantified by a hemagglutination assay as described previously.[25]

An adenovirus serotype 5-based vector carrying the GFP gene was constructed as reported.[26] It contained the cytomegalovirus (CMV) promoter, simian virus (SV)-40 intron, and SV-40 polyadenylation signal. An AAV serotype 2-based vector expressing the GFP gene under the control of the chicken β-actin promoter with the CMV immediate-early enhancer (a gift from Dr J Miyazaki) was prepared as described previously.[27] Gene transfer experiments were performed using 3.4×10^2 genome copies (g.c.)/cell of the adenoviral vector or 2.4×10^4 g.c./cell of the AAV vector. The period of exposure was 48 h.

Flow cytometry

GFP and SSEA-4 expression was analyzed on a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA) using the

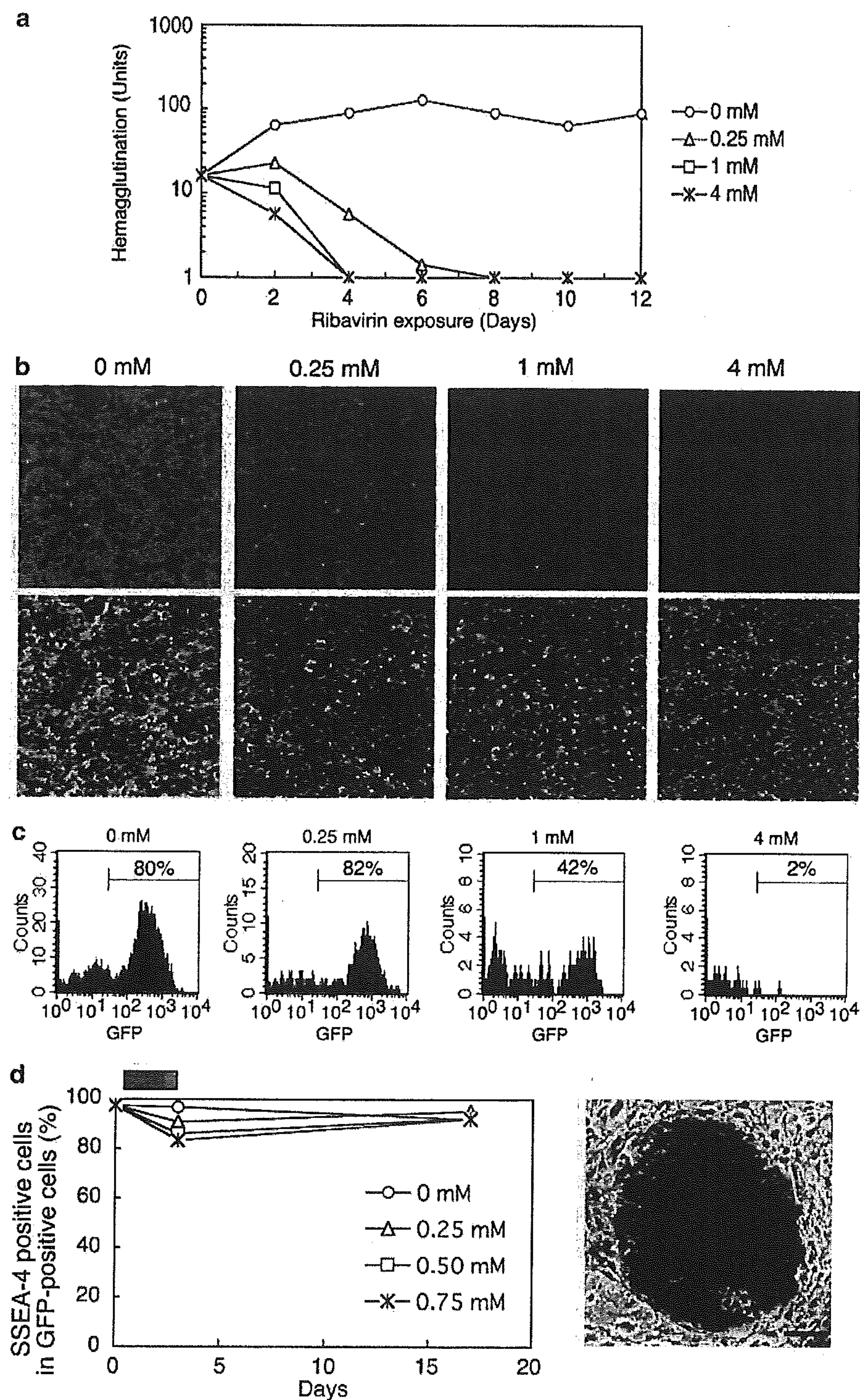

Figure 6 *Ribavirin-regulated transgene expression. (a) A rhesus kidney cell line (LLC-MK2) was infected with the SeV vector at 3 TU/cell. Ribavirin was started at various concentrations on day 2 after the infection. The formation of viral particles in the infected LLC-MK2 cells was examined by the hemagglutination assay. (b) The ribavirin-treated LLC-MK2 cells were observed under a fluorescent microscope after an 8-day exposure of ribavirin (upper, dark field; lower, bright field). (c) Ribavirin was added at various concentrations to the SeV-infected, fluorescent cynomolgus ES cells. The GFP expression was assessed by flow cytometry after a 3-day exposure of ribavirin. (d) The fractions of SSEA-4-positive ES cells were assessed by flow cytometry with anti-SSEA-4 before and after a 3-day exposure of ribavirin and are shown as a function of time (days) in the left panel. A gray bar indicates ribavirin treatment. ES cells were stained for alkaline phosphatase (in red) at day 21 after a 3-day exposure of 0.75 mM ribavirin and are shown in the right panel. Bar = 100 μm.*

CellQuest software (Becton Dickinson). For SSEA-4 staining, cells were incubated with a primary antibody, anti-SSEA-4 (MC-813-70; Chemicon, Temecula, CA, USA), and then a secondary antibody, PE-conjugated F(ab')2 fragment of rabbit anti-mouse immunoglobulins (DakoCytomation, Glostrup, Denmark). Cocultured BALB/c feeder cells could be distinguished from cynomolgus ES cells by using PE-conjugated anti-mouse

H-2d (SF1-1.1; PharMingen, San Diego, CA, USA), which does not react to cynomolgus cells but does react to BALB/c cells.

Teratoma formation

