TABLE 1. CYES CELL ENGRAFTMENT AT TERM AFTER IN UTERO TRANSPLANTATION IN SHEEP

Animal number	*Transplanted ES cells*	*Gestational day at transplantation*	*Transplanted cell number per site*	*Engraftment*
1	CMK6G	43	2.0×10^7	–
			2.0×10^6	–
2	CMK6G	45	2.5×10^7	+
			5.0×10^6	+
3	CMK6	47	2.6×10^6	+
4	CMK6	47	1.4×10^7	+
			1.4×10^6	+
			1.4×10^5	–
			1.4×10^4	–
5	CMK6	49	7.5×10^6	+
			7.5×10^5	–
6	CMK6	49	7.5×10^6	–
7	CMK6	50	1.1×10^7	–
			1.1×10^6	–
			1.1×10^5	–
			1.1×10^4	–
8	CMK6G	50	6.0×10^6	–
			6.0×10^5	–
			6.0×10^4	–
			6.0×10^3	–
9	CMK6	50	5.0×10^6	–
			5.0×10^5	–
			5.0×10^4	–
			5.0×10^3	–
10	CMK6	53	2.6×10^7	–
11	CMK6	55	5.0×10^7	–
12	CMK6G	63	4.5×10^6	–
			4.5×10^5	–
			4.5×10^4	–
			4.5×10^3	–
13	CMK6	64	9.0×10^6	–
			9.0×10^5	–
			9.0×10^4	–
			9.0×10^3	–
14	CMK6	66	1.4×10^7	–
15	CMK6	67	5.0×10^7	–

for instance at 1.5 versus 6.5 months of age, as shown in Fig. 4C. In one of the engrafted sheep, a quantitative DNA-PCR analysis of the grafts showed that the cynomolgus portion decreased from 78% at 1.5 months to 42% at 6.5 months. Thus, the cynomolgus tissues were replaced by host-derived granulated tissue over months, although they remained engrafted for longer than half a year after birth (9 months post-transplant). The cyES cell-derived grafts after birth were no longer positive for the pluripotent marker Oct-3 (data not shown). Although cultured graft cells were all negative for Oct-3 (Fig. 4D), they could be propagated for more than 6 passages.

Innate immune responses

Although xenograft rejection requires T cells [34], it has been recognized that innate immune responses precede and drive adaptive immune responses in xenograft rejection [35]. Therefore, we examined natural killer (NK) cells (as $CD11b^+CD14^-CD5^-$) and monocytes (as $CD11b^+CD14^+CD5^-$) in the fetal leukocytes at transplantation by flow cytometry. The NK cell fraction was $2.2 \pm 0.1\%$ at 48 days and $1.9 \pm 0.6\%$ at 60 days. The monocyte fraction was $0.1 \pm 0.0\%$ at 48 days, and $0.2 \pm 0.1\%$ at 60 days (Fig. 5A). There were no statistical dif-

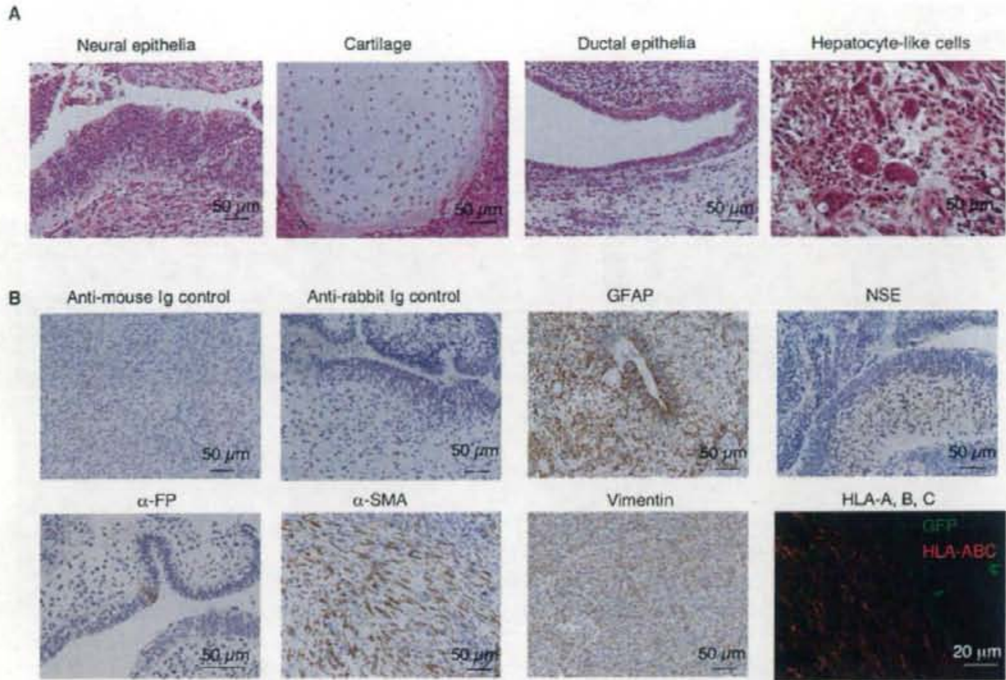

FIG. 2. Cynomolgus grafts in sheep contained mature tissue structures. (**A**) Hematoxylin & Eosin staining revealed that the grafts contained three germ layer cells with mature tissue structures. (**B**) Immunohistochemistry revealed that the grafts contained various kinds of mature cells derived from three germ layers. Duct cells were stained positively with anti-GFAP, anti-NSE, and anti-α-FP. Spindle cells in the interstitial areas were stained positively with anti-αSMA and anti-vimentin. Control immunostaining with anti-mouse IgG and anti-rabbit IgG is also shown. Some of the transplanted cyES cell progeny (GFP^+, green, *lower right*) still remained negative for HLA-A, B, and C, although most cells became positive (red, *lower right*).

ferences in the percentages of NK cells or monocytes between 48 (<50) days and 60 (>50) days of gestation. The very low prevalence of monocytes was also described in a previous study [32]. Neutrophils did not appear at this gestational age [32]. However, macrophages were infiltrated in the transplanted progeny two weeks later when transplanted at 60 days of gestation (Fig. 3G, lysozyme-positive cells), although they were not infiltrated when transplanted at 48 days of gestation (Fig. 3B, lysozyme-positive cells). Thus, the premature (absent or weak) innate immune responses before 50 days of gestation might be one of the reasons for the survival of xenogeneic ES cells.

Adaptive immune responses

Next, we examined adaptive immune responses. To examine cellular immune responses in the newborn sheep (at 3 months of age), we performed MLRs ($n = 3$). The reactivity against cyES cells and graft cells was considerably higher in the cyES cell-engrafted sheep than in the nonengrafted (transplanted but not engrafted) and nontransplanted (naive) sheep (Fig. 5B), suggesting that the sheep engrafted with cyES cells were sensitized to the ES cells. The low reactivity to ES and graft cells as compared to cynomolgus PBMCs might be a reflection of low immunogenicity of ES cells and their progeny [34,36]. The low reactivity in the nonengrafted sheep suggests that cyES cells and their progeny were cleared away before the development of conventional T cell memory, which was not established yet at this time of gestation [37,38].

Regarding the humoral immunity (Fig. 5C), considerable levels of IgM against cyES cells were detected at 2 weeks post-transplant when the transplantation was conducted at 60 days of gestation (day 60 + 14). The slight increase in $CD45R^+$ fraction (as B cells) at 60 days of gestation might support the B cell development after 50 days of gestation (Fig. 5A). Anti-cyES cell IgM was, however, no longer detectable at birth in the engrafted ($n = 4$) or nonengrafted sheep ($n = 3$). Although anti-cyES cell IgG xenoantibodies were not detected at 2 weeks post-transplant regardless of the transplant days (either 48 or 60 days of gestation), they were detected in

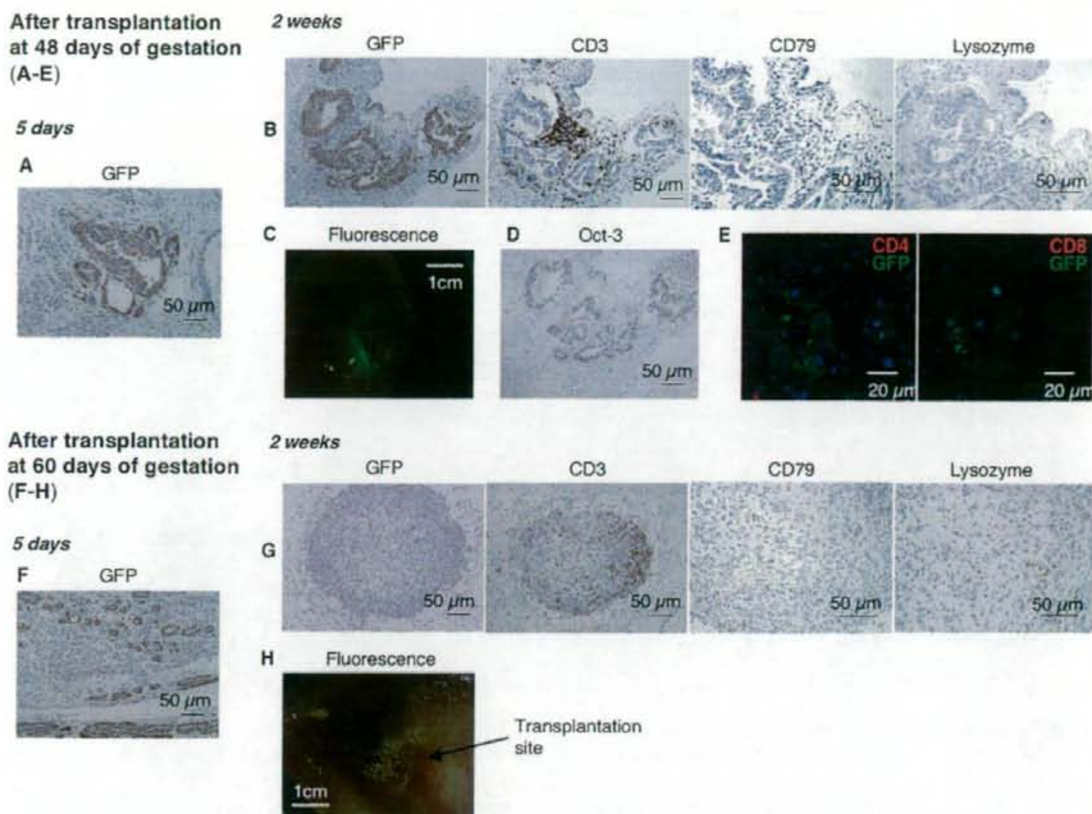

FIG. 3. Cynomolgus grafts in sheep during the fetal period. (**A–E**) When cyES cells expressing GFP were transplanted at 48 days of gestation, GFP^+ transplanted cell progeny were clearly detected both at 5 days (**A**) and at 2 weeks post-transplant (**B** and **C**). (**B**) According to the immunostaining of serial sections, a considerable number of $CD3^+$ T cells and a small number of $CD79^+$ B cells were observed around GFP^+ transplanted cell progeny, but lysozyme-positive macrophages were not observed. (**D**) Transplanted cell progeny at 2 weeks post-transplant were still positive for Oct-3. (**E**) Staining of frozen sections showed that T cells in **B** were $CD4^+$ (red, *left*) and $CD8^-$ (red, *right*). (**F–H**) When cyES cells expressing GFP were transplanted at 60 days of gestation, GFP^+ transplanted cell progeny were observed at 5 days post-transplant (**F**), but they were no longer detected at 2 weeks post-transplant (**G** and **H**). (**G**) According to the immunostaining of serial sections, GFP^- host-derived granulated tissue infiltrated with $CD3^+$ T cells, $CD79^+$ B cells, and lysozyme-positive macrophages was observed, suggesting that the transplanted cells were cleared in 2 weeks when the transplantation was conducted at 60 days of gestation.

all of the cyES cell-engrafted sheep at birth ($n = 4$). Thus, humoral immune responses against cyES cells also occurred during the pregnancy in the engrafted sheep.

We then tried an additional transplantation into the engrafted sheep to examine the immunological tolerance. cyES cells (1×10^7 cells /site) were transplanted subcutaneously into the engrafted ($n = 2$) and nonengrafted sheep ($n = 3$), which were already tested in the MLR and xenoantibodies detection studies, at more than 6 months after birth, and the sheep were examined 3 months later. No additional engraftment or tumor was observed in any of the sheep, suggesting that immunological tolerance was not achieved. Thus, both cellular and humoral immune responses against cyES cells were generated in the cyES cell-engrafted sheep, despite the sustained macroscopic engraftment of cynomolgus tissues in immunocompetent sheep.

Involvement of regulatory T cells

Because cyES cell-derived grafts could survive long term in xenogeneic sheep despite immune responses, some mechanisms should be involved in the sustained engraftment. Although $CD4^+$ T cells surrounded the transplanted cyES cell progeny at 2 weeks post-transplant, the cyES cell-derived orderly structures were not affected at all (Fig. 3B). Such a circumferential distribution of T cells was reminiscent of 'peri-insulitis'; regu-

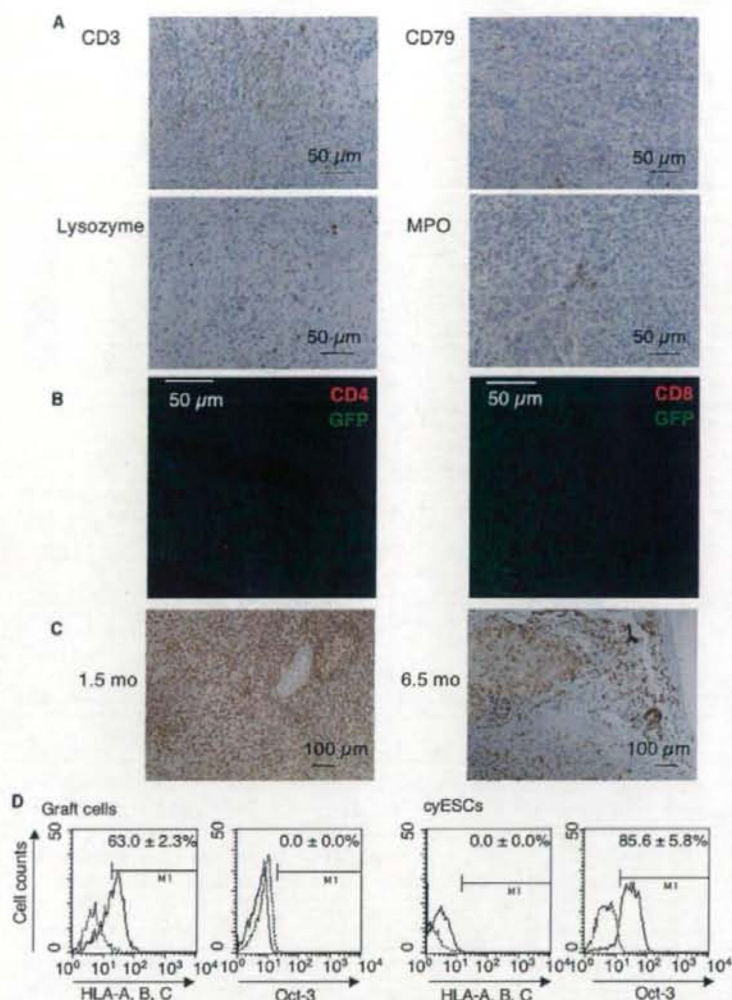

FIG. 4. Cynomolgus grafts in sheep after birth. (**A**) Immunostaining shows infiltration by T cells (positive for CD3), B cells (positive for CD79), macrophages (positive for lysozyme), and neutrophils (positive for MPO) in the cynomolgus-derived grafts in sheep after birth. (**B**) Most of the T cells in the grafts were $CD4^+$ (red, *left*), but some were $CD8^+$ (red, *right*). (**C**) In situ hybridization to detect cynomolgus cells showed that the graft in sheep no. 3 (the longest graft-surviving sheep) at 6.5 months of age (*right*) consisted of more granulated tissues and less cynomolgus components as compared to the graft at 1.5 months (*left*). (**D**) Cultured graft cells were all negative for Oct-3 and 63.0 ± 2.3% of the cells were positive for HLA-A, -B, and -C. On the other hand, cyES cells were all negative for HLA-A, -B, and -C and nearly 90% of the cells were positive for Oct-3. Dotted lines show the staining with the isotype-matched, fluorescence-conjugated, irrelevant control Abs.

latory T (T_{reg}) cells were distributed around islets in nonobese diabetic mice in which normoglycemia had been restored [39]. Therefore, we considered that T cells surrounding the cynomolgus tissues in Fig. 3B might be T_{reg} cells, which possibly suppressed immune rejection.

Although sheep T_{reg} cells have not been characterized, the transcription factor Foxp3 is known to be one of the most specific markers of T_{reg} cells that is highly conserved among species [40,41]. Therefore, we first cloned and sequenced the ovine ortholog of *foxp3*. The translated amino acid sequence showed a high homology to other known orthologs of Foxp3 (Fig. 6A, left; human, 90%; mouse, 88%; cattle, 99%). We next examined the cross-reactivity of an anti-mouse Foxp3 Ab to ovine Foxp3. Cells transfected with the cloned ovine *foxp3* were stained positively with the anti-mouse Foxp3 Ab by flow cytometry (Fig. 6A, right). We then stained a fetal sheep spleen at 64 days of gestation with this anti-Foxp3 Ab,

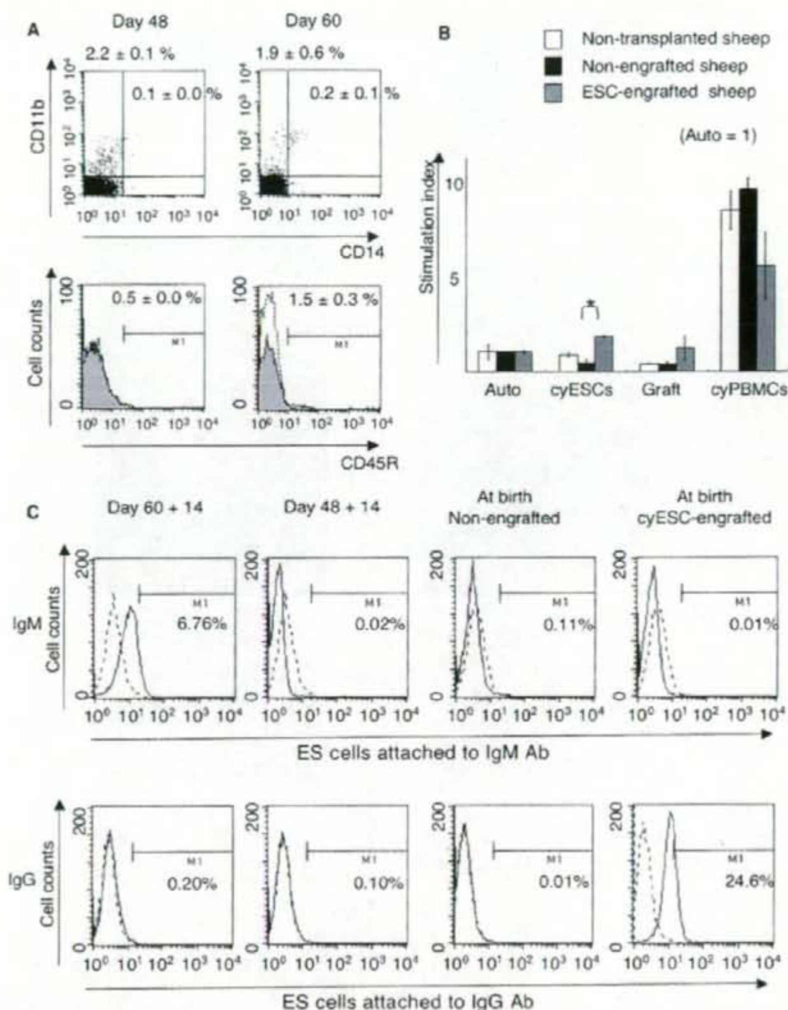

FIG. 5. Host immune responses. (**A**) Cell-surface antigens of fetal sheep peripheral blood leukocytes are compared between 48 days and 60 days of gestation. CD11b and CD14 double staining of the $CD5^-$ cells are shown (*upper*). There were no statistical differences in the percentages of NK cells ($CD11b^+CD14^-CD5^-$) between 48 days and 60 days of gestation. Monocytes ($CD11b^+CD14^+CD5^-$) were scarcely detected at both gestational days. $CD45R^+$ cells (as B cells) are slightly increased at 60 days of gestation (*lower*). Dotted lines show the staining with isotype-matched, fluorescence-conjugated, irrelevant control Abs. (**B**) The MLR against cyES cells and graft cells was higher in the cyES cell-engrafted sheep than in the nonengrafted or non-transplanted sheep. Statistical differences with the *t*-test were indicated (* $p < 0.01$). (**C**) IgM (*upper*) and IgG (*lower*) xenoantibodies against cyES cells were determined by flow cytometry. (Dotted lines) Negative control sera from nontransplanted adult sheep; (solid lines) sample sera. Day 60 + 14; transplanted with cyES cells at 60 days of gestation and examined at 14 days post-transplant. Day 48 + 14; transplanted with cyES cells at 48 days of gestation and examined at 14 days post-transplant.

and found that cells positive for Foxp3 were always positive for CD4 (Fig. 6B). We have also shown that $CD4^+CD25^{high}$ cells of adult sheep PBMCs were mostly $Foxp3^+$ just like human T_{reg} cells (42) (data not shown). These results indicate that this Ab can be used to detect sheep $Foxp3^+$ T cells, namely sheep T_{reg} cells. Using this Ab (Fig. 6C), more than half of the T cells around the grafts at 2 weeks post-transplant were found to be positive for Foxp3. At birth, 10–20% of the T cells in the grafts were positive for Foxp3. These data suggest that T_{reg} cells might be involved in the sustained engraftment of cynomolgus tissues in sheep. To characterize ovine fetal T_{reg} cells further, it would be ideal to isolate T_{reg} cells from the specimen at 2 weeks post-transplant in Fig. 6C

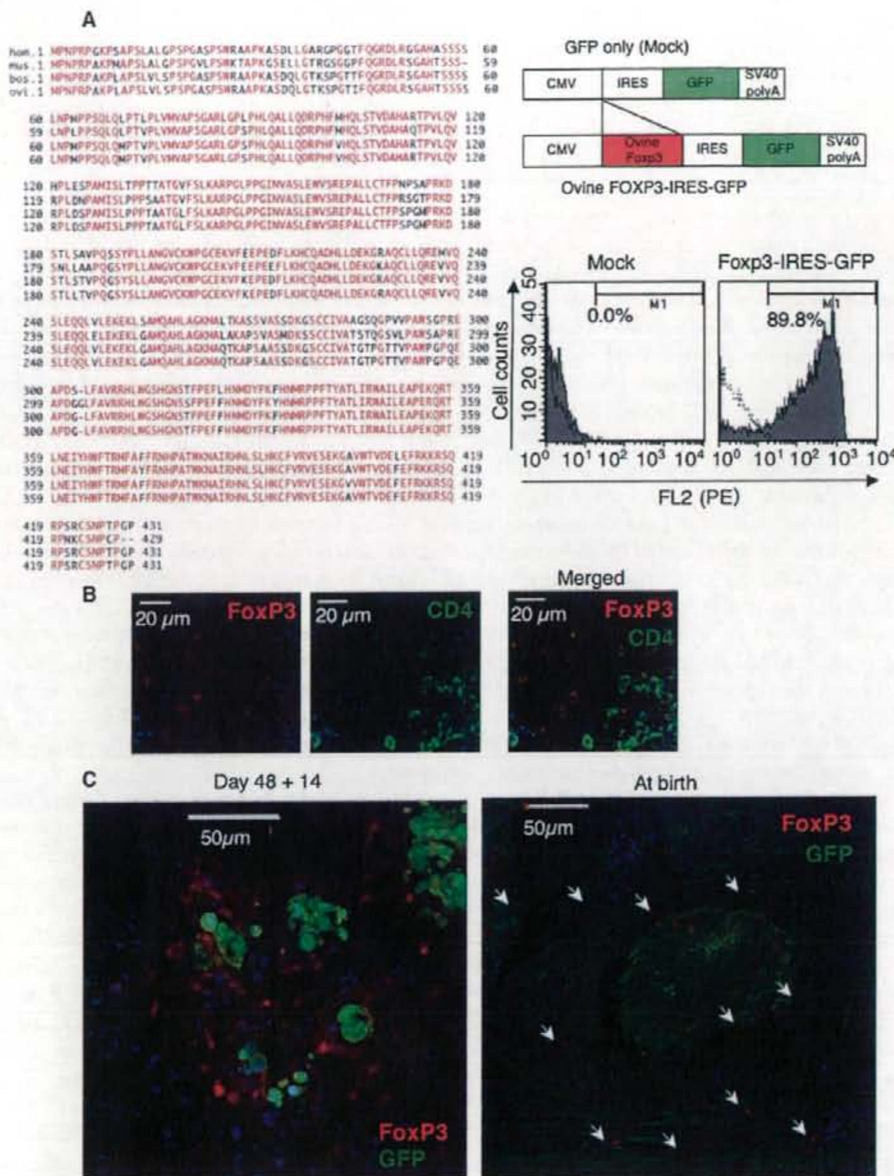

FIG. 6. Detection of Foxp3$^+$ T_{reg} cells. (**A**) Amino acid sequences of Foxp3 in four mammals. Foxp3 of sheep (ovi) is compared with that of human (hom), mouse (mus), and cattle (bos) (GenBank accession numbers; NM014009, NM054039, and DQ322170, respectively). Conserved amino acids are indicated in red (*left*). The ovine *foxp3* cDNA was introduced into 293T cells. The plasmid inserts are shown (*upper right*). Flow cytometric analyses of 293T cells transfected with the mock plasmid and with the plasmid expressing the ovine *foxp3* are shown (*lower right*). Gray areas indicate GFP-expressing cells stained with the PE-conjugated anti-mouse Foxp3 Ab. Dotted lines show GFP-expressing cells stained with PE-conjugated isotype-matched irrelevant Ab. (**B**) Fetal sheep spleen at 64 days of gestation was stained positively with the anti-mouse Foxp3 Ab (red, *left*) and with anti-ovine CD4 (green, *middle*). The merged image revealed that Foxp3$^+$ cells were always CD4$^+$ and they were considered as T_{reg} cells (*right*). (**C**) As assessed with this Ab, when transplanted at 48 days of gestation, more than half of the surrounding T cells at 2 weeks post-transplant (Fig. 3B) were positive for Foxp3 (stained in red, *left*). Ten to 20% of the T cells in the grafts at birth (Fig. 4A) were positive for Foxp3 (stained in red, arrows, *right*).

and demonstrate the suppressive function. However, it was not possible to collect sufficient viable immune cells from the tiny subcutaneous tissues.

DISCUSSION

In the setting of nonprimate-to-primate xenotransplantation, very rapid and vigorous immune rejection occurs because of the interaction between the Gal α1-3Gal epitope abundantly expressed on nonprimate cells and the primate natural anti-Gal α1-3Gal antibody [43–46]. In contrast, primate-to-nonprimate xenotransplantation does not evoke such rejection because primate cells do not express the Galα1-3Gal epitope [47]. Regarding this point, our strategy to generate primate ES cell-derived grafts in sheep has cleared one hurdle of xenotransplantation. In addition, hES cells are less immunogenic even in xenotransplantation settings [34,36]. For instance, when hES cells were transplanted in the leg muscle of immunocompetent mice, no leukocytic infiltration was observed 48 h later, although human mature cells induced rapid granulocytic infiltration within 48 h [36]. Our MLR results showing much lower stimulation evoked by cyES cells or their progeny than by cynomolgus PBMCs might be explained by the less immunogenicity of cyES cells.

In the present study, we transplanted cyES cells into sheep fetuses under several different conditions. Only when transplanted with more than 1×10^6 cyES cells at $<$50 days of gestation did cyES cell progeny show sustained engraftment even after birth. To our knowledge, this is the first report describing the long-term macroscopic engraftment of xenogeneic ES cells after in utero transplantation.

One issue to be discussed is why cynomolgus tissues can engraft for such a long time in sheep. There are several possible explanations for this. First, we showed that the premature innate immunity before 50 days of gestation might be one of the reasons for the survival of the ES cells from early xenorejection. Second, the adaptive immune system during the early fetal period is so premature that even xenogeneic cynomolgus cells introduced in this period can be recognized as a sort of "self." However, both cellular and humoral immune responses against cyES cells were detected in the cyES cell-engrafted sheep, and additional engraftment was not successful in the animals. Therefore, despite their sustained engraftment, the cynomolgus tissues in sheep are recognized as foreign. Third, mixed hematopoietic chimerism (existence of both donor and recipient hematopoiesis) would induce donor-specific T cell tolerance even across a xenogeneic barrier [48–50]. In the mouse allogeneic setting, the transplantation of ES cells is shown to generate such mixed hematopoietic chimerism [51]. Similarly, transplanted cyES cells possibly generated mixed hematopoietic chimerism in sheep, serving to induce cynomolgus-specific tolerance. However, no cynomolgus cells were detectable in the peripheral blood of the cyES cell-engrafted sheep ($n = 4$) as assessed by a sensitive PCR analysis; that is, there was no mixed hematopoietic chimerism in the sheep (data not shown). Thus, the sustained engraftment of cynomolgus cells in sheep was not attributable to mixed hematopoietic chimerism.

Finally, T_{reg} cells would serve to induce transplant tolerance [52]. When transplanted at $<$50 days of gestation, $CD4^+$ T cells were found mobilized around transplanted cyES cell progeny, many of which were $Foxp3^+$ T_{reg} cells. For allografts to survive, T_{reg} cells had to promote tolerance in mice [53,54]. In a xenogeneic setting, host T_{reg} cells were shown to suppress immune responses to donor antigens in athymic mice that were grafted with neonatal porcine thymus [55]. In the human fetus, preterm cord blood is known to contain a high proportion of T_{reg} cells that declines with gestational age to the level in adult peripheral blood [56]. Fetal $CD4^+$ $CD25^{high}$ T_{reg} cells were reported to play an important role in the suppression of immature fetal T cell responses during early development, which might suppress the auto-reactive T cells or alloreactivity to maternal antigens [57]. Therefore, it is possible that an adequate number of T_{reg} cells were mobilized in the early fetal period and contributed to the engraftment of cynomolgus tissues in sheep.

In this study, however, additional engraftment after birth was not successful. Although further investigation of the fetal immune system is necessary, one plausible possibility is that with the maturation of immune system, the immune responses against xenogeneic cynomolgus tissues might eventually exceed the ability of T_{reg} cells to suppress the immune responses. To provide a sufficient supply of therapeutic cells or tissues by this in vivo differentiation method, further interventions for successful additional transplantation would be necessary. One might be to somehow enhance the ability of T_{reg} cells to suppress the immune responses after birth. Another one might be the induction of immunological tolerance or unresponsiveness through mixed hematopoietic chimerism by in utero co-transplantation of congenic hematopoietic stem cells or those derived from the ES cells. Given that xenograft rejection requires T cells [34], administration of immunosuppressive drugs to the fetal and cyES cell-engrafted sheep might be of help, although it should be considered that T_{reg} cells may also be suppressed.

In conclusion, when a certain quantity of cyES cells are transplanted before 50 days of gestation, $Foxp3^+$ T_{reg} cells are mobilized and cyES cell-derived mature cells are able to survive long term in sheep, although immunological tolerance is not achieved. This finding suggests a possibility of generating sheep with human grafts after in utero transplantation of hES cells, although ma-

jor challenges remain, especially with respect to the in vivo regulation of hES cell differentiation to functional cells. Because the differentiation of ES cells in vivo is influenced by the microenvironment at transplantation sites [31,58], transplantation into specific sites might be of help in regulating the differentiation. Genetic manipulation of ES cells should be of help, for instance transduction with *hoxb4* for in vivo hematopoiesis [59]. Differentiation of ES cells to certain precursor cells in vitro prior to transplant might be also helpful [60]. Because in vivo ES cell-derived grafts after birth were no longer at all positive for the pluripotent marker Oct-3, they are free of undifferentiated ES cells, implying their potential utility for clinical cell preparations [61,62]. In view of clinical application, however, there is concern about potential risk of horizontal infection between species [63]. Although closed housing and breeding of carefully selected specific pathogen-free sheep herd could possibly control infections, the risk of unknown pathogens cannot be eliminated [64]. Therefore, further study and constant vigilance are inevitable.

ACKNOWLEDGMENTS

We thank Norio Nakatsuji (Kyoto University, Kyoto, Japan) and Yasushi Kondo (Tanabe Seiyaku Co., Ltd., Osaka, Japan) for providing cyES cells. We acknowledge Chieko Ohno for technical assistance. This study was supported by grants (JMS 21st Century COE Program, High-tech Research Center Program, and KAKENHI) from the Ministry of Education, Culture, Sports, Science and Technology of Japan as well as grants (KAKENHI) from the Ministry of Health, Labor and Welfare of Japan.

REFERENCES

1. Thomson JA, J Itskovitz-Eldor, SS Shapiro, MA Waknitz, JJ Swiergiel, VS Marshall and JM Jones. (1998). Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147.
2. Reubinoff BE, MF Pera, CY Fong, A Trounson and A Bongso. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nature Biotechnