ENGRAFTMENT AND TUMOR FORMATION AFTER ALLOGENEIC IN UTERO TRANSPLANTATION OF PRIMATE EMBRYONIC STEM CELLS[1]

TAKAYUKI ASANO,[3,5] NAOHIDE AGEYAMA,[7] KOICHI TAKEUCHI,[4] MIKIO MOMOEDA,[6] YOSHIHIRO KITANO,[8]
KYOKO SASAKI,[2,5] YASUJI UEDA,[9] YUTAKA SUZUKI,[10] YASUSHI KONDO,[10] RYUZO TORII,[11]
MAMORU HASEGAWA,[9] SHIGEO OOKAWARA,[4] KIYONORI HARII,[5] KEIJI TERAO,[7] KEIYA OZAWA,[3] AND
YUTAKA HANAZONO[2,12]

Background. To achieve human embryonic stem (ES) cell-based transplantation therapies, allogeneic transplantation models of nonhuman primates would be useful. We have prepared cynomolgus ES cells genetically marked with the green fluorescent protein (GFP). The cells were transplanted into the allogeneic fetus, taking advantage of the fact that the fetus is so immunologically immature as not to induce immune responses to transplanted cells and that fetal tissue compartments are rapidly expanding and thus providing space for the engraftment.

Methods. Cynomolgus ES cells were genetically modified to express the GFP gene using a simian immunodeficiency viral vector or electroporation. These cells were transplanted in utero with ultrasound guidance into the cynomolgus fetus in the abdominal cavity (n=2) or liver (n=2) at the end of the first trimester. Three fetuses were delivered 1 month after transplantation, and the other, 3 months after transplantation. Fetal tissues were examined for transplanted cell progeny by quantitative polymerase chain reaction and in situ polymerase chain reaction of the GFP sequence.

Results. A fluorescent tumor, obviously derived from transplanted ES cells, was found in the thoracic cavity at 3 months after transplantation in one fetus. However, transplanted cell progeny were also detected (~1%) without teratomas in multiple fetal tissues. The cells were solitary and indistinguishable from surrounding host cells.

Conclusions. Transplanted cynomolgus ES cells can be engrafted in allogeneic fetuses. The cells will, however, form a tumor if they "leak" into an improper space such as the thoracic cavity.

[2] Division of Regenerative Medicine, Jichi Medical School, Tochigi, Japan.

[3] Division of Genetic Therapeutics, Jichi Medical School, Tochigi, Japan.

[4] Department of Anatomy, Jichi Medical School, Tochigi, Japan.

[5] Department of Plastic and Reconstructive Surgery, University of Tokyo, Tokyo, Japan.

[6] Department of Obstetrics and Gynecology, University of Tokyo, Tokyo, Japan.

[7] Tsukuba Primate Center, National Institute of Infectious Diseases, Ibaraki, Japan.

[8] Department of Surgery, National Center for Child Health and Development, Tokyo, Japan.

[9] DNAVEC Research Inc., Ibaraki, Japan.

[10] Discovery Research Laboratory and Safety Research Laboratory, Tanabe Seiyaku Co., Ltd., Osaka, Japan.

[11] Research Center for Animal Life Science, Shiga University of Medical Science, Shiga, Japan.

[12] Address correspondence to: Yutaka Hanazono, M.D., Ph.D., Associate Professor, Division of Regenerative Medicine, Center for Molecular Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi, Kawachi, Tochigi 329-0498, Japan. E-mail: hanazono@jichi.ac.jp.

Because human embryonic stem (ES) cell lines have dual abilities to proliferate indefinitely and differentiate into multiple tissue types (*1,2*), human ES cell-based transplantation therapies are considered to hold great promise in the treatment of a variety of diseases and injuries. To address the safety and efficacy of these therapies, allogeneic transplantation models of large animals, especially nonhuman primates, would be useful. However, there have been no reports on the transplantation of primate ES cells or their derivatives into allogeneic hosts thus far. There are two major reasons for this. First, the efficient and stable marking of primate ES cells has been difficult. It is necessary to distinguish transplanted allogeneic ES cell progeny from surrounding host cells. Second, the immune rejection of transplanted cells must be circumvented for a sustained engraftment. The cells would otherwise be cleared by immune responses.

We have previously reported a highly efficient gene transfer into cynomolgus ES cells using a lentiviral vector derived from the simian immunodeficiency virus (SIV) (*3*). Lentiviral transgene expression in ES cells is stable with minimal levels of transcriptional silencing (*4,5*). In addition, we have established cynomolgus ES cell sublines stably expressing green fluorescent protein (GFP) by electroporation of a GFP-expressing plasmid (*6*). The GFP expression in the cells is stable even after the terminal differentiation of the cells. By using cynomolgus ES cells genetically modified to express GFP, it is now possible to distinguish transplanted allogeneic ES cell progeny from surrounding host cells because GFP will serve as a good genetic tag.

The early gestational fetus may be a good recipient with which to circumvent immune rejection, because the immune system is so immature as not to induce an immune response. Furthermore, in the animal fetus, "space" would be relatively available as compared with the adult, because of the rapid expansion of the fetal tissue compartments. Thus, transplanted cells could be engrafted without any conditioning of

recipients such as irradiation or immunosuppressive treatment. This strategy was indeed successful in achieving human and sheep chimeras (up to 10–30% human blood cells) after in utero human hematopoietic stem cell transplantation into fetal sheep (7,8). The engraftment of human mesenchymal stem cells in fetal sheep has also been reported (9). In addition, the engraftment of human hematopoietic stem cells was observed in the setting of in utero transplantation in other animals including nonhuman primates (10,11) and canines (12). In the present study, we have transplanted nonhuman primate (cynomolgus macaque) ES cells stably expressing GFP into allogeneic fetuses in utero and examined the in vivo fate of the transplanted cells using GFP as a genetic tag.

MATERIALS AND METHODS

Vectors

An SIV vector carrying the enhanced GFP (Clonetech, Palo Alto, CA) was used for transduction of cynomolgus ES cells (3). The vector was constructed as previously reported (13). The vector was self-inactivating because it was constructed with a deletion of the U3 region in the 3' long terminal repeat and was pseudotyped with the vesicular stomatitis virus glycoprotein. The GFP gene was driven by the cytomegalovirus promoter.

A plasmid expressing the enhanced GFP (pCE-EGFP) was used for transfection of cynomolgus ES cells (6). The plasmid was derived from pEGFP-1 (Clontech) and contains the human elongation factor 1 alpha promoter to express GFP.

Genetically Modified Cynomolgus ES Cells

Cynomolgus ES cells (CMK10) were transduced using the SIV vector at 10 transducing units per cell as previously described (3). The transduced cells were cultured in an undifferentiated state for more than 1 month before transplantation. A subline stably expressing GFP (CMK6G) was established from the parental cynomolgus ES cell line (CMK6) by electroporation of the plasmid pCE-EGFP as previously described (6).

All cynomolgus ES cells were cultured on a mouse embryonic fibroblast (MEF) feeder layer as previously described (14). MEF cells were obtained from fetal BALB/c mice that have the H-2^d haplotype (Clea, Tokyo, Japan). Cynomolgus ES cells were dissociated from MEF cells using 0.05% trypsin/phosphate-buffered saline (PBS), washed with Hanks' balanced salt solution (Gibco, Gaithersburg, MD), resuspended in 0.4 mL of Hanks' balanced salt solution, and used for transplantation.

Flow Cytometry

The expression of GFP by ES cells was assessed before transplantation by flow cytometry using a FACScan (Becton Dickinson, Frank-

lin Lakes, NJ) with excitation at 488 nm and fluorescence detection at 530±30 nm. The co-cultured BALB/c MEF feeder cells could be distinguished from cynomolgus ES cells by using the phycoerythrin-conjugated mouse anti-mouse H2K^d monoclonal antibody (PharMingen, San Diego, CA), which does not react to cynomolgus cells but reacts to BALB/c cells.

Animals and Transplant Procedures

Four pregnant cynomolgus monkeys aged 18 years (IA10-1, IA10-2, IH10-1, and IH6G-1) were obtained by mating monkeys and were bred in the Tsukuba Primate Center (Ibaraki, Japan) as previously described (15). The animals were certified free of intestinal parasites and were seronegative for simian type-D retrovirus, herpes virus B, varicella zoster-like virus, measles virus, and SIV (16). All animals were individually housed.

Monkeys were anesthetized by intramuscular administration of ketamine hydrochloride (Ketalar, 10 mg/kg; Sankyo, Tokyo, Japan). Genetically modified cynomolgus ES cells (3.6–4.8×10^6 cells/fetus, approximately 2.0×10^8 cells/kg) were injected into the fetal abdominal cavity (IA10-1's fetus [IA10-1ft] and IA10-2ft) or liver (IH10-1ft and IH6G-1ft) through a 23-gauge needle using an ultrasound-guided technique at approximately the end of the first trimester (Table 1). The full term is 165 days. The weight of the fetus at the time of transplantation was estimated at 20 g (17). All surgical procedures and postoperative care of animals were performed in accordance with the Rules for Animal Care and Management of Tsukuba Primate Center (18) and Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan (19). The protocol of experimental procedures was approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases (Tokyo, Japan).

Tissue Sampling

Three of the four pregnant monkeys (IA10-1, IA10-2, and IH10-1) underwent a cesarean section 1 month after transplantation. The other monkey (IH6G-1) underwent a cesarean section 3 months after transplantation. All operations were conducted under isoflurane (A.D.S.1000; Shin-ei, Tokyo, Japan)-induced general anesthesia. Each of the pregnant monkeys carried one fetus. The sex of the fetus was confirmed by examining the morphology of external genitalia. The three fetuses (IA10-1ft, IA10-2ft, and IH10-1ft) delivered at 1 month after transplantation were cannulated through the umbilical vessel, and umbilical blood was collected. The other fetus (IH6G-1ft) delivered 3 months after transplantation was cannulated through the intracardiac cavity, and fetal blood was collected. After collection of the blood, the fetuses were irrigated with natural saline for mercy killing and fetal blood was completely washed out.

Fetal tissues of brain, lung, heart, thyroid, thymus, liver, spleen, kidney, small intestine, skeletal muscle, and cartilage were collected from each of the fetuses. Tissue samples were fixed for 4 hr at 4°C in 4% paraformaldehyde in PBS and embedded in paraffin. Tissue

TABLE 1. Allogeneic in utero transplantation of genetically modified cynomolgus ES cells

Fetus	IA10-1ft	IA10-2ft	IH10-1ft	IH6G-1ft
Sex	Male	Female	Male	Male
Transplanted ES cell line	CMK10	CMK10	CMK10	CMK6G
% GFP expression	53.5	43.4	43.5	91.4
Copy number of GFP gene per cell	1.89	1.89	1.89	1.00
Number of transplanted cells	3.6×10^6	3.9×10^6	4.8×10^6	3.9×10^6
Gestational age				
At transplantation	61 (0.37)	54 (0.33)	50 (0.30)	49 (0.30)
At delivery	90 (0.55)	88 (0.53)	84 (0.51)	150 (0.91)
Days of incubation	29 (0.18)	34 (0.21)	34 (0.21)	101 (0.61)
Injection site	Abdominal cavity	Abdominal cavity	Liver	Liver

Values in parentheses represent ratios of days to the full term (165 days).

samples were also embedded in the OCT compound (Sakura, Tokyo, Japan) and frozen in liquid nitrogen and stored at −80°C. In addition, raw samples were frozen in liquid nitrogen and stored at −80°C for subsequent cellular DNA extraction.

