ABSTRACT

Cynomolgus monkey embryonic stem cell (cyESC)-derived in vivo hematopoiesis was examined in an allogeneic transplantation model. cyESCs were induced to differentiate into the putative hematopoietic precursors in vitro and the cells were transplanted into the fetal cynomolgus liver around the end of first trimester (n = 3). Although cyESC-derived hematopoietic colony-forming cells were detected in the newborns (4.1 and 4.7%), a teratoma developed in all newborns. The risk of tumor formation was high in this allogeneic transplantation model, given that tumors were hardly observed in immunodeficient mice or fetal sheep that had been xeno-transplanted with the same cyESC-derivatives. It turned out that the cyESC-derived donor cells included a residual undifferentiated fraction positive for stage-specific embryonic antigen (SSEA)-4 (38.2 ± 10.3%) despite the rigorous differentiation culture. When an SSEA-4-negative fraction was transplanted (n = 6), the teratoma was no longer observed while the cyESC-derived hematopoietic engraftment was unperturbed (2.3-5.0%). SSEA-4 is therefore a clinically-relevant pluripotency marker of primate embryonic stem (ES) cells. Purging pluripotent cells with this surface marker would be a promising method of producing clinical progenitor cell preparations using human ES cells.

Key Words. Non-human primate embryonic stem cell • Primate allogeneic transplantation•In utero transplantation•Hematopoiesis•Teratoma•Tumor prevention

INTRODUCTION

Human embryonic stem cells (hESCs) hold great potential in the treatment of a variety of diseases and injuries, because embryonic stem (ES) cells have the ability to proliferate indefinitely in culture and to differentiate into any cell type [1, 2]. Since ES cells are able to form teratomas when transplanted into immunodeficient mice, safety concerns would be raised against the clinical application of hESCs [3, 4]. It will be necessary to test the safety of these cells in animal transplantation models before clinical application. Nonhuman primate transplantation models would be desirable for this purpose, however, there have been only a few reports on these models [5-7]. The successful engraftment of transplanted cells in primates will not be achieved unless the immune rejection of transplanted cells is circumvented, for instance through immunosuppressive treatment [6]. The early gestational fetus may be a good recipient with which to circumvent immune rejection because the immune system is premature [8]. In addition, in the animal fetus, transplanted cells would engraft without conditioning of recipients such as irradiation or immunosuppressive treatment [9-12]. We have previously established a system for allogeneic transplantation of cynomolgus ES cells (cyESCs) using preimmune fetal monkeys as recipients [5].

We have also reported a novel method for hematopoietic engraftment from cyESCs in sheep [13]. The method is a combination of three steps: i) differentiation in vitro to generate the putative hematopoietic precursors [14], ii) transplantation of the cells in utero [15], and iii) development into hematopoietic cells in vivo utilizing the hematopoietic microenvironment of the fetal liver [16]. In the present study, we have examined the safety as well as the efficacy of hematopoietic engraftment of cells derived from cyESCs in the allogeneic transplantation model

MATERIALS AND METHODS

Animals

Pregnant cynomolgus monkeys (16-22 years old) were obtained by mating and were reared at the Tsukuba Primate Research Center in accordance with Rules for Animals Care and Management set forth by the Research Center and Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan. Experimental procedures were approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases. The animals were free of intestinal parasites and were seronegative for herpes virus B, varicella-zoster-like virus, measles virus, and simian immunodeficiency virus.

Cell Preparation

A cyESC line (CMK6G) stably expressing green fluorescent protein (GFP) was established after transfection of the parental cyESC line (CMK6) with the enhanced GFP gene (Clontech, Rockey Hill, HJ) [17]. cyESCs were maintained on a feeder layer of mitomycin C (Kyowa, Tokyo, Japan)-treated mouse (ICR or BALB/c, Clea Japan, Tokyo, Japan) embryonic fibroblasts as previously described [18]. The mouse bone marrow stromal cell line OP9 was maintained in α-minimum essential medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal calf serum (FCS; Invitrogen) [19].

cyESCs were induced to differentiate into the putative hematopoietic precursors as previously described [13]. Briefly, undifferentiated cyESCs were transferred onto mitomycin C-treated confluent OP9 cells and cultured for 6 days in

Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 8% FCS, 8% house serum (Invitrogen), 5 x 10^{-6} M hydrocortisone (Sigma, St. Louis, MO), and multiple cytokines including 20 ng/ml of recombinant human (rh) bone morphogenetic protein-4 (R&D systems, Minneapolis, MN), 20 ng/ml of rh stem cell factor (Biosource, Camarillo, CA), 20 ng/ml of rh vascular endothelial growth factor (VEGF; R&D systems), 20 ng/ml of rh Flt-3 ligand (PeproTech, Rocky Hill, NJ), 20 ng/ml of rh interleukin-3 (PeproTech), 10 ng/ml of rh interleukin-6 (PeproTech), 20 ng/ml of rh granulocyte colony-stimulating factor (PeproTech), and 2 IU/ml of rh erythropoietin (Roche, Basel, Switzerland). The cells were resuspended in 0.1% human serum albumin (Sigma)/ Hanks' balanced salt solution (Sigma) for transplantation.

Flow Cytometry

Primary antibodies (Abs) used in the present study were anti-human CD34 monoclonal antibody (mAb) (Pharmingen, San Diego, CA), anti-human CD31 mAb (Pharmingen), anti-human CD45 mAb (Pharmingen), anti-human vascular endothelial (VE) cadherin mAb (Pharmingen), rabbit anti-human VEGF receptor (VEGFR)-2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-stage-specific embryonic antigen (SSEA)-4 mAb (Chemicon, Temecula, CA). All of them cross-reacted to cynomolgus counterparts as previously demonstrated [18, 20-22]. Secondary Abs were phycoerythin (PE)-conjugated rabbit anti-mouse immunoglobulins (Igs) Ab (DakoCytomation, Glostrup, Denmark) and Alxa Fluor 647-conjugated goat anti-mouse IgG Ab (Molecular Probes, Eugene, OR). Cells stained with unlabeled primary Abs were incubated with fluorescence-labeled secondary Abs. Cells were incubated with either primary or secondary Ab for 20-60 minutes at 4°C. Regarding staining with the anti-VEGFR-2 Ab,

the cells were incubated with biotin-conjugated goat anti-rabbit IgG Ab (Beckman Coulter, Miami, FL), followed by PE-conjugated streptavidin (Beckman Coulter). Fluorescence-labeled cells were analyzed with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). Data analysis was performed using the CellQuest software (Becton Dickinson). Isotype-matched, irrelevant mAbs (DakoCytomation or Beckman Coulter) served as negative controls. Non-viable cells were excluded from analysis by propidium iodide (Sigma) co-staining.

Cell Sorting

Cell sorting was performed to purge SSEA-4^+ cells from among the cultured cyESCs in vitro. Cells were incubated with the anti-SSEA-4 mAb for 1 hour at 4°C, and washed twice with Dulbecco's modified Eagle's medium supplemented with 10% FCS. The cells were then incubated with the PE-conjugated anti-mouse Igs Ab for 1 hour at 4°C and washed twice again. GFP-positive and SSEA-4-negative cells were sorted using an Epics Elite cell sorter (Beckman Coulter). Data acquisition was performed using the Expo2 software (Beckman Coulter).

Transplantation and Delivery

Transplant procedures were previously described [5]. Briefly, animals were anesthetized via an intramuscular administration of ketamine hydrochloride (Ketalar, 10 mg/kg; Sankyo, Tokyo, Japan) and received 0.5-1.0% isoflurane by inhalation by way of an endotracheal tube. Cells (0.16-46 x 10^6 cells/fetus; Table 1) were injected into the fetal liver through a 23-gauge needle using an ultrasound-guided technique around the end of

the first trimester. The fetuses were delivered by cesarean section at 2-3 months post-transplant (120-157 days gestation/full term 165 days).

Colony Polymerase Chain Reaction (PCR)

Cynomolgus clonogenic hematopoietic colonies were produced as previously described [20]. After cells were cultured in methylcellulose medium for 10-14 days, well-separated individual colonies were plucked into 50 μl of distilled water and digested with 20 μg/ml of 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 a nested polymerase chain reaction (PCR) to detect the GFP gene sequence. The outer primer set was 5'-AAGGACGACGGCAACTACAA-3' and 5'-ACTGGGTGCTCAGGTAGTGG-3', and the inner primer set was 5'-GCATCGACTTCAAGGAGGAC-3' and 5'-GTTGTGGCGGATCTTGAAGT-3'. Amplification conditions for both the outer and inner PCR were 30 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. The outer PCR products were purified using a QIA quick PCR purification kit (Qiagen, Chatsworth, CA). Simultaneous PCR for the β-actin sequence was also performed to certify DNA amplification of the sample in each colony. The primer set for β-actin was 5'-CATTGTCATGGACTCTGGCGACGG-3' and 5'-CATCTCCTGCTCGAAGTCTAGGGC-3'. Amplification conditions for β-actin PCR were 40 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. Amplified GFP (131 bp) and β-actin (234 bp) products were resolved on 2% agarose gel (Sigma) and visualized by ethidium bromide (Invitrogen) staining.

RNA-PCR

Total RNA was extracted from cells of interest using the EZ1 RNA universal tissue kit (Qiagen). RNA was reverse-transcribed at 50°C for 30 minutes using the RNA LA PCR kit (Takara) with oligo dT primer. The resulting cDNA was then subjected to PCR. Regarding PCR for Oct-4, the primer set was 5'-GGACACCTGGCTTCGGATT-3' and 5'- TTCGCTTTCTCTTTCGGGC-3'. The PCR conditions were 35 cycles of 95°C for 30 seconds, 67°C for 45 seconds, and 68°C for 1.5 minutes. Regarding PCR for Scl, the primer set was 5'-GGGCGGAAAGCTGTTTGCGATT-3' and 5'-TCGCTGAGAGGCCTGCAGTT-3'. The PCR conditions were 35 cycles of 95°C for 30 seconds, 63°C for 1 minute, and 72°C for 1 minute. A simultaneous PCR for β-actin was also conducted on each cDNA sample as an internal control as described above. Amplified Oct-4 (697 bp), Scl (201 bp), and β-actin (234 bp) products were resolved on 2% agarose gel and visualized by ethidium bromide staining.

RESULTS

In Utero Transplantation and Delivery

cyESCs stably expressing GFP were used in this study [17]. In the setting of allogeneic transplantation, GFP was used as a genetic tag to track transplanted cell progeny. We employed the OP9 stromal cell coculture method instead of the embryoid body formation method to facilitate the hematopoietic differentiation [19, 23, 24] (Fig. 1A, B). According to the flow cytometric analysis, CD34, CD31 (PECAM-1), CD144 (VE-cadherin) and VEGFR-2 (Flk-1) were all up-regulated on day 6 but decreased thereafter (Fig. 1C, D, E, G). Among the markers examined, CD34 is a widely-used surface marker of hematopoietic stem cells in both human and monkey subjects [25-27]. The others are key markers of hemangioblasts (which generate endothelial and hematopoietic lineages) in both mice and humans [14, 28]. Cells positive for both VEGFR-2 and VE-cadherin emerged on day 6 (Fig. 1H). CD45, however, was not detected until day 12 (Fig. 1F). Despite the hemangioblast marker expression on day 6, the hematopoietic Scl gene was up-regulated at this time point as assessed by RNA-PCR (Fig. 1I), implying that the hematopoietic commitment might have already occurred on day 6 [29, 30]. We therefore designated the day 6 cESC-derived progenitor cells as putative hematopoietic precursors. The time course profiles presented here were similar to those of hESCs [14, 24]. The GFP expression was stable during the 6-day culture (Fig. 1A, B) and even thereafter (data not shown).

Teratoma Formation

The undifferentiated cyESCs (n = 3) or cyESC-derived putative hematopoietic precursors (n = 3) were transplanted in utero into allogeneic fetuses in the liver under ultrasound guidance around the end of the first trimester (49-66 days/full term 165 days) (Table 1). Whether the undifferentiated cyESCs or putative hematopoietic precursors were transplanted, tumors were found in the thoracic or abdominal cavities in all the six animals at 2-3 months post-transplant (Table 1 and Fig. 2A). The tumors fluoresced (Fig. 2B) and consisted of three germ layer cells. Thus, they were teratomas derived from transplanted cells. Tumors were, however, hardly observed in fetal sheep (1/10; ref. 13 and our unpublished data) (Table 1) and immunodeficient (NOD/SCID) mice (3/10; our unpublished data) after the same putative hematopoietic precursors were transplanted.

In Vivo cyESC-derived Hematopoiesis

Regarding the newborn monkeys that had been transplanted with the putative hematopoietic precursors, we harvested cells from the femur, cord blood and liver, and plated the cells in methylcellulose medium to produce clonogenic hematopoietic colonies (colony-forming units, CFU) (Fig. 3A). The monkey cells generated colonies of clear hematopoietic morphology in this assay (Fig. 3B). To detect transplanted cell-derived, GFP-positive colonies, we tried to observe GFP fluorescence of colonies under a fluorescent microscope, but were hampered by the high auto-fluorescence. We then conducted PCR for the GFP gene sequence in DNA isolated from each colony (colony PCR) (Fig. 3C). The transplanted cell-derived CFU were clearly detected in the animals (4.1 and 4.7%; Table 1). We repeated the colony PCR and confirmed that the

results were reproducible. We detected both granulocytic and erythroid cynomolgus CFU. In the peripheral blood, however, we were not able to detect cells expressing GFP by flow cytometry. It turned out that the fractions of GFP-positive cells in the peripheral blood were very small (< 0.1%) as assessed by quantitative PCR. Low peripheral "chimerism" has been reported more than once in other in utero transplantations of ES or hematopoietic stem cells such as in mice, sheep, and pigs [13, 31-33].

Purging SSEA-4$^+$ Cells of the Putative Hematopoietic Precursors

We examined the expression of an undifferentiated primate ES cell marker, SSEA-4, in the undifferentiated cyESCs (day 0) and putative hematopoietic precursors (day 6). The proportion of SSEA-4$^+$ cells was 93.4 ± 8.1% and 38.2 ± 10.3% among the day-0 and -6 cells, respectively (Fig. 4A). A substantial number of cells were still positive for SSEA-4 after the rigorous differentiation culture. In addition, a considerable number of cells expressing another undifferentiated marker, Oct-4, remained among the day-6 population as assessed by RNA-PCR (Fig. 4B). Those residual undifferentiated cells might be responsible for the formation of teratomas in the recipients.

To prevent teratomas from forming in recipients, we purged SSEA-4$^+$ cells of the putative hematopoietic precursors and transplanted the SSEA-4$^-$ population into the fetal monkey liver (n = 6) (Fig. 4C). At delivery, tumors were no longer observed in the six animals that had been transplanted with the sorted SSEA-4$^-$ cells (Fig. 4D). The transplanted cell-derived CFU were clearly detected in the newborns and the fraction was not spoiled (2.3-5.0%; Table 1), although the removed SSEA-4$^+$ fraction included some CD34$^+$ cells (data not shown).

DISCUSSION

We have previously described a method for hematopoietic engraftment from cyESCs [13]. cyESCs were first cultured for 6 days in vitro and the day-6 cyESC-derived putative hematopoietic precursors were transplanted in vivo into fetal sheep liver after the first trimester, generating sheep with cynomolgus hematopoiesis. We transplanted the day-6 cells because the CD34 expression level was highest at this time point (Fig. 1C). We transplanted the cells into the liver because the liver is the major hematopoietic organ at this stage of gestation in sheep [34]. In the present study, we tested this method in a cynomolgus monkey allogeneic transplantation model and successfully detected cyESC-derived hematopoietic cells in cynomolgus recipients albeit at low levels. cyESC-derived chimerism was, however, higher in the primate allogeneic transplantation model (2.3-5.0%) than in our recently reported sheep xeno-transplantation model (1.1-1.6%; ref. 13) (Table 1). To enhance ES cell-derived hematopoiesis, further consideration is required of the in vitro culture conditions (the cytokine milieu, coculture- or embryoid body-associated cellular microenvironment, culture period, and genetic manipulation) and the in utero transplantation conditions (the preconditioning, route, and timing).

Teratomas developed in all animals even after the transplantation of ES cell-derived progenitor cells that had been cultured for 6 days in the differentiation medium. The risk of tumor formation was high, given that we could hardly detect tumors in immunodeficient mice or fetal sheep that had been transplanted with the same day-6 cyESC-derivatives (ref. 13 and our unpublished data). Innate immune responses against cynomolgus-derived tumors might be more rigorous in xeno-transplanted mice and sheep than in allo-transplanted monkeys, resulting in a failure to detect

tumorigenesis in the xeno-transplantation models. Similarly, Erdo et al. reported that tumors developed after ES cell-derived progenitor cell transplantation in the mouse-to-mouse setting, but not in the mouse-to-rat setting [35]. Our monkey allogeneic transplantation setting would therefore allow the strict evaluation of the in vivo safety of transplantation therapies using ES cells. However, given that teratomas indeed form when undifferentiated cyESCs alone are xeno-transplanted into immunodeficient mice, it is unclear why residual undifferentiated cells included among the day-6 cyESC-derivatives did not form teratomas in immunodeficient mice or fetal sheep.

SSEAs that are developmentally regulated during early embryogenesis are widely used as markers to monitor the differentiation of both mouse and human embryos and ES cells [36-38]. Undifferentiated ESCs of both human and cynomolgus origin are characterized by the expression of SSEA-4, and by a lack of SSEA-1 [1, 2, 18]. We have therefore used SSEA-4 as a marker for the negative selection of an undifferentiated fraction. As a result of this negative selection, tumors were no longer detected in the monkeys after transplantation. On the other hand, Bieberich et al. recently developed a method for selective apoptosis of residual pluripotent stem cells using the transcription factor Oct-4 as a pluripotency marker to prevent teratoma formation [39]. They found that the expression of Oct-4 is colocalized with that of prostate apoptosis response-4 (PAR-4), a protein mediating ceramide-induced apoptosis. Treatment of ES cell-derived neural precursors with ceramide resulted in selective elimination of residual Oct-4-positive pluripotent cells. Our method, however, utilizes a cell surface marker to purge pluripotent cells. With this method, one can see the purging efficiency in real-time. This would be meritorious for clinical applications. Although we

utilized a cell sorter to obtain the SSEA-4$^-$ fraction in the present study, selection with beads would be easier and more appropriate for clinical applications.

To generalize the use of SSEA-4 for eliminating undifferentiated cells from among donor cells, we differentiated cyESCs into neural stem cells. After the culture, around 10% of cells were still positive for SSEA-4. When all the cells were transplanted into the striatum of Parkinson's cynomolgus monkeys, teratomas developed. We then transplanted cyESC-derived neural stem cells without an SSEA-4$^+$ fraction into the cynomolgus striatum and successfully detected the engraftment without tumor formation (our unpublished data). The removal of SSEA-4$^+$ cells is useful at least for hematopoietic and neural lineages.

In conclusion, we are now able to prevent the formation of tumors in nonhuman primate recipients by purging SSEA-4$^+$ cells from among ES cell-derived progenitor cells without spoiling the engraftment. SSEA-4 is therefore a clinically-relevant pluripotency marker of primate ES cells. Purging pluripotent cells with this marker would be a promising method for producing clinical progenitor cell preparations using hESCs to improve safety in vivo.

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