Cynomolgus ES cells (approximately 10^6 cells per site) were injected subcutaneously into the hind leg of 6- to 8-week-old nonobese diabetic/severe combined immunodeficient mice (Jackson Laboratory, Bar Harbor, ME, USA). The resulting tumors (usually 9–12 weeks after the injection) were dissected and fixed in 4% paraformaldehyde. For histological analysis, samples from the tumors were embedded in paraffin and stained with hematoxylin and eosin. To observe GFP fluorescence, samples were embedded in OTC compound (Sakura, Zoeterwoude, Netherlands), frozen, sectioned, and examined under a fluorescence microscope.

Hematopoietic differentiation

The mouse bone marrow stromal cell line OP9 was maintained in α-modified minimum essential medium (Invitrogen) supplemented with 20% FCS as described previously.[28] For induction of hematopoietic differentiation, ES cells were seeded onto a mitomycin C-treated confluent OP9 cell layer in six-well plates. Medium to support the differentiation was described elsewhere.[29] Cells at day 18 were placed in Methocult GF+ media (StemCell Technologies, Vancouver, Canada) at 1×10^4 and 1×10^5 cells per plate and clonogenic hematopoietic colonies were produced. After 14 days, individual colonies were removed and spun onto glass slides. Cells were stained with the Wright–Giemsa method. The nitro blue tetrazolium (NBT, Sigma) reduction test was performed on the cells as a granulocyte functional assay according to a previously described method.[30]

Neural differentiation

The induction of neural differentiation was carried out as described previously.[31] Day-4 embryoid bodies were plated onto tissue culture dishes and nestin-positive cells were selected in DMEM/F12 medium supplemented with 5 μg/ml of insulin (Sigma), 50 μg/ml of transferrin (Sigma), 30 nM selenium chloride (Sigma), and 5 μg/ml of fibronectin (Sigma) for 5 days. Cells were then trypsinized and plated in polyornithine-coated dishes (15 μg/ml) and expanded in N2 medium[32] supplemented with 1 μg/ml of laminin (Sigma) and 10 μg/ml of basic fibroblast growth factor (bFGF; Roche, Basel, Switzerland) for 6 days. Differentiation was induced by removal of bFGF. To confirm the neural differentiation, cells were stained with anti-human MAP-2. Briefly, cells were fixed in 4% paraformaldehyde in PBS and incubated with anti-human MAP-2 (HM-2; Sigma; diluted 1:4000) and then by Alexa Fluor 594-labeled antibody (diluted 1:500; Molecular Probe, Eugene, OR, USA). The samples were examined under a fluorescence microscope.

DNA-PCR

DNA-PCR for the SeV genome and GFP sequences was carried out as follows. DNA was extracted using the QIAamp DNA mini kits (Qiagen, Hilden, Germany) and 250 ng was used for each PCR with ExTaq (Takara, Shiga, Japan). Amplification conditions were 30 cycles of 94°C for 1 min, a variable annealing temperature (noted below) for 1 min, and 72°C for 1 min. The amplified products were run on 2% agarose gel and visualized by ethidium bromide staining. Primer sequences, annealing temperatures and product sizes were as follows: the SeV vector genome sequence: 5′-AGA GAA CAA GAC TAA GGC TAC C-3′ and 5′-ACC TTG ACA ATC CTG ATG TGG-3′ (55°C, 580 bp); the GFP sequence: 5′-CGT CCA GGA GCG CAC CAT CTT C-3′ and 5′-GGT CTT TGC TCA GGG CGG ACT-3′ (60°C, 356 bp). the cynomolgus β-actin sequence: 5′-CAT TGT CAT GGA CTC TGG CGA CGG-3′ and 5′-CAT CTC CTG CTC GAA GTC TAG GGC-3′ (60°C, 234 bp).

RNA-PCR

RNA-PCR for the SeV RNA genomic sequence was carried out as follows. Total RNA was extracted using RNA STAT-60 (Tel-Test, Friendswood, TX, USA). Reverse transcription was conducted by using Taqman reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). The product (250 ng) after the reverse transcription was used for the subsequent PCR as described above.

Acknowledgements

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Assessment of the International Prognostic Scoring System for Determining Chemotherapeutic Indications in Myelodysplastic Syndrome: Japanese Retrospective Multicenter Study

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Abstract

To standardize a rational therapeutic strategy of chemotherapy using the International Prognostic Scoring System (IPSS), we retrospectively analyzed 292 high-risk myelodysplastic syndrome (MDS) patients in 20 hospitals in Japan. Results of multivariate analysis of the data on patients who received all types of chemotherapy indicated that poor cytogenetics as shown by the IPSS was the only significant risk factor ($P = .047$). We then focused on the IPSS composition of each patient. The intermediate 2 (Int-2) category consisted of a heterogeneous group. We attempted to subdivide the category into Int-2A and Int-2B. Patients with good or intermediate cytogenetics had ≥5% bone marrow (BM) blasts (Int-2A), and most of the other patients had poor cytogenetics and ≤10% BM blasts (Int-2B). In the Int-2B category, overall survival for patients who received chemotherapy was significantly worse than for those who did not receive chemotherapy ($P = .005$). Most patients in the High category who had the diagnosis of MDS according to the World Health Organization classification had poor overall survival with or without chemotherapy. We propose the Int-2B and High categories may be considered possible high risk, whereas all patients in the Int-2A category and patients with more than 5% BM blasts in the Int-1 category may be categorized as being at possible intermediate risk. Our proposition may be useful for developing a chemotherapeutic strategy for patients with MDS in Japan.

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Key words: Myelodysplastic syndrome; Chemotherapy; Survival; Cytogenetics; Practice guidelines

1. Introduction

A therapeutic strategy for patients with myelodysplastic syndrome (MDS) is still controversial. In Europe, practice guidelines have recently been proposed in Italy and the United Kingdom [1,2]. In the United States, the National Comprehensive Cancer Network (NCCN) recently updated its guidelines, according to which stem cell transplantation is now one of the major options for treating MDS [3]. Although the upper age limit for stem cell transplantation has been gradually raised, large numbers of patients with MDS are too old for treatment with stem cell transplantation. New agents for MDS are being evaluated in preclinical and clinical studies, but the effectiveness of these agents is still questionable [4-9]. Many patients who cannot be treated with stem cell transplantation can be treated with chemotherapy using antileukemic agents.

The International Prognostic Scoring System (IPSS) is the most common instrument for prognosis [10]. Both European practice guidelines and the NCCN guidelines use the IPSS to determine therapeutic indications [1,2]. In the European practice guidelines, the indications for chemotherapy are determined with the IPSS (Table 1). According to the IPSS the Int-2 and High categories are considered indications for chemotherapy. Each risk group in the IPSS, however, contains heterogeneous patient characteristics. Thus the effectiveness of chemotherapy may vary among patients within a single risk group in the IPSS [11]. In devising a strategy for chemotherapy, it may be necessary to subdivide each risk group in the IPSS. In addition, we may have to verify whether the IPSS is an appropriate basis on which to select the type of chemotherapy. We retrospectively analyzed the cases of

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Table 1.
Chemotherapeutic Indications of the International Practice Guidelines for Myelodysplastic Syndromes*

	Italy	United Kingdom	United States (NCCN)
Publication year of the newest version	2002	2003	2005
IPSS category	Int-2/high	Int-2/high	Not recommended†
Age, y	≤54 or 55-64 (if ECOG 0-1)	≤65	Not recommended†
Induction therapy prior to stem sell transplantation	No	Yes	No

*IPSS indicates International Prognostic Scoring System; ECOG, Eastern Cooperative Oncology Group performance status.
†National Comprehensive Cancer Network (NCCN) guideline does not recommend standard chemotherapy for patients with myelodysplastic syndromes.

high-risk MDS patients from multiple centers in Japan to determine whether it is appropriate to use the IPSS to make decisions on chemotherapy and to standardize therapeutic strategy.

2. Methods

2.1. Patients

Our questionnaire regarding chemotherapy for MDS patients included information on patient age, sex, diagnosis, percentage of bone marrow (BM) blasts, number of lineages showing cytopenia, cytogenetic character, initial chemotherapy regimen, result of therapy, and survival. Patients were categorized into separate groups using 3 variables of the IPSS, that is, number of lineages showing cytopenia, percentage of BM blasts, and cytogenetics [10]. The definition of remission described by Okamoto et al was used [12]. We also inquired about treatment with stem cell transplantation. The questionnaire was sent to 27 institutes belonging to the Japanese Study Group for Intractable Hematopoietic Diseases. Responses were obtained from 20 institutes describing 326 MDS cases newly diagnosed between 1998 and 2003 (Table 2). Data were collected regarding all patients in the Int-2 and High risk categories and those in the Int-1 category who were to be treated with intensive therapy at each institute. On the basis of these criteria, we analyzed the cases of 292 evaluable patients. We excluded 28 patients lacking key data and 6 patients who did not fit a specific category. The study included 58 cases of refractory anemia (RA), 1 case of RA with ringed sideroblasts, 150 cases of RA with excess blasts (RAEB), 68 cases of RAEB in transformation (RAEBt), and 15 cases of chronic myelomonocytic leukemia according to the French-American-British (FAB) criteria. We also tried to establish a diagnosis for each patient by World Health Organization (WHO) classification [13,14]. There were 200 men and 92 women in the study.

2.2. Treatment Subgroups

We categorized patients into 3 groups according to their initial therapy (Table 3), that is, acute myeloid leukemia (AML)-like chemotherapy, low-dose chemotherapy, and supportive care. AML-like therapy included idarubicine (IDR) plus cytarabine (ara-C) [15], behenoylcytarabine (BHAC), 6-mercaptopurine (6-MP) plus daunorubicin (DNR) (BHAC-DM), DNR plus ara-C, and various other regimens. In some patients treated with BHAC-DM, etoposide was also administered, and those with DNR plus ara-C sometimes received oral 6-MP. Low-dose therapy included low-dose ara-C (10 mg/m^2 every 12 hours for 21 days), CAG regimen (low dose ara-C, aclarubicin plus granulocyte colony-stimulating factor) [16], low-dose ara-C plus anthracyclines, cytarabine ocfosfate, and other types of therapy. Some patients with CAG also received etoposide concurrently. We refer to the third group as having supportive care, which included all types of therapy without anticancer agents, that is, no therapy, vitamin K_2 [17] (plus vitamin D_3 in some cases [18]), anabolic steroids [19] (plus corticosteroids in some cases), prednisone, and various other types of therapy.

2.3. Statistics

Univariate probabilities of overall survival were calculated according to the method of Kaplan-Meier, and log-rank test was used for comparisons. In this study, 43 patients were

Table 2.
Patient Characteristics*

	n
Data collected	326
Evaluable	292
Lack of key data	28
Not classifiable	6
Among evaluable cases	
Male	200
Female	92
Age, y	
≤40	30
41-50	41
51-60	59
61-70	90
71-80	58
≥81	14
FAB criteria	
RA	58
RARS	1
RAEB	150
RAEBt	68
CMML	15
IPSS	
Int-1	75
Int-2	97
High	120

*FAB indicates French-American-British; RA, refractory anemia; RARS, RA with ringed sideroblasts; RAEB, RA with excess blasts; RAEBt, RAEB in transformation; CMML, chronic myelomonocytic leukemia; IPSS, International Prognostic Scoring System; Int, intermediate.

Table 3.
Initial Therapy*

Initial therapy	n
Chemotherapy	154
AML-like chemotherapy	
IDR + ara-C	34
BHAC-DM (± etoposide)	15
DNR + ara-C (± 6-MP)	10
Other	6
Unknown (lack of data)	10
Low-dose chemotherapy	
CAG (± etoposide)	32
Low dose ara-C	16
Low dose ara-C + anthracycline	13
SPAC	4
Other	5
Unknown (lack of data)	9
Supportive therapy	138
Symptomatic therapy	56
Vitamin K_2 (± vitamin D_3)	26
Anabolic steroids (± corticosteroids)	18
Corticosteroids	9
Cyclosporine	7
Other	20
Unknown	2

*Patients who received stem cell transplantation as initial therapy were included in the category of symptomatic therapy. Survival duration was censored at the time of stem cell transplantation. AML indicates acute myeloid leukemia; IDR, idarubicine; ara-C, cytarabine; BHAC-DM, behenoylcytarabine and 6-mercaptopurine (6-MP) plus daunorubicin (DNR); CAG, low-dose ara-C, aclarubicin plus granulocyte colony-stimulating factor; SPAC, cytarabine ocfosfate.

treated with allogeneic stem cell transplantation and 2 with autologous transplantation. In the analysis of the difference in survival for each category, patients treated with stem cell transplantation were censored at the time of transplantation. The remission rate also was calculated for each category of chemotherapy, and the χ^2 test was used to evaluate the significance of difference. To find poor prognostic factors for patients who received chemotherapy as initial therapy, we performed multivariate Cox proportional hazards regression analysis.

3. Results

3.1. Prognostic Variables in Survival

Table 4 shows the prognostic variables including each variable of the IPSS for patients who received any type of chemotherapy. We used data on patients receiving chemotherapy to find prognostic factors of relevance for determining therapeutic strategy. No significant differences were seen among either the FAB criteria or the IPSS categories. Among 3 IPSS variables, that is, percentage of BM blasts, karyotype, and number of lineages showing cytopenia, poor cytogenetics was the only variable that revealed a significant difference. To confirm the results of multivariate analysis, we analyzed Kaplan-Meier curves for all patients (Figure 1A) and for patients who received chemotherapy (Figure 1B) to determine the overall survival of patients in each cytogenetic category. The overall survival of patients with poor cytogenetics was significantly worse than that of patients with good cytogenetics ($P < .001$) and that of patients with intermediate cytogenetics ($P = .006$ for all patients, $P = .001$ for patients who received chemotherapy).

3.2. Int-2 MDS Subdivision

Patients were evaluated for each combination of the 3 variables (percentage of BM blasts, karyotype, and number of lineages showing cytopenia) to clarify the heterogeneity of each risk group of the IPSS (Figure 2). The Int-2 category consisted of a heterogeneous group including all types of cytogenetics. When we focused on MDS of the WHO classification (WHO-MDS), most patients in the High category had poor cytogenetics. In the Int-1 category, most patients had good or intermediate cytogenetics. We tried to divide the Int-2 category into Int-2A and Int-2B (Figure 3). As shown in Table 4, patients in the Int-2B category, who have poor cytogenetics, are not considered suitable for receiving chemotherapy, whereas those in the Int-2A category may be considered candidates. The Int-2A category consisted of patients with 5% to 30% blasts and good or intermediate cytogenetics. The Int-2B category contained patients with 0% to 10%

Table 4.
Multivariate Analysis with Cox Proportional Hazards Regression Model*

Variable	n	Odds Ratio	P
Age, y	154	1.01	.22
Sex			
Male	104	1.00	NA
Female	49	1.00	1.00
FAB criteria			
RAEB	53	1.00	NA
RAEBt	88	0.68	.24
CMML	10	1.03	.96
IPSS			
Int-1	11	1.00	NA
Int-2	48	0.87	.83
High	94	2.80	.28
BM blasts, %			
0-4	8	1.00	NA
5-9	28	1.10	.94
10-19	60	0.47	.36
20-29	57	0.44	.40
Cytopenia lineage			
1	25	1.00	NA
2	60	1.09	.82
3	68	0.71	.32
Cytogenetics			
Good	54	1.00	NA
Intermediate	28	1.07	.73
Poor	71	2.27	.047

*The cases of only 154 patients who received chemotherapy as initial therapy were analyzed. In sex, 1 case with missing data was not included. In French-American-British (FAB) criteria, 3 patients with refractory anemia (RA) are not shown. In cytopenia lineage, 1 case with no cytopenia lineage was not included. NA indicates not applicable; RAEB, RA with excess blasts; RAEBt, RAEB in transformation; CMML, chronic myelomonocytic leukemia; IPSS, International Prognostic Scoring System; Int, intermediate; BM, bone marrow.

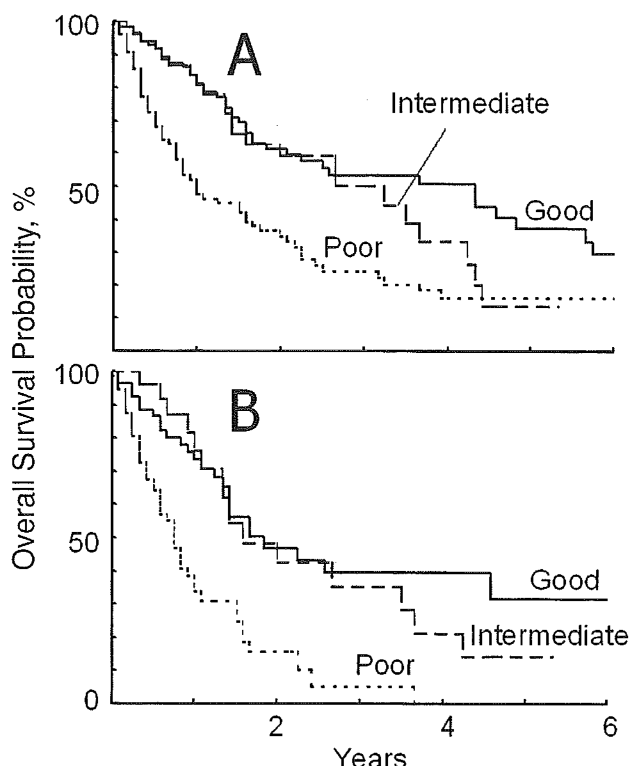

Figure 1. Kaplan-Meier estimates of each cytogenetic category of the International Prognostic Scoring System. Overall survival probability for all patients (A) and patients who received chemotherapy was categorized for the good (solid line; n = 118 for A and n = 53 for B), intermediate (large dashed line; n = 52 for A and n = 26 for B), and poor (small dashed line; n = 119 for A and n = 72 for B) cytogenetics groups. Three patients lacking data for survival duration were excluded. The survival probability of the poor cytogenetics group was significantly worse than for the good group ($P < .001$ for A and B) and the intermediate group ($P = .006$ for A and $P = .001$ for B).

blasts and poor cytogenetics. We compared the overall survival of patients who received either type of chemotherapy or supportive therapy as the initial therapy. In the Int-2A category, the overall survival of patients with chemotherapy was better but not significantly better (Figure 4A). In the Int-2B category, the overall survival of patients with chemotherapy was significantly worse than that of patients with supportive therapy ($P = .005$) (Figure 4B). The overall survival was not significantly different between the types of therapy in the High group of the WHO-MDS classification (Figure 4C). Although we found similar phenomena of survival by subcategorization with cytogenetics in patients with AML by the WHO classification, no statistical significance was seen because of numbers (data not shown).

We propose 3 possible risk groups based on IPSS subclassification (Figure 5). Our proposition may be reflected by the effectiveness of chemotherapy. The high-risk group consists of the Int-2B category and the High category in WHO-MDS. It is recommended that these patients not receive chemotherapy as initial therapy. The intermediate-risk group includes the Int-2 category and patients in the Int-1 category with more than 5% BM blasts. These patients may be treated with chemotherapy if applicable after consideration of the indications for stem cell transplantation. The low-risk group consists of patients with less than 5% BM blasts and should be treated with noncytotoxic agents.

4. Discussion

According to the Italian and British practice guidelines, patients in the Int-2 or High categories may be treated with chemotherapy [1,2]. Although the NCCN guidelines do not recommend standard remission induction chemotherapy for the majority of MDS patients, both European guidelines indicate that patients older than 65 years should not be treated with chemotherapy. The Italian guidelines suggest that chemotherapy is to be considered in the care of patients who do not qualify for stem cell transplantation, but the guidelines do not provide definite criteria for chemotherapy prior to stem cell transplantation. In contrast to the NCCN guidelines, the British guidelines recommend considering chemotherapy for all patients younger than 65 years in the Int-2 and High categories. If complete remission is attained, the British guidelines recommend stem cell transplantation if possible [20]. In Japan, patients who cannot be treated with stem cell transplantation may be treated with chemotherapy.

In this study, only 43 of 292 patients received stem cell transplantation, and the others received either chemotherapy or supportive therapy. The IPSS includes 3 kinds of variables to estimate risk: cytopenia, percentage of BM blasts, and cytogenetics [10]. Because multivariate analysis showed no significant difference among the IPSS risk groups, each risk group naturally consists of heterogeneous patients. We investigated whether these 3 variables are appropriate for determining the type of therapy. Multivariate analysis of the significance of IPSS score with regard to outcome among

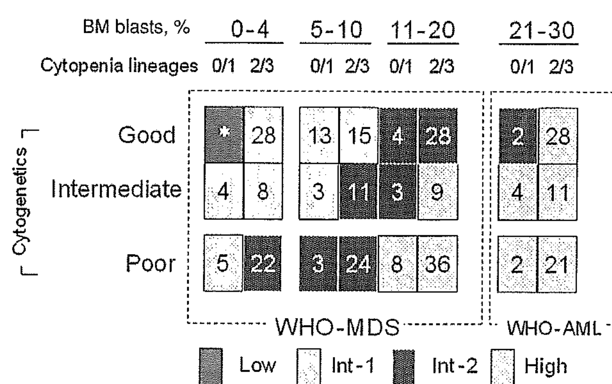

Figure 2. Columns show number of patients for corresponding combination of the 3 variables of the International Prognostic Scoring System (IPSS). The background of each column is shaded according to IPSS category. Among patients with myelodysplastic syndrome (MDS) according to the French-American-British criteria, numbers are presented separately between MDS (WHO-MDS) and acute myeloid leukemia (WHO-AML) according to the World Health Organization (WHO) classification. BM indicates bone marrow.

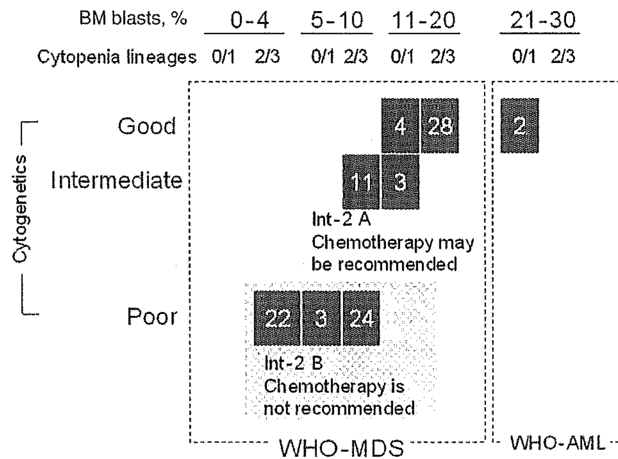

Figure 3. Number of patients in the intermediate 2 (Int-2) category. Owing to the heterogeneity of the Int-2 category, this category may be subdivided into 2 groups (Int-2A and Int-2B) on the basis of cytogenetics (good/intermediate and poor) according to the International Prognostic Scoring System variables. BM indicates bone marrow; WHO, World Health Organization; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia.

the patients treated with chemotherapy showed no significant difference among the different IPSS categories. To explain this phenomenon, we used multivariate analysis to analyze the outcome impact of each IPSS variable among patients who received chemotherapy. The IPSS gives a higher score to patients with a larger percentage of BM blasts. Our results suggest that patients with higher IPSS scores have better survival results when treated with chemotherapy. Similarly, the IPSS gives 0.5 points for patients with 2 or 3 lineages showing cytopenia. Multivariate analysis failed to demonstrate the prognostic significance of number of cytopenia lineages.

Cases with poor cytogenetics according to the IPSS definition had significantly poor results. It seems reasonable to separate patients into different groups according to the results of our cytogenetic study to recognize different possible patterns of response to chemotherapy. The Int-2 category is the most heterogeneous group among the 4 IPSS risk groups. Separation of the Int-2 category into 2 subgroups, Int-2A (good or intermediate cytogenetics) and Int-2B (poor cytogenetics) helped identify a subgroup of patients with poor survival. We should hesitate to use chemotherapy in the treatment of patients in the Int-2B category.

The Int-1 category consisted mainly of patients with good cytogenetics. The proportion of BM blasts in all patients in the Int-1 category was 10% or less. The therapeutic strategy may be based on blast percentages in the marrow. Patients with less than 5% blasts may be treated with methods other than chemotherapy, such as immunosuppressive therapy. The recommended therapy for Int-1 patients with more than 5% blasts is unknown, because we did not have enough data in this study (data not shown). Many patients in this category were censored at the time of treatment with stem cell transplantation without chemotherapy. Reclassification by percentage of BM blasts may therefore be useful for determining therapy for Int-1 patients.

The age of patients is another important factor for therapy selection. Both European practice guidelines demonstrate age as an independent factor [1,2] and recommend using 65 years as the upper age limit for chemotherapy. However, our analysis using multivariate estimate failed to demonstrate the significance of survival according to age. Therapeutic indications may be based on variables such as performance status (PS), organ dysfunction, and infectious status in addition to age.

We used a questionnaire to analyze survival, but there is the possibility of bias in interpreting the data. The age of patients treated with AML-like chemotherapy was lower

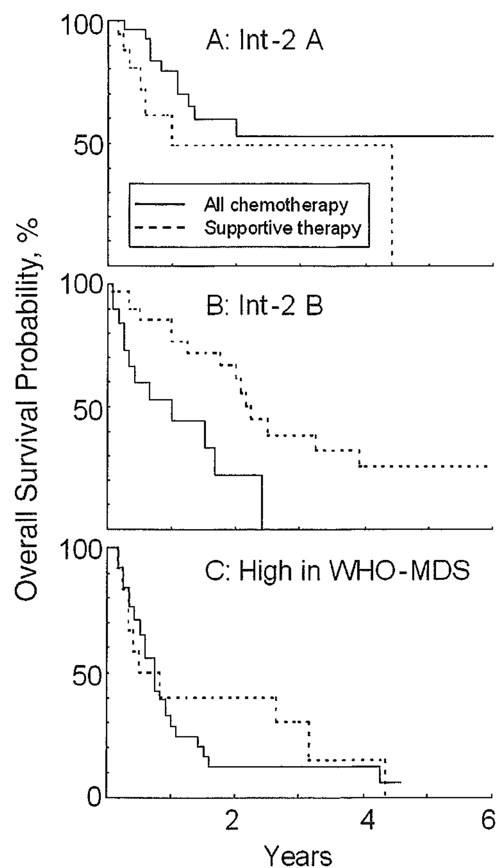

Figure 4. Kaplan-Meier estimates showing overall survival probability of patients in the intermediate 2A (Int-2A) and Int-2B categories. Patients were categorized according to cytogenetic category (good/intermediate and poor) of the International Prognostic Scoring System variables. Patients were further categorized according to initial therapy, that is, all types of chemotherapy (solid lines) and supportive care (dashed lines). We included 28 patients with chemotherapy and 19 with supportive therapy in the Int-2A category, 19 with chemotherapy and 30 with supportive therapy in the Int-2B category, and 38 with chemotherapy and 14 with supportive therapy in the analysis. In the Int-2B category, the survival of patients with chemotherapy was significantly worse than that of patients who received supportive therapy (P = .005). WHO indicates World Health Organization; MDS, myelodysplastic syndrome.