Quantitative polymerase chain reaction (PCR)

Cellular DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). To assess the fraction of transplanted cell progeny in each fetal tissue, quantitative DNA PCR for the GFP sequence was performed using 250 ng of DNA as template. The GFP standards consisted of DNA extracted from the MGirL22Y cells (which contain one copy of the GFP sequence per cell) serially diluted with control cynomolgus genomic DNA. The negative control was DNA extracted from naive cynomolgus peripheral blood. PCR of the β-actin sequence was also performed to document initial DNA amounts of samples. The primer set for the GFP sequence was 5'-CGT CCA GGA GCG CAC CAT CTT C-3' and 5'-GGT CTT TGC TCA GGG CGG ACT-3'. The primer set for the β-actin sequence was 5'-CAT TGT CAT GGA CTC TGG CGA CGG-3' and 5'-CAT CTC CTG CTC GAA GTC TAG GGC-3'. Each PCR cycle consisted of four steps (denaturing, annealing, extension, and data acquisition). Amplification conditions for the GFP sequences were 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and 80°C for 15 sec with 40 cycles. Amplification conditions for the β-actin sequence were 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and 75°C for 15 sec with 40 cycles. The final step was inserted to suppress the generation of primer dimers. The temperature of the final step was optimized for each PCR.

PCR was performed in the presence of SYBR Green using the ABI PRISM 7700 sequence detection system (Perkin-Elmer, Foster, CA) with buffers, nucleotides, and Taq polymerase from the QuantiTect SYBR Green PCR Kit (Qiagen) as directed. All quantitative PCRs were certified with each run to yield a linear amplification in the range of the intensity of the positive control series (0.0001–1.0 copy/cell, correlation coefficient >0.98). The threshold cycle (Ct) value of the GFP sequence was normalized based on the Ct value of the internal control β-actin sequence in the same sample. To calculate the fraction (%) of transplanted cell progeny in each tissue, the copy number of the GFP gene per tissue cell was divided by the copy number of the GFP gene per transplanted cell. Amplification products were also analyzed on 2% agarose gel followed by ethidium-bromide staining.

In Situ PCR

In situ detection of transplanted cell progeny was performed by amplifying the GFP sequence as previously reported (20). The primer sequences were the same as used for the quantitative PCR described above. The reaction mixture consisted of 420 μM dATP, 420 μM dCTP, 420 μM dGTP, 378 μM dTTP, 42 μM digoxigenin-labeled dUTP (Roche, Mannheim, Germany), 0.8 μM of each GFP primer, 4.5 mM MgCl$_2$, PCR buffer (Mg^{2+} free), and 4 U of Takara Taq DNA polymerase (Takara, Tokyo, Japan). Slides were covered with the Takara Slide Seal for in situ PCR (Takara). PCR was performed using the PTC100 Peltier Thermal Cycler (MJ Research, Watertown, MA) with the following conditions: 94°C for 1 min and 55°C for 1 min with 15 cycles. After amplification, cover seals were lifted off and slides were washed with PBS at room temperature three times each for 5 min.

The digoxigenin incorporated-DNA fragments were detected using horseradish peroxidase (HRP)-conjugated rabbit F(ab') anti-digoxigenin antibody (Dako, Glostrup, Denmark) followed by histochemical staining. Briefly, slides were incubated at 37°C for 3 hr with the HRP-conjugated rabbit F(ab') anti-digoxigenin antibody solution diluted (1:100) in blocking solution (2% bovine serum albumin and 5% normal horse serum in PBS) and rinsed with PBS at room temperature three times each for 5 min. Slides were then stained for HRP using the Vector SG substrate kit (Vector, Burlingame, CA). The reaction was terminated by transferring slides into a water bath

upon development of a dark blue or black color (usually requiring 3–10 min to develop the signal). Finally, sections were counterstained with the Kernechtrot that stains nucleotides, washed with water, mounted in glycerol, and examined under a light microscope.

RESULTS

In Utero Transplantation

Genetically modified cynomolgus ES cells that stably express GFP were obtained either by transduction with an SIV vector carrying the GFP gene or by transfection with a GFP-expressing plasmid.

The transduction of cynomolgus ES cells (CMK10) using the SIV vector was conducted once at 10 transducing units per cell. The transduction efficiency was about 50% (Table 1). Each transduced cell was estimated to contain 1.89 copies of the GFP gene by quantitative PCR (data not shown). The transduced ES cells were cultured in an undifferentiated state for more than 1 month before transplantation to confirm that GFP expression was stable. These cells were used for transplantation (to IA10-1ft, IA10-2ft, and IH10-1ft; Table 1) without further enrichment of GFP-positive cells. Thus, the fraction of GFP-positive cells remained about 50% at transplantation.

On the other hand, a cynomolgus ES subline (CMK6G) was a cloned, stable transfectant expressing the GFP gene. It was established from the parental cynomolgus ES cell line (CMK6) by electroporation of the GFP-expressing plasmid. Virtually all (>90%) CMK6G cells expressed the GFP gene at transplantation (to IH6G-1ft; Table 1). Each ES cell contained one copy of the GFP gene (data not shown).
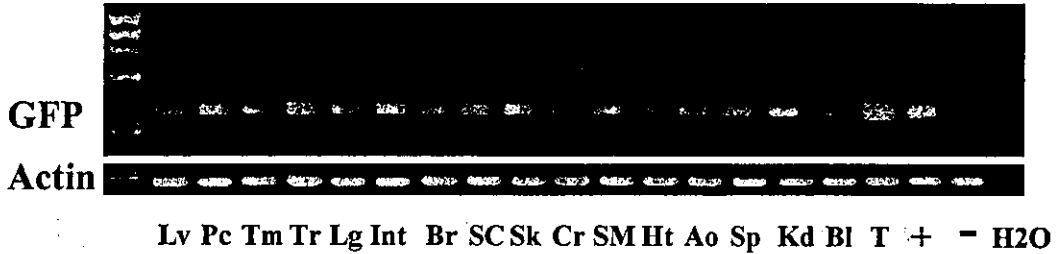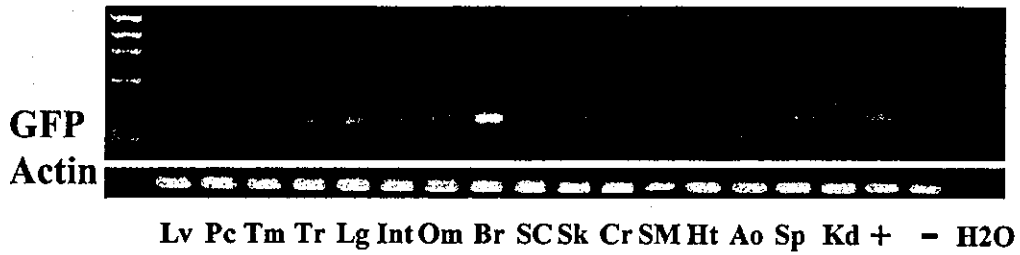
The GFP-expressing cynomolgus ES cells ($3.6–4.8\times10^6$ cells/fetus, approximately 2.0×10^8 cells/kg) were transplanted in utero into four allogeneic fetuses in the abdominal cavity (IA10-1ft and IA10-2ft) or liver (IH10-1ft and IH6G-1ft) under ultrasound guidance at approximately the end of the first trimester (Table 1). There were no complications during the pregnancies of all four mother monkeys. The three fetuses (IA10-1ft, IA10-2ft, and IH10-1ft) that received CMK10 cells were delivered by cesarean section 1 month after transplantation. The other fetus (IH6G-1ft), which re-

TABLE 2. Fractions (%) of transplanted cell progeny in fetal tissues

Fetus	IA10-1ft	IA10-2ft	IH10-1ft	IH6G-1ft
Lv-Liver	0.00	0.00	0.01	1.24
Pc-Pancreas	0.10	0.00	0.01	1.54
Tm-Thymus	0.01	0.00	0.00	0.01
Tr-Thyroid	0.02	0.00	0.01	0.34
Lg-Lung	0.01	0.00	0.02	0.12
Int-Small intestine	0.00	0.25	0.00	0.09
Om-Omentum	1.23	0.00	0.02	N.D.
Br-Brain	0.01	0.00	0.02	0.08
SC-Spinal cord	0.02	0.14	0.01	0.38
Sk-Skin	0.02	0.01	0.03	0.18
Cr-Cartilage	0.02	0.05	0.00	0.82
SM-Skeletal muscle	0.01	0.00	0.00	1.04
Ht-Heart	0.00	0.00	0.01	0.83
Ao-Aorta	0.03	0.00	0.00	0.16
Sp-Spleen	0.05	0.00	0.01	0.24
Kd-Kidney	0.00	0.00	0.00	1.20
Bl-Blood	ND	ND	ND	0.21

ND, not determined.

a

GFP

Actin

Lv Pc Tm Tr Lg Int Om Br SC Sk Cr SM Ht Ao Sp Kd +   − H2O

GFP

Actin

Lv Pc Tm Tr Lg Int Br SC Sk Cr SM Ht Ao Sp Kd Bl T +   − H2O
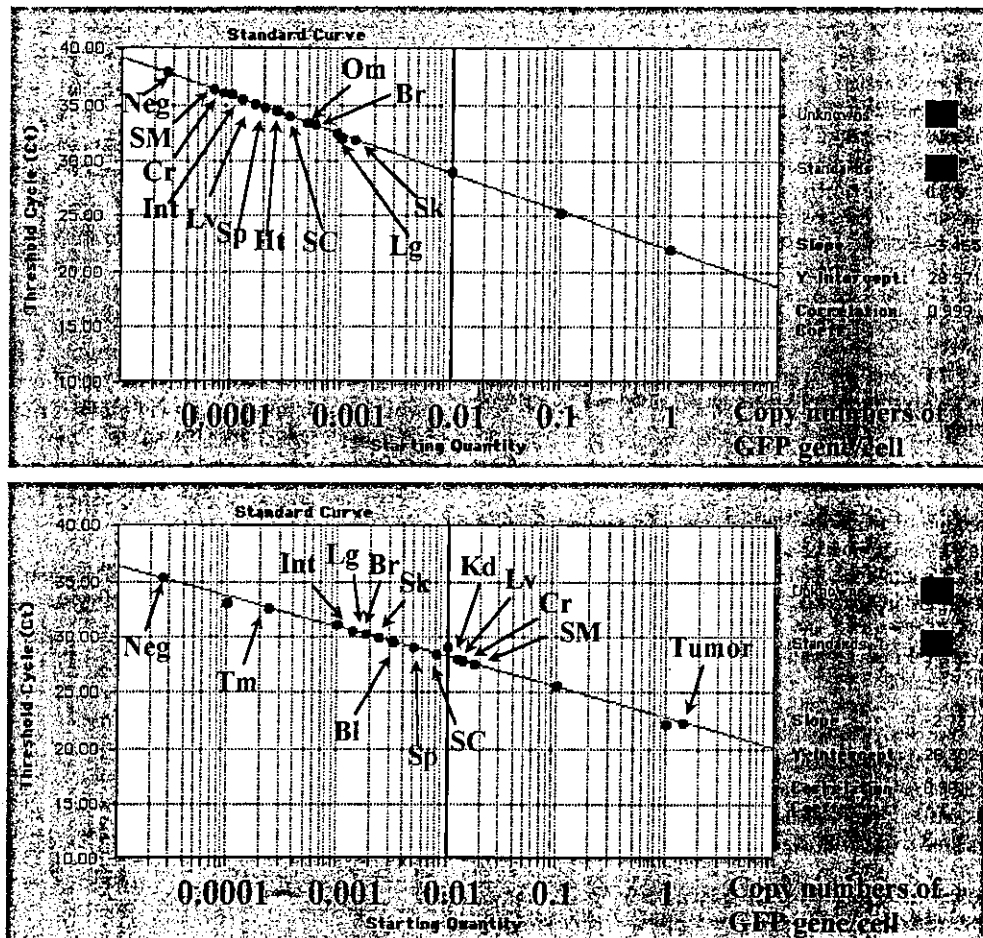
b

FIGURE 1. Detection of transplanted cell progeny by PCR of the GFP sequence. (a) Gels represent the samples from IH10-1ft at 1 month (34 days) after transplantation (*upper*) and IH6G-1ft at 3 months (101 days) after transplantation (*lower*). PCR of the β-actin sequence was simultaneously performed as controls. +, CMK6G; −, naive cynomolgus cells; T, tumor. Tissue abbreviations are listed in Table 2. (b) Standardized amplification curves of the quantitative PCR for the GFP sequence are shown. Curves represent the PCR results of IH10-1ft at 1 month (34 days) after transplantation (*upper*) and IH6G-1ft at 3 months (101 days) after transplantation (*lower*). Black dots represent the standards and grey dots represent each fetal tissue. Neg, negative control (naive cynomolgus cells).

ceived CMK6G cells, was delivered by cesarean section 3 months after transplantation, just 2 weeks before the expected delivery date.

In Vivo Detection of Transplanted Cell Progeny

Fetal tissues were collected as listed in Table 2. To detect transplanted cell progeny in fetal tissues, we extracted cellular DNA from each tissue and performed PCR of the GFP transgene sequence. Figure 1a shows representative results at 1 month (IH10-1ft, upper panel) and at 3 months (IH6G-1ft, lower panel) after transplantation. Transplanted cell progeny were widely distributed in multiple fetal tissues. The fraction of transplanted cell progeny in each tissue was estimated at 0.01 to 1% by quantitative PCR for the GFP sequence (Fig. 1b; the same monkeys as those in Fig. 1a). The limit of detection was 1 in 10,000 cells (0.01%). The quantitative PCR results of all the four monkeys are summarized in Table 2.

The transplanted cell distribution considerably varied among individual animals, as was the case with a previous report using human mesenchymal stem cells (9). The fractions of transplanted cell progeny in IH6G-1ft that received CMK6G cells were one-log higher than those in the other fetuses that received CMK10 cells (Table 2). IA10-1ft and IA10-2ft received cells in the abdominal cavity, and large numbers of transplanted cell derivatives were detected in tissues in the abdominal cavity such as the omentum (IA10-1ft, 1.23%) and small intestine (IA10-2ft, 0.25%). IH10-1ft and IH6G-1ft received cells in the liver, and considerable numbers of transplanted cell derivatives were detected in tissues distant from the injection site (liver) such as skin (IH10-1ft, 0.03%; IH6G-1ft, 0.18%), lung (IH10-1ft, 0.02%; IH6G-1ft, 0.12%), and brain (IH10-1ft, 0.02%; IH6G-1ft, 0.08%).

We then examined the tissue sections by in situ PCR, which amplified the GFP sequence. Figure 2 shows success-
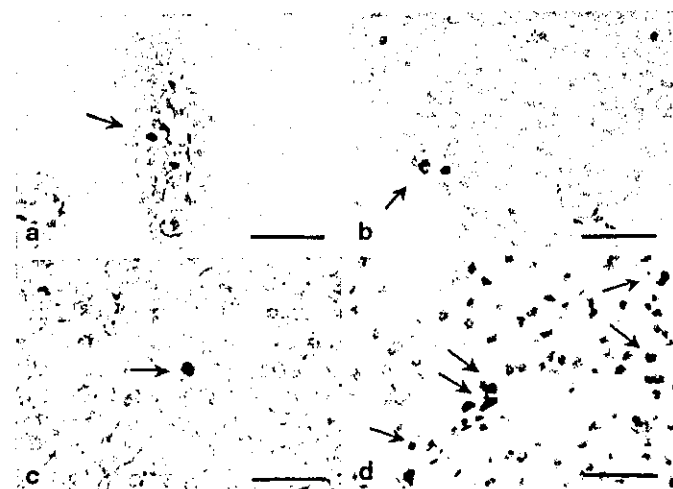
ful in situ detection of transplanted ES cell derivatives in the small intestine (a), spinal cord (b and d), and liver (c). All cells detected were solitary and indistinguishable from surrounding host cells by microscopic examination. Unfortunately, we could not further characterize the cells by staining specific surface markers, because the procedures for the in situ PCR impaired cell membranes. We tried to detect GFP fluorescence under a fluorescent microscope but were hampered by the high autofluorescence of tissue samples. We also tried to immunostain the samples using anti-GFP antibody (Clontech), but GFP-specific immunostaining could not be detected.

Tumor Formation

Although no tumors were observed in the fetuses delivered at 1 month after transplantation, a cystic tumor of $4 \times 3 \times 2.5$ cm was observed in the thoracic cavity in the fetus delivered at 3 months after transplantation (Fig. 3a). Both lungs remained deflated after the tumor resection (Fig. 3b). The tumor was derived from transplanted ES cells, because GFP fluorescence in the tumor was clearly observed under a fluorescent scope (Fig. 3, c and d) and because the GFP gene was detected by in situ PCR (Fig. 3e). The tumor consisted of many types of epithelial cells (Fig. 3f), but cells derived from other embryonic germ cell layers were rare. Thus, strictly speaking, the tumor may not be a teratoma but a teratoma-related tumor, because teratomas should consist of all three embryonic germ layer cells. Of note, no tumor was found in any other tissues of the animal even at the injection site (liver), although transplanted cell derivatives were detected at the site by in situ PCR (Fig. 2c).

DISCUSSION

Transplanted ES cell progeny were detected in multiple fetal tissues at approximately 1% or higher at 3 months after transplantation. They were even observed in the central nervous system, which was distant from the injection site. All cells detected were solitary and indistinguishable from surrounding host cells by microscopic examination. The fusion of transplanted ES cells with preexisting host cells may in part account for such a change of phenotype. Although the cell fusion frequency was recently optimized up to 1% under special conditions (heat-shocked epithelial cells vs. mesenchymal stem cells) (21), cell fusion usually occurs at one per 10^4 to 10^6 cells (22,23). Therefore, it is unlikely that all the detected cells were attributable to fusion events in our study. The transplanted cell fraction is considerably high, given that relatively limited numbers of ES cells were transplanted into the early gestational fetus. Transplanted ES cells might effectively compete with host cells to achieve significant expansion after transplantation.

To deliver transplanted cells to fetal organs, direct injection of cells into the circulation (such as intracardiac injection or injection by umbilical vein) might be more effective than intrahepatic or intraperitoneal injection (24). Unfortunately, cynomolgus monkey fetuses at the first trimester were so small (hearts were about 1 cm long) that such injection methods seemed difficult even under the finest ultrasound guidance. We have tried intrahepatic injection as well as intraperitoneal injection. We have performed intrahepatic injection for two fetal monkeys (in this study) and they were

FIGURE 2. Detection of transplanted cell derivatives by in situ PCR of the GFP sequence. Typical GFP sequence-positive cells (*arrows*). (a) A villus of small intestine at 1 month after transplantation (IA10-2ft). (b) Spinal cord at 1 month after transplantation (IA10-2ft). (c) Liver at 3 months after transplantation (IH6G-1ft). (d) Spinal cord at 3 months after transplantation (IH6G-1ft). Bars=50 μm.
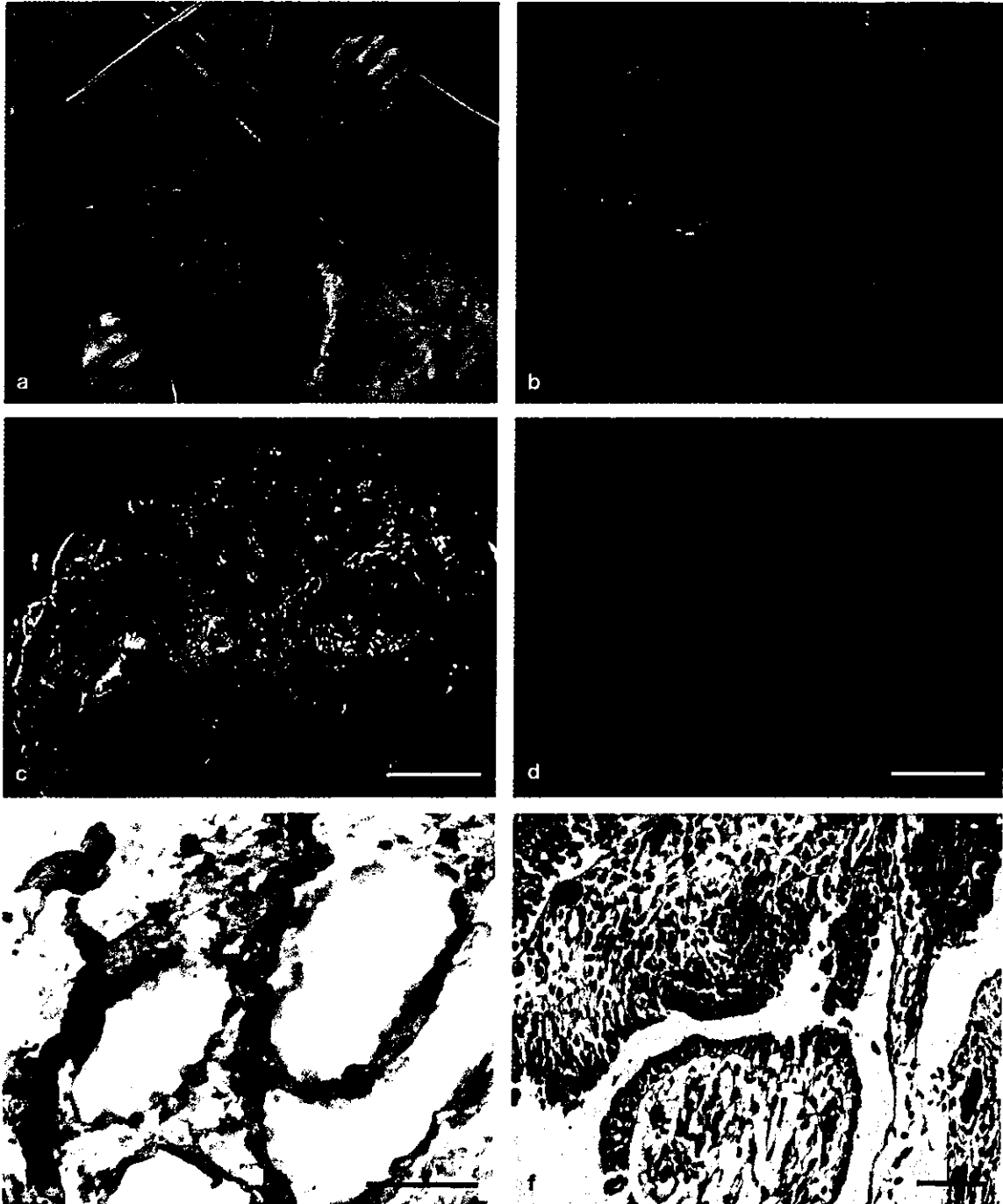
FIGURE 3. Tumor formation in a cynomolgus fetus transplanted with allogeneic ES cells. (a) A tumor (4×3×2.5 cm) was detected in the thoracic cavity at 3 months after transplantation (IH6G-1ft). (b) Both lungs remained deflated after the tumor resection. The tumor was observed in a bright field (c) and in a dark field (d) under a fluorescent scope. Bars in c and d=1 cm. (e) The GFP gene was detected in the tumor cells by in situ PCR. (f) The tumor mainly consisted of epithelial cells (hematoxylin-eosin staining). Bars in e and f=50 μm.

alive until delivery. We have also performed intrahepatic injection for nine fetal sheep (in another series of experiments), and only two of them resulted in abortion (our unpublished data). The abortion rate was not higher than that by the intraperitoneal injection. Thus, the intrahepatic approach was as safe as the intraperitoneal one.

It is well known that primate ES cells form teratomas after transplantation into immunodeficient mice (1,2). In the present study, a tumor was found in the thoracic cavity of a cynomolgus fetus at 3 months after the transplantation of allogeneic ES cells. The tumor was obviously derived from the transplanted ES cells, because it expressed GFP. To our knowledge, this is the first report of teratoma-related tumor formation in primates after allogeneic transplantation of primate ES cells. From this observation, it is obvious that successful therapeutic use of ES cell-derived donor cells would

require the generation of essentially pure differentiated cell cultures. No tumor was found, however, at other sites including the injection site. In explanation of the tumor site (the thoracic cavity), some ES cells might have been directly injected or leaked into the thoracic cavity during the transplantation procedure, although we carefully targeted the cells inside the liver under ultrasound guidance. Of note, no tumor was detected in any of the fetuses delivered at 1 month after transplantation. One month might be too short to allow teratoma formation. In fact, we have observed teratoma formation at 9 to 12 weeks after transplantation into immunodeficient (NOD/SCID) mice (our unpublished data).

From our observation, it is likely that there was "space" in the expanding fetal tissues that was available for homing and engraftment of transplanted ES cells. The allogeneic fetal host generated space in each tissue from which ES cells could obtain proper growth and regulatory signals. Thus, when ES cells are lodged in such a proper space, they can be engrafted and adapted. On the other hand, when transplanted cells "leak" into improper spaces such as the thoracic cavity (from which the cells cannot obtain proper signals), then the cells may form tumors.

ES cell-based transplantation into adult hosts requires immunosuppression of the host for the sustained engraftment of transplanted cells (25,26). In this study, ES cells were transplanted into four different fetal hosts and transplanted cell progeny were detected in all the animals without immunosuppressive treatment. In addition, we found a large teratoma-related tumor 3 months after transplantation in the fetus without immune rejection. These results suggest that the fetuses were tolerant of the transplanted ES cells. Another group has reported that transplantation of human peripheral blood stem cells into rhesus monkey fetuses resulted in the generation of chimeric rhesus infants (9), although Lindton et al. (27) have reported that the primate fetus may be more immune competent than experimental animals. If transplanted ES cells were to become permanent components of the host body without forming teratomas, we would be able to obtain immunocompetent adult recipients that are tolerant of grafts of the same ES cell origin. This would provide ideal primate recipients for ES cell-based transplantation studies.

REFERENCES

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998; 282: 1145.
2. Reubinoff BE, Pera MF, Fong C, et al. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 2000; 18: 399.
3. Asano T, Hanazono Y, Ueda Y, et al. Highly efficient gene transfer into primate embryonic stem cells with a simian lentivirus vector. Mol Ther 2002; 6: 162.
4. Lois C, Hong EJ, Pease S, et al. Germline transmission and tissue-specific expression of transgenesis delivered by lentiviral vectors. Science 2002; 295: 868.
5. Pfeifer A, Ikawa M, Dayn Y, et al. Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. Proc Natl Acad Sci USA 2002; 99: 2140.
6. Takada T, Suzuki Y, Kondo Y, et al. Monkey embryonic stem cell lines expressing green fluorescent protein. Cell Transplant 2002; 11: 631.
7. Flake AW, Harrison MR, Adzick NS, et al. Transplantation of fetal hematopoietic stem cells in utero: the creation of hematopoietic chimeras. Science 1986; 233: 776.
8. Zanjani ED, Pallavicini MG, Flake AW, et al. Engraftment and long term expression of human fetal hemopoietic stem cells in sheep following transplantation in utero. J Clin Invest 1992; 89: 1178.
9. Liechty KW, MacKenzie TC, Shaaban AF, et al. Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. Nat Med 2000; 6: 1282.
10. Harrison MR, Slotnick RN, Crombleholme TM, et al. In-utero transplantation of fetal liver haemopoietic stem cells in monkeys. Lancet 1989; 2: 1425.
11. Tarantal AF, Goldstein O, Barley F, et al. Transplantation of human peripheral blood stem cells into fetal rhesus monkeys (*Macaca mulatta*). Transplantation 2000; 69: 1818.
12. Omori F, Lutzko C, Abrams-Ogg A, et al. Adoptive transfer of genetically modified human hematopoietic stem cells into preimmune canine fetuses. Exp Hematol 1999; 27: 242.
13. Nakajima T, Nakamaru K, Ido E, et al. Development of novel simian immunodeficiency virus vectors carrying a dual gene expression system. Hum Gene Ther 2000; 11: 1863.
14. Suemori H, Tada T, Torii R, et al. Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. Dev Dyn 2001; 222: 273.
15. Honjo S, Cho F, Terao K. Establishing the cynomolgus monkey as a laboratory animal. Adv Vet Sci Comp Med 1984; 28: 51.
16. Buchl SJ, Keeling ME, Voss WR. Establishing specific pathogen-free (SPF) nonhuman primate colonies. ILAR J 1997; 38: 22.
17. Tarantal AF. Interventional ultrasound in pregnant macaques: embryonic/fetal applications. J Med Primatol 1990; 19: 47.
18. Honjo S. The Japanese Tsukuba Primate Center for Medical Science (TPC): an outline. J Med Primatol 1985; 14: 75.
19. Primate Society of Japan. Guiding principles for animal experiments using nonhuman primates. Primate Res 1986; 2: 111.
20. Hasse AT, Retzel EF, Staskus KA. Amplifications and detection of lentiviral DNA inside cells. Proc Natl Acad Sci USA 1990; 87: 4971.
21. Spees JL, Olson SD, Ylostalo J, et al. Differentiation, cell fusion, and nuclear fusion during ex vivo repair of epithelium by human adult stem cells from bone marrow stroma. Proc Natl Acad Sci USA 2003; 100: 2397.
22. Ying QL, Nichols J, Evans EP, et al. Changing potency by spontaneous fusion. Nature 2002; 416: 545.
23. Terada N, Hamazaki T, Oka M, et al. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 2002; 416: 542.
24. Westgren M, Ek S, Bui T, et al. Tissue distribution of transplanted fetal liver cells in the human fetal recipient. Am J Obstet Gynecol 1997; 176: 49.
25. Reubinoff BE, Itsykson P, Turetsky T, et al. Neural progenitors from human embryonic stem cells. Nat Biotechnol 2001; 19: 1134.
26. Zhang SC, Wernig M, Duncan ID, et al. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 2001; 19: 1129.
27. Lindton B, Markling L, Ringden O, et al. Mixed lymphocyte culture of human fetal liver cells. Fetal Diagn Ther 2000; 15: 71.

RESEARCH ARTICLE

Collection and Analysis of Hematopoietic Progenitor Cells From Cynomolgus Macaques (*Macaca fascicularis*): Assessment of Cross-Reacting Monoclonal Antibodies

HIROAKI SHIBATA[1], YUTAKA HANAZONO[2], NAOHIDE AGEYAMA[3], TAKEYUKI NAGASHIMA[4], YASUJI UEDA[4], MAMORU HASEGAWA[4], KEIYA OZAWA[2], YASUHIRO YOSHIKAWA[5], AND KEIJI TERAO[1*]
[1]*Tsukuba Primate Center, National Institute of Infectious Diseases, Ibaraki, Japan*
[2]*Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, Tochigi, Japan*
[3]*Corporation for Production and Research of Laboratory Primates, Ibaraki, Japan*
[4]*DNAVEC Research, Inc., Ibaraki, Japan*
[5]*Department of Biomedical Science, Graduate School of Agriculture and Life Science, University of Tokyo, Tokyo, Japan*

Previous studies have shown that hematopoietic progenitor cells can be isolated from human or nonhuman primate bone marrow (BM) cells. In the present study, we studied the cross-reactivity of 13 anti-human CD34, two anti-human c-Kit, and one anti-human CD133 monoclonal antibodies (mAbs) with cynomolgus macaque (*Macaca fascicularis*) BM cells, using flow cytometric analysis, cell enrichment, and clonogenic assay. Among the 13 anti-human CD34 mAbs assessed, six cross-reacted as previously reported by other groups. However, only three of these six mAbs (clones 561, 563, and 12.8) recognized cynomolgus $CD34^+$ cells that formed progenitor colonies when grown in methylcellulose culture. Similarly, of the two anti-human c-Kit mAbs (clones NU-c-kit and 95C3) that were previously reported to cross-react with cynomolgus BM cells, only one (clone NU-c-kit) resulted in a similar outcome. The anti-human CD133 mAb (clone AC133) also cross-reacted with cynomolgus BM cells, although these cells did not give rise to colonies when grown in culture. These results suggest that antibodies that cross-react with nonhuman primate cells may not identify the hematopoietic cells of interest. In addition, while the CD34 mAb (clone 561) results in the selection of hematopoietic progenitor cells of all lineages when assessed in methylcellulose culture, the c-Kit^{high} fraction (NU-c-kit) exclusively identifies erythroid-specific progenitor cells after growth in culture. It is important to consider these findings when selecting cross-reacting mAbs to identify cells of hematopoietic lineages in macaque species. Am. J. Primatol. 61:3–12, 2003. © 2003 Wiley-Liss, Inc.

INTRODUCTION

The CD34 antigen is widely used as a marker for the positive selection of human and macaque hematopoietic stem/progenitor cells, both in research and in clinical hematopoietic stem cell (HSC) transplantation and gene therapy [Berenson et al., 1991; Dunbar et al., 1995; Andrews et al., 1999; Cavazzana-Calvo et al., 2000; Ageyama et al., 2002]. Several anti-human CD34 monoclonal antibodies (mAbs) have previously been shown to cross-react with macaque bone marrow (BM) cells. Clones 12.8, 561, 563, 581, and QBEnd10 have been reported to cross-react with rhesus BM cells [Guadernack & Egeland, 1995; Sopper et al., 1997; Rosenzweig et al., 2001; http://research.bidmc.harvard.edu/v_path/v_pathogens.asp], whereas clones 12.8, 563, 581, QBEnd10, and NU-4A1 have been shown to cross-react with cynomolgus BM cells [Yoshino et al., 2000; http://research.bidmc.harvard.edu/v_path/v_pathogens.asp]. Among the cross-reacting CD34 mAbs, only two (clones 12.8 and 561) have been successfully used for the purpose of hematopoietic progenitor cell enrichment and HSC transplantation in macaques [Donahue et al., 1996; Banerjee et al., 1997; Ageyama et al., 2002]. The other mAbs have been evaluated for cross-reactivity by immunophenotyping. However, results from flow cytometry alone may be misleading as a result of the methods used for "gating," or the positive and negative controls used.

The c-Kit (CD117) antigen is a transmembrane tyrosine kinase receptor, the ligand of which is stem cell factor (SCF). c-Kit is expressed in immature hematopoietic cells [Kawashima et al., 1996; D'Arena et al., 1998; Ratajczak et al., 1998]. Yoshino et al. [2000] have shown by flow cytometric analysis that three anti-human c-Kit mAbs (clones 95C3, 104D2, and NU-c-kit) cross-react with macaque BM cells, whereas Rosenzweig et al. [2001] reported that clone 95C3 does not cross-react with macaque BM cells. Thus, there is some discordance among assessments by flow cytometry alone. The CD133 mAb is another candidate for the positive selection of hematopoietic stem/progenitor cells [Yin et al., 1997; Miraglia et al., 1997]. To our knowledge, however, the cross-reactivity of human CD133 mAb with macaque cells has not yet been reported.

In the present study, we examined the cross-reactivity of human CD34, c-Kit, and CD113 mAbs with cynomolgus macaque cells derived from BM using flow cytometry, cell selection and sorting, and subsequent hematopoietic progenitor clonogenic assay. In contrast to previous studies, the current results show that some cross-reaction mAbs with cynomolgus cells may not necessarily identify the cell of interest.

METHODS

Animals

Sixty-six healthy cynomolgus macaques (22 males and 44 females, 2–15 years old, 2.1–6.4 kg body weight) were reared at the Tsukuba Primate Center, and housed in accordance with the rules for animal care and management set forth by the Tsukuba Primate Center [Honjo, 1985] and the *Guiding Principles for Animal Experiments Using Nonhuman Primates* formulated by the Primate

Society of Japan [1986]. The animals were free of intestinal parasites and were seronegative for simian type-D retrovirus (SRV), herpes virus B, varicella-zoster-like virus, measles virus, and simian immunodeficiency virus (SIV) [Buchl et al., 1997]. BM collection (see below) was performed under general anesthesia by intramuscular injection of ketamine hydrochloride (Ketalar, 10 mg/kg; Sankyo, Tokyo, Japan). After the BM was harvested, the animals were administered butorphanol tartrate (0.5 mg/kg IM) daily for 3 days to alleviate any discomfort associated with the procedure.

Preparation of BM Cells

BM aspirates (10–20 ml) were collected once from the femur, iliac crest, or ischial tuberosity of each animal. Aspirates were collected into syringes with attached needles (Illinois bone marrow aspiration/intraosseous infusion needle 18G; Baxter, Deerfield, IL) containing preservative-free heparin (Sigma, St. Louis, MO). Mononuclear cells (MNCs) were isolated by density-gradient centrifugation using Ficoll Paque (1.077 g/ml; Pharmacia, Piscataway, NJ) followed by red blood cell lysis with ACK buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA; Wako, Osaka, Japan). MNCs were washed twice with phosphate-buffered saline (PBS; Sigma) containing 2% human serum type AB (Sigma) and 100 µg/ml DNase I (Sigma), and were suspended in α(–)-minimum essential medium (α(–)-MEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS; Intergen, Purchase, NY) and antibiotics (100 U/ml penicillin (Banyu, Tokyo, Japan) and 0.1 mg/ml streptomycin (Meiji, Tokyo, Japan)). Nonadherent cells were collected after 1-hr incubation in tissue-culture flasks at 37°C under a 5% CO_2 condition.

Flow Cytometric Analysis and Sorting

All of the antibodies (except clone H-140) used in the present study were mouse anti-human mAbs (see Table I). The anti-CD34 antibody, H-140, is a rabbit polyclonal antibody. All antibodies were obtained from Becton Dickinson (Franklin Lakes, NJ), PharMingen (San Diego, CA), Beckman Coulter (Miami, FL), CellPro (Bothell, WA), Nichirei (Tokyo, Japan), Medical & Biological Laboratories (MBL; Nagoya, Japan), Santa Cruz Biotechnology (Santa Cruz, CA), or Miltenyi Biotec (Bergisch Gladbach, Germany), as shown in Table I. Unlabeled mAbs, ICO115 (anti-CD34) and H-140 (anti-CD34) were detected with fluorescein isothiocyanate (FITC)-conjugated goat (Fab')$_2$ anti-mouse IgG/M mAb (Biosource, Camarillo, CA) and FITC-conjugated anti-rabbit immunoglobulins antibody (Dako, Copenhagen, Denmark), respectively. To block nonspecific binding via Fc receptors, aggregated human IgG (Sigma) was included at a concentration of 100 µg/ml in the blocking buffer. Biotinylated anti-CD34 mAb (clone 12.8) was detected with phycoerythrin (PE)-conjugated streptavidin (Beckman Coulter). Isotype-matched, irrelevant mAbs (Dako) served as controls. The cells were incubated with each antibody in the washing medium (PBS with 2% FCS and 0.1% NaN_3) for 30 min at 4°C, and were washed with the washing medium twice followed by fixation with 1% paraformaldehyde (Wako)-PBS. Flow cytometric analysis was performed using a FACS Calibur flow cytometer (Becton Dickinson) equipped with an argon-ion laser set at 488 nm. Data acquisition and analysis were performed using the CellQuest software (Becton Dickinson). Each antibody was evaluated for cross-reactivity using MNCs from at least three

TABLE I. Cross-Reactivity of Anti-Human Monoclonal Antibodies With Cynomolgus Macaque Bone Marrow Cells

Antigen	Clone	Source	Cross-reactivity[a]
CD34	My10	Becton Dickinson	–
	8G12	Becton Dickinson	–
	QBEnd10	Beckman Coulter	±
	Immu-133	Beckman Coulter	–
	Immu-409	Beckman Coulter	, –
	12.8	CellPro	+
	561	Dynal	+[b]
	563	PharMingen	+
	581(formerly ICH-3)	Beckman Coulter	±
	NU-4A1	Nichirei	–
	H-140[c]	Santa Cruz	–
	ICO115	Santa Cruz	–
	AC136	Miltenyi Biotec	–
CD117 (c-kit)	NU-c-kit	Nichirei	+
	95C3	MBL	–
CD133	AC133	Miltenyi Biotec	±

[a]Evaluated by flow cytometry: +, positive reaction; ±, weak staining; –, negative reaction.
[b]Evaluated by the immuno-magnetic separation method (see Methods).
[c]Rabbit polyclonal antibody.

different animals. To ensure that small populations were reliably detected, more than 10,000 events were acquired for analysis.

For cell sorting, nonadherent MNCs were incubated with each mAb (anti-CD34, anti-c-Kit, or anti-CD133) for 1 hr at 4°C. They were then washed and resuspended in PBS containing 2% human serum type AB and 0.1% NaN_3. All of the cells were stained with propidium iodide (PI; 5 μg/ml) for 5 min at 4°C so that viable cells could be enumerated prior to sorting. Experiments were performed using MNCs from two or three different animals. The cells were sorted using a FACS Vantage (Becton Dickinson) or EPICS ELITE (Beckman Coulter) cell sorter, each of which was equipped with an argon-ion laser. Data acquisition and analysis were performed using CellQuest or EXPO2 software (Beckman Coulter), respectively.

Immunomagnetic Cell Selection

BM cells were rosetted with Dynabeads M450 directly coated with the anti-CD34 mAb clone 561 (Dynal, Oslo, Norway) for 45 min at 4°C on an apparatus that provided tilting and gentle rotation (Dynal). A cell density of 1–2 × 10^8 cells/ml (beads to cell ratio = 1:1) was found to be optimal. The rosetted cells and beads were suspended in a tube containing 8 ml of chilled PBS with 0.5% bovine serum albumin (BSA; Sigma) and 5 mM EDTA, and the tube was attached to a magnet stand (Dynal). Non-rosetted (CD34⁻) cells were removed by aspiration, and rosetted cells that were retained in the tube were washed five times. The beads were detached from rosetted cells by incubation with 100 μl DETACHaBEAD (Dynal) in a final volume of 300 μl for 15 min at 37°C with gentle shaking. After incubation, 8 ml of the above-mentioned buffer was added and beads were removed by the magnets (repeated five times). To completely remove the beads,

resuspended cells were passed through the MACS separation column (Miltenyi Biotec). $CD34^+$ cells were washed with the buffer and counted.

Clonogenic Hematopoietic Progenitor Assay

The cells (100–1,000 sorted cells by each mAb) were plated in a 35-mm petri dish in 1 ml of α(-)-MEM containing 1.2% methylcellulose (Shin-Etsu Chemicals, Tokyo, Japan) supplemented with 2 U/ml recombinant human erythropoietin (Roche, Basel, Switzerland), 100 ng/ml recombinant human interleukin-3 (PeproTech, Rocky Hill, NJ), 100 ng/ml recombinant human interleukin-11 (PeproTech), 100 ng/ml recombinant human SCF (Biosource, Camarillo CA), 20% FCS, 1% deionized BSA, 5×10^{-5} M 2-mercaptoethanol (Sigma), and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). After incubation for 10–14 days at 37°C with 5% CO_2, colonies containing >50 cells were counted using an inverted light microscope (Nikon, Tokyo, Japan). Experiments were performed in triplicate. The average and the standard deviation (SD) of colony numbers per 500 cells were calculated.

RESULTS

Antibody Cross-Reactivity Assessed by Flow Cytometry

We first examined the cross-reactivity of anti-human CD34, c-Kit, and CD133 mAbs with the cynomolgus macaque samples by flow cytometric analysis. Isotype-matched mAb staining served as the negative control. As shown in Table I, although four anti-human CD34 mAbs (QBEnd10, 12.8, 563, and 581) cross-reacted with cynomolgus BM cells, the intensity of cross-reactivity varied considerably among the mAbs. Clones 12.8 and 563 cross-reacted strongly, while clones QBEnd10 and 581 showed weak or inconsistent results. Clone NU-4A1 did not cross-react with cynomolgus BM cells. Clone 561 was examined for cross-reactivity by immunomagnetic separation only, since this mAb is not commercially available for use in flow cytometric analyses.

Of the two anti-human c-Kit mAbs, clone NU-c-kit was shown to cross-react with cynomolgus BM cells by flow cytometry, but clone 95C3 resulted in a negative outcome. The anti-human CD113 mAb, clone AC133, was shown to cross-react with cynomolgus BM cells, albeit weakly.

Clonogenic Assay of Cynomolgus BM Cells Sorted or Immunoselected by Anti-Human CD34 and CD133 mAbs

We examined whether clonogenic progenitor cells could be grown after being sorted by the CD34 and CD133 mAbs that were shown to cross-react by flow cytometric analysis or immunomagnetic cell selection. Experiments showed similar results between animals (two or three animals per antibody), as demonstrated in Table II. Although the 561^+ and 563^+ cells showed a significant growth of clonogenic progenitor cells (colony-forming units (CFUs)) in culture, the 581^+, $QBEnd10^+$, and $AC133^+$ cells resulted in no detectable CFUs. These results suggest that antibodies that cross-react with macaque cells may not identify the hematopoietic progenitor cells of interest.

Some differences in CFU numbers between $CD34^+$ fractions (561^+ and 563^+ fractions) were found and attributed to individual differences between monkeys. We were not able to examine whether the 12.8^+ cells included CFU, since this clone is no longer commercially available.

TABLE II. Colony Formation From Sorted or Selected Bone Marrow Cells

Antigen	mAb[a]	Fraction	CFU/500 cells[b]
CD34	QBEnd10	−	160±5
		+	0
	561	−	0
		+	325±40
	563	−	10±5
		+	460±95
		−	115±10
		+	0
	581	−	55±10
CD133	AC133	+	0

[a]Sorted by flow cytometry, except the clone 561⁺ cells that were immunoselected by magnetic beads.
[b]Experiments were conducted in triplicate and repeated two or three times using samples from different animals. Data represent the mean ± SD of a representative experiment.

TABLE III. Colony Formation From Cynomolgus Bone Marrow CD34⁺ Cells

Cell fractions[b]	Colonies per 500 sorted cells[a]			
	CFU-GM[c]	BFU-E[d]	GMEmix[e]	Total
CD34^negative	1.5±1.0	0	0	1.5±1.0
CD34^positive	48.0±5.2	13.7±1.5	1.0±1.7	62.7±3.1

[a]Experiments were conducted in triplicate and repeated three times using samples from different animals. Data represent the mean ±SD of a representative experiment.
[b]Sorted by clone 563.
[c]Colony dorming unit-granulocyte, macrophage.
[d]Burst forming unit-erythroid.
[e]Granulocyte, macrophage, and erythroid.

We conducted clonogenic assays with cynomolgus BM cells fractionated using clone 561. Three separate experiments showed that this clone almost exclusively results in the growth of progenitor cells of all hematopoietic lineages (CFU-GM, BFU-E, and GMEmix) from the CD34⁺ fraction (Table III).

Clonogenic Assay of Cynomolgus BM Cells Sorted by Anti-Human c-Kit mAbs

Cynomolgus BM cells were analyzed for the expression of c-Kit using the clone NU-c-Kit (see Fig. 1). In Fig. 1A, a gate was set on live (PI⁻) mononuclear cells. In Fig. 1B, the gated cells were divided into three subgroups according to the expression of c-Kit; c-Kit^high (8.0% ± 4.2%; mean ± SD, n = 5), c-Kit^low (8.0% ± 2.6%), and c-Kit⁻ (84.0% ± 4.8%). Figure 1C–E show profiles of sorted c-Kit⁻, c-Kit^low, and c-Kit^high cells, with a purity of 97%, 90%, and 90%, respectively. Clonogenic assays were conducted on each sorted subgroup. Experiments were repeated using BM cells from three different animals, and similar results were obtained (see Table IV). The c-Kit⁻ fraction included no detectable CFUs. On the other hand, both c-Kit^low and c-Kit^high fractions resulted in significant numbers of CFUs, although there were more CFUs formed from the c-Kit^high fraction than from the c-Kit^low fraction. Of note, the c-Kit^high fraction included only erythroid
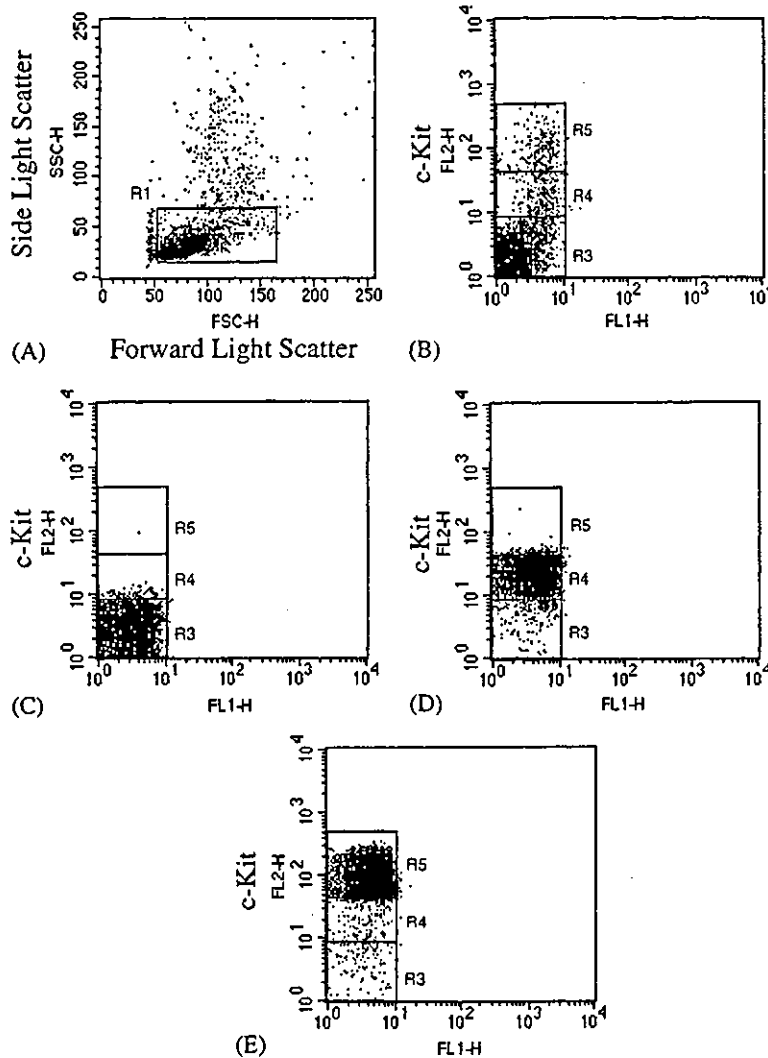
Fig. 1. Flow cytometric analysis of c-Kit expression in cynomolgus BM cells. Three independent experiments were conducted, and one representative dot-plot profile is shown. A: R1 indicates the gate for mononuclear cells. B: R3, R4, and R5 indicate the gates for the c-Kit⁻ fraction (85%), c-Kit^{low} fraction (8%), and c-Kit^{high} fraction (7%), respectively, derived from R1 and PI⁻ cells. BM cells were sorted based on c-Kit expression: (C) c-Kit⁻, (D) c-Kit^{low}, and (E) c-Kit^{high} cells. The purity was 97%, 90%, and 90%, respectively.

progenitor cells (BFU-E), and multipotential CFUs (GMEmix) were detected only in the c-Kit^{low} fraction.

DISCUSSION

Although six anti-human CD34 mAb clones have been reported to cross-react with macaque BM cells, as assessed by flow cytometry, we have shown that one of these antibodies does not result in CFU growth when assessed in culture. Thus, immunophenotyping alone may be misleading. These findings may be a result of the gating methods used, or may be due to inappropriate controls. We have thus confirmed that three anti-human CD34 mAbs (clones 561, 563, and 12.8) truly recognize cynomolgus CD34⁺ cells, which, when grown in methylcellulose

TABLE IV. Colony Formation of Cynomolgus Bone Marrow Cells Separated on the Basis of c-Kit Expression

Cell fractions[b]	Colonies per 500 sorted cells[a]			
	CFU-GM[c]	BFU-E[d]	GMEmix[e]	Total
c-Kit negative	0	0	0	0
c-Kit[low]	50 ± 10	30 ± 10	5 ± 5	85 ± 25
c-Kit[high]	0	135 ± 20	0	135 ± 20

[a]Experiments were conducted in triplicate and repeated three times using samples from different animals. Data represent the mean ±SD of a representative experiment.
[b]Sorted by clone NU-c-kit.
[c]Colony forming unit-granulocyte, macrophage.
[d]Burst forming unit-erythroid.
[e]Granulocyte, macrophage, and erythroid.

culture, result in erythroid and myeloid progenitor colonies. Among these clones, only clones 561 and 563 are commercially available.

The CD34 molecule has many O- and N-linked glycosylation sites that give rise to different epitopes [Sutherland & Keating, 1992]. These epitopes can be grouped into three classes [Greaves et al., 1992]. Glycosylation of the macaque CD34 is considerably different from that of the human CD34 antigen (unpublished data), and this may be why only a few anti-human CD34 mAbs cross-react with the macaque CD34 antigen. In fact, the cross-reacting CD34^{+} mAbs clones 561 and 563, which give rise to colonies in culture, recognize the same epitope class (group III) [Gaudernack & Egeland, 1995]. The tertiary structure of this particular epitope may be similar in humans and macaques.

When we used cross-reacting c-Kit mAbs (NU-c-kit), we found that only erythroid-specific progenitor cells could be grown in methylcellulose culture from the sorted cynomolgus c-Kit[high] fraction. Although some groups have also reported that erythroid progenitor cells are mostly included in the c-Kit[high] fraction in humans [Sakaba et al., 1997], others concluded that erythroid progenitor cells are mainly found in the c-Kit[low] fraction in humans [Gunji et al., 1993]. The discordance among these results may be explained by species differences and differences in the antibodies chosen. In addition, because some c-Kit mAbs may inhibit the growth of c-Kit^{+} cells [Broudy et al., 1992; Gunji et al., 1993], it is possible that the c-Kit^{+} cells did not develop adequately in culture post selection.

In conclusion, the current results suggest that mAbs used for immunophenotyping may not necessarily identify the cells of interest. When cynomolgus BM cells were sorted or immunoselected using defined cross-reacting CD34 and c-Kit mAbs, different hematopoietic progenitor cell populations resulted after growth in methylcellose culture. It is important to consider these findings when selecting cross-reacting mAbs to identify cells of hematopoietic lineages in macaque species.

ACKNOWLEDGMENTS

REFERENCES

Ageyama N, Hanazono Y, Shibata H, Ohto K, Ono F, Nagashima T, Ueda Y, Donahue RE, Hasegawa M, Ozawa K, Yoshikawa Y, Terao K. 2002. Safe and efficient methods of autologous hematopoietic stem cell transplantation for biomedical research in cynomolgus monkeys. Comp Med 52:445–451.

Andrews RG, Briddell RA, Hill R, Gough M, McNiece IK. 1999. Engraftment of primates with G-CSF mobilized peripheral blood CD34$^+$ progenitor cells expanded in G-CSF, SCF and MGDF decreases the duration and severity of neutropenia. Stem Cells 17:210–218.

Banerjee PT, Ierino FL, Kaynor GC, Giovino M, Hawley R, Rosa MD, LeGuern C, Sachs DH, Monroy R. 1997. Expression of swine MHC class II genes in a cynomolgus monkey: retrovirus-mediated gene therapy in a preclinical transplantation model. Xenotransplantation 4:174–185.

Berenson RJ, Bensinger WI, Hill RS, Andrews RG, Garcia-Lopez J, Kalamasz DF, Still BJ, Spitzer G, Buckner CD, Bernstein ID, Thomas ED. 1991. Engraftment after infusion of CD34$^+$ marrow cells in patients with breast cancer or neuroblastoma. Blood 77:1717–1722.

Broudy VC, Lin N, Zsebo KM, Birkett NC, Smith KA, Bernstein ID, Papayannopoulou T. 1992. Isolation and characterization of a monoclonal antibody that recognizes the human c-kit receptor. Blood 79:338–346.

Buchl SJ, Keeling ME, Voss WR. 1997. Establishing specific pathogen-free (SPF) nonhuman primate colonies. ILAR J 38:22–27.

Cavazzana-Calvo M, Hacein-Bey S, Basile GDS, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova JL, Bousso P, Deist FL, Fischer A. 2000. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 288:669–672.

D'Arena G, Musto P, Cascavilla N, Carotenuto M. 1998. Thy-1 (CD90w) and c-kit receptor (CD117) expression on CD34$^+$ hematopoietic progenitor cells: a five dimensional flow cytometric study. Haematologica 83:587–592.

Donahue RE, Kirby MR, Metzger ME, Agricola BA, Sellers SE, Cullis HM. 1996. Peripheral blood CD34$^+$ cells differ from bone marrow CD34$^+$ cells in Thy-1 expression and cell cycle status in nonhuman primates mobilized or not mobilized with granulocyte colony-stimulating factor and/or stem cell factor. Blood 87:1644–1653.

Dunbar CE, Cottler-Fox M, O'Shaughnessy JA, Doren S, Carter C, Berenson R, Brown S, Moen RC, Greenblatt J, Stewart FM, Letiman SF, Wilson WH, Cowan K, Young NS, Nienhuis AW. 1995.

Retrovirally marked CD34-enriched peripheral blood and bone marrow cells contribute to long-term engraftment after autologous transplantation. Blood 85:3048–3057.

Guadernack G, Egeland T. 1995. Epitope mapping of 33 CD34 mAb, including the Fifth Workshop panel. In: Schlossman SF, Boumsell L, Gilks W, Harlan JM, Kishimoto T, Morimoto C, Ritz J, Shaw S, Silverstein R, Springer T, Tedder TF, Todd RF, editor. Leukocyte typing V. New York: Oxford University Press, Inc. p 861–864.

Greaves MF, Brown J, Molgaard HV, Spurr NK, Robertson D, Delia D, Sutharland DR. 1992. Molecular feature of CD34: a hematopoietic progenitor cell-associated molecule. Leukemia 6(Suppl 1):31–36.

Gunji Y, Nakamura M, Osawa H, Nagayoshi K, Nakauchi H, Miura Y, Yanagisawa M, Suda T. 1993. Human primitive hematopoietic progenitor cells are more enriched in KIT^{low} cells than in KIT^{high} cells. Blood 82:3283–3289.

Honjo S. 1985. The Japanese Tsukuba Primate Center for Medical Science (TPC): an outline. J Med Primatol 14:75–89.

Kawashima I, Zanjani ED, Alamida-Porada G, Flake AW, Zeng H, Ogawa M. 1996. CD34$^+$ human marrow cells that express low levels of kit protein are enriched for long-term marrow-engrafting cells. Blood 87:4136–4142.

Miraglia S, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, Bray RA, Waller EK, Buck DW. 1997. A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. Blood 90:5013–5021.

Primate Society of Japan. 1986. Guiding principles for animal experiments using nonhuman primates. Primate Res 2:111–113.

Ratajczak MZ, Plectcher CH, Marlicz W, Machalinski B, Moore J, Wasik M, Ratajczak J, Gewirtz AM. 1998. CD34+, kit+, rhodamine 123(low) phenotype identifies a marrow cell population highly enriched for human hematopoietic stem cells. Leukemia 12:942–950.

Rosenzweig M, Marks DF, DeMaria MA, Connole M, Johnson RP. 2001. Identification of primitive hematopoietic progenitor cells in the rhesus macaque. J Med Primatol 30:36–45.

Sakaba H, Ohmizono Y, Tanimukai S, Kimura T, Mori JK, Abe T, Sonoda Y. 1997. Functional differences between subpopulations of mobilized peripheral blood-derived CD34$^+$ cells expressing different levels of HLA-DR, CD33,

CD38 and c-kit antigens. Stem Cells 15: 73–81.

Sopper S, Stahl-Hennig C, Demuth M, Johnston ICD, Dörries R, Meule VT. 1997. Lymphocyte subsets and expression of markers in blood and lymphoid organs of rhesus monkeys. Cytometry 29: 351–362.

Sutherland DR, Keating A. 1992. The CD34 antigen: structure, biology, and potential clinical applications. J Hematother 1: 115–129.

Yin AH, Miraglia S, Zanjani ED, Almeida-Porada, Ogawa M, Leary AG, Olweus J, Kearny J, Buck DW. 1997. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 90:5002–5012.

Yoshino N, Ami Y, Terao K, Tashiro F, Honda M. 2000. Upgrading of flow cytometric analysis for absolute counts, cytokines and other antigenic molecules of cynomolgus monkeys (Macaca fascicularis) by using anti-human cross-reactive antibodies. Exp Anim 49:97–110.

Short Communication

Positive and negative effects of adeno-associated virus Rep on AAVS1-targeted integration

Masashi Urabe,[1,2,4] Katsuhiro Kogure,[1,2] Akihiro Kume,[1,2] Yuko Sato,[3] Kiyotake Tobita[4] and Keiya Ozawa[1,2]

Correspondence
Masashi Urabe (at Division of
Genetic Therapeutics)
murabe@jichi.ac.jp

[1]Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, 3311-1 Yakushiji, Minami-Kawachi, Tochigi 329-0498, Japan

[2]CREST, Japan Science and Technology Corporation (JST), Tochigi 329-0498, Japan

[3]Department of Intractable Diseases, Research Institute, International Medical Center of Japan, Tokyo 162-8655, Japan

[4]Department of Virology, Jichi Medical School, Tochigi 329-0498, Japan

Adeno-associated virus type 2 integrates preferentially into the AAVS1 locus on chromosome 19 of the human genome. It was reported previously that transfection with two plasmids, one for Rep and the other carrying a transgene flanked by inverted terminal repeats (ITRs), enables preferential integration of the latter into AAVS1. Aiming at increasing the frequency of AAVS1-specific integration, the Rep- to transgene-plasmid ratio necessary to achieve a higher frequency of site-specific integration was examined. 293 cells were co-transfected with the Rep78 plasmid and an ITR-flanked Neo gene at different ratios. G418-resistant clones were selected randomly. Extensive Southern blot analysis showed an optimum range of Rep78 expression. In that range, approximately 20 % of clones harboured the Neo gene at AAVS1. Excess Rep expression, however, resulted in 'abortive' integration of the Neo gene, a rearrangement of AAVS1 without transgene integration. Rep78 appeared to cause abortive integration more extensively than Rep68. Deleterious effects of the Rep protein on the AAVS1 locus should be considered to develop an improved AAVS1-targeted system.

Retrovirus vectors are used widely for gene therapy applications. However, the random integration of retrovirus vector sequences may cause insertional mutagenesis, and the accidental activation of proto-oncogenes cannot be prevented. Adeno-associated virus type 2 (AAV) is a non-pathogenic parvovirus being considered as a gene transfer vehicle (Berns & Giraud, 1996; Kotin, 1994; Muzyczka, 1992). The AAV genome, a linear single-stranded DNA of 4·7 kb long, integrates preferentially into a defined locus in the human genome, AAVS1, on chromosome 19 (19q13·3-qter) (Kotin et al., 1990, 1992; Samulski et al., 1991). AAV can provide a potentially ideal gene delivery system for site-specific integration.

Each end of the AAV genome consists of inverted terminal repeats (ITRs), which are required in cis for AAVS1-specific integration. The AAV rep gene encodes four overlapping non-structural proteins, Rep78, Rep68, Rep52 and Rep40, while the cap gene encodes structural Cap proteins. The unspliced and spliced transcripts from the p5 promoter encode Rep78 and Rep68. Either Rep78 or Rep68 plays a key role in AAVS1-specific integration, binding ITRs (Im & Muzyczka, 1989) and AAVS1 (Weitzman et al., 1994) via tandem repeats of the GAGC tetramer (McCarty et al.,

1994). The mechanism of AAVS1-specific integration of AAV has not been elucidated fully. However, a model whereby integration proceeds via a circular intermediate of the AAV genome by a deletion-substitution mechanism has been proposed (Dyall & Berns, 1998; Linden et al., 1996).

A structural difference between Rep78 and Rep68 is that Rep78 possesses a zinc finger-like motif at its carboxyl terminus. Both Rep proteins share essentially the same functions: strand-specific DNA binding (Im & Muzyczka, 1989), site-specific nicking and ATP-dependent helicase activity (Im & Muzyczka, 1990). Either Rep protein alone is sufficient for replication of the AAV genome (Hölscher et al., 1994) and for AAVS1-specific integration (Surosky et al., 1997). The multifunctional Rep proteins inhibit cellular transformation by heterologous genes (Labow et al., 1987; Yang et al., 1992) and suppress heterologous promoters, including the c-fos, c-myc, H-ras and LTR of human immunodeficiency virus type 1 (HIV-1) (Hermonat, 1991, 1994; Oelze et al., 1994). The Rep proteins also modulate cell cycle-regulating proteins (Hermanns et al., 1997). These results indicate that overexpression of Rep proteins has negative effects on cells and is, on occasion, lethal to cells.

AAV vectors lacking the *rep* gene fail to integrate into AAVS1, showing apparent random integration into the host chromosomal DNA (Kearns *et al.*, 1996). A non-viral plasmid-based system capable of integrating a transgene specifically into AAVS1 has been described; this was achieved by transferring the transgene flanked by the ITRs with transient expression of Rep78 or Rep68 (Balagué *et al.*, 1997; Pieroni *et al.*, 1998; Shelling & Smith, 1994; Surosky *et al.*, 1997; Tsunoda *et al.*, 2000). Thus, this system is safer than integrating retrovirus and AAV vectors randomly. A strategy utilizing two plasmids, one harbouring the transgene cassette between the ITR sequences and the other for Rep expression, allows only the transgene plasmid to integrate into the AAVS1 locus (Surosky *et al.*, 1997). This method successfully introduced the transgene into AAVS1 in haematopoietic K562 cells (Kogure *et al.*, 2001).

The frequency of AAVS1-specific integration by the plasmid-based methods has differed among studies. Shelling & Smith (1994) reported that 9 of 12 cell clones (75 %) obtained by transfecting HeLa or 293 cells with an AAV vector plasmid on which the Neo gene was placed under the control of the p40 promoter, the original promoter for Cap proteins, had rearranged AAVS1 and mentioned that approximately 50 % of the rearranged bands also hybridized to an AAV probe. Another strategy using one plasmid on which both a Rep cassette and an ITR-flanked transgene cassette were placed has targeted the transgene to AAVS1 in 6 of 21 (29 %) 293 cell clones (Balagué *et al.*, 1997). Similar methods applied to other cell lines, HeLa and Huh-7 cells, have been able to insert the transgene to AAVS1 in up to 20 % of clones (Lamartina *et al.*, 1998; Pieroni *et al.*, 1998). All the studies mentioned here used a one plasmid system and the p5 promoter for Rep expression.

Aiming at increasing the frequency of AAVS1-directed integration, we first examined whether AAVS1-specific integration depended on the levels of Rep protein expressed in cells. To control the expression of the cytotoxic Rep proteins, we chose to vary the amount of Rep plasmid DNA. 293 cells were transfected using the calcium phosphate precipitation method with 2, 0·4, 0·2, 0·04, 0·02 or 0 µg pCMVR78, which expresses Rep78 under the control of the CMV promoter (Surosky *et al.*, 1997), and 2 µg pWNeo (Rep:Neo ratio of 1, 0·2, 0·1, 0·02, 0·01 or 0). pWNeo bears a Neo gene under the control of the CMV promoter between the ITRs. To monitor the amount of plasmid DNA incorporated, extrachromosomal DNA was analysed by Southern blot with a plasmid backbone probe (Fig. 1a). As the amount of Rep plasmid decreased, signal intensities corresponding to pCMVR78 decreased gradually, whereas those corresponding to pWNeo changed little, indicating that the amount of plasmid DNA incorporated into the cells correlated with that used for transfection. Western analysis of the transfected 293 cells confirmed that the expression level of the Rep protein was a function of the amount of pCMVR78 (Fig. 1b).
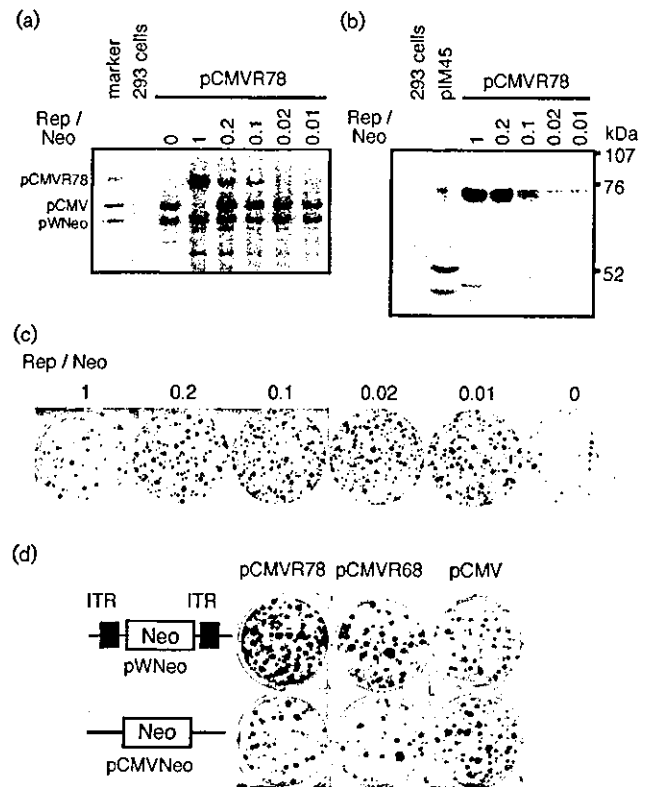

Fig. 1. (a) Quantification of plasmid DNAs incorporated into 293 cells. 293 cells (2×10^5 cells per well) in 6-well plates were transfected with 2 µg pWNeo and various amounts of pCMVR78 (0, 2, 0·4, 0·2, 0·04 or 0·02 µg) at a Rep to Neo plasmid ratio of 0, 1, 0·2, 0·1, 0·02 or 0·01 by the calcium phosphate precipitation method. The amount of plasmid DNA transfected per well was made up to a total of 4 µg with a plasmid devoid of a Rep cassette (pCMV). Following transfection, extrachromosomal DNA was isolated and treated with *Bam*HI. *Bam*HI digestion generates a 5·9, 3·4 or 4·0 kb band, derived from pCMVR78, pWNeo or pCMV, respectively, that hybridizes to a plasmid backbone probe. (b) Expression of Rep78 in 293 cells transfected with various amounts of pCMVR78. The Rep to Neo ratio is indicated above each lane. pIM45 harbours the AAV *rep* and *cap* genes (McCarty *et al.*, 1991). Anti-Rep antibody 294.4 (a gift from J. Kleinschmidt) was used. (c) Comparison of the number of G418-resistant colonies obtained using various amounts of pCMVR78. Following transfection with pCMVR78 and pWNeo at different Rep to Neo ratios (1, 0·2, 0·1, 0·02, 0·01 or 0 µg), a 1/500 fraction of transfected cells was replated onto a 10 cm dish in triplicate and cultured for 10 days in the presence of G418. (d) Representative comparison of the number of G418-resistant colonies generated using pCMVR78 or pCMVR68. After transfection with 0·8 µg pCMVR78, pCMVR68 or pCMV and 3·2 µg pWNeo or pCMVNeo, a 1/500 fraction of transfected cells was replated onto 6-well plates in triplicate and cultured for 10 days in the presence of G418. When either Rep plasmid was co-transfected with pWNeo, a larger number of colonies was formed.

Fig. 1(c) compares G418-resistant colonies grown after transfection with pCMVR78. The number of colonies increased significantly when pCMVR78 was added to the transfection solution. The number of colonies observed did not differ significantly at the Rep to Neo ratios of 0·2–0·01. On transfection at the Rep to Neo ratio of 1, however, the number of colonies decreased, probably due to the strong cytotoxicity of Rep78. To estimate the frequency of integration of the Neo gene to AAVS1, we extensively analysed clones by Southern blot. From each group, 14–20 clones were expanded and their genomic DNA was digested with HindIII or EcoRV, enzymes that do not cleave plasmid pWNeo or the proximal portion of AAVS1 where integration of the AAV genome occurs predominantly (Giraud et al., 1994; Kotin et al., 1992). The presence of co-migrating bands that hybridized to both AAVS1 and Neo probes on both HindIII- and EcoRV-blots was a criterion to conclude that the Neo gene was integrated into AAVS1. Table 1 summarizes the result of Southern blot analysis of the 293 cell clones. When the pCMVR78 to pWNeo ratio was 1 or 0·2, approximately 95 % of clones showed rearrangement of AAVS1. The frequency of rearrangement of AAVS1 decreased gradually as the amount of pCMVR78 was reduced. Unexpectedly, integration of the Neo gene into AAVS1 was observed only in 1 of 16 clones (6 %) at the Rep to Neo ratio of 1. In contrast, transfection at the Rep to Neo ratio of 0·2–0·02 produced approximately 20 % of clones that delivered the Neo gene to AAVS1. These results indicated that a high-level expression of the Rep proteins increased the frequency of AAVS1 rearrangement and rather decreased the frequency of AAVS1-specific integration of the transgene.

In the second experiment, we compared Rep78 with Rep68 by transfecting 0·8 μg pCMVR78, pCMVR68 or pCMV along with 3·2 μg pWNeo or pCMVNeo (Rep to Neo ratio of 0·25). pCMVR68 expresses Rep68 alone (Surosky et al.,

1997) and pCMVNeo is the same as pWNeo except for the absence of ITRs. Fig. 1(d) is a representative comparison of G418-resistant colony formation. The results of the Southern blot analysis of clones selected randomly are summarized in Table 1. Rep78 generated rearrangement of AAVS1 in approximately 90 % of clones and 24 % of clones had the Neo gene at AAVS1. The frequency of rearrangement of AAVS1 is 65 % with the use of pCMVR68 and 40 % of clones integrated the Neo gene to AAVS1. This result suggested that Rep78 appeared to cause more 'abortive' integration of the Neo gene, rearrangement of AAVS1 without integration of transgene, although the difference between Rep78 and Rep68 was not statistically significant.

Fig. 2 shows Southern blot analysis of representative clones with the Neo gene at AAVS1. Fig. 2(a, b) is the HindIII- or EcoRV-digest probed with an AAVS1-specific probe (upper panel) or a Neo probe (lower panel). Each clone presented here has an upshifted band(s) other than a basal band (arrow). Common bands that hybridized to both AAVS1 and Neo probes are indicated by arrowheads. Fluorescent in situ hybridization (FISH) analysis confirmed the integration of the Neo gene into chromosome 19 in 11 of 12 clones. A representative chromosomal analysis is shown in Fig. 2(c). The 293 cells used in the present study have four copies of chromosome 19 labelled with Cy-3-conjugated chromosome 19-specific probe (arrowheads). The left panel shows a metaphase spread of clone C6/6. Fluorescein Neo signals are localized to chromosome 19 and another unidentified site (arrows). In the right panel showing analysis of clone C6/18, one chromosome 19 harbours the Neo signals at its terminal portion.

'Abortive' integration into AAVS1, rearrangement of AAVS1 without foreign gene insertion, has been described in 293 or HeLa cells (Balagué et al., 1997; Shelling & Smith,

Table 1. Summary of Southern blot analysis

Rep plasmid	Experiment 1 — pCMVR78									Experiment 2			
										pCMVR78		pCMVR68	
Rep to Neo ratio	1		0·2		0·1		0·02		0·01	0·25		0·25	
No. of clones analysed	16		20		19		14		14	17		20	
Enzyme used	HindIII	EcoRV	HindIII	EcoRV	HindIII	EcoRV	HindIII	EcoRV	HindIII	HindIII	EcoRV	HindIII	EcoRV
Rearranged AAVS1 band*	14	14	19	18	14	8	9	7	6	13	15	12	13
Common band†	4	6	7	4	8	5	4	4	0	9	4	8	8
AAVS1 rearrangement (%)‡	15 (94)		19 (95)		14 (74)		9 (64)		6 (43)	15 (88)		13 (65)	
Neo at AAVS1(%)§	1 (6)		4 (20)		4 (21)		3 (21)		0 (0)	4 (24)		8 (40)	
Neo signal on chromosome 19‖										3		8	

*Number of clones with rearrangement of AAVS1.
†Number of clones with common bands hybridizing to both AAVS1 and Neo probes.
‡Number of clones with rearrangement of AAVS1 on either the HindIII or the EcoRV blot.
§Number of clones with common bands on both HindIII and EcoRV blots.
‖Number of clones with the Neo signal on chromosome 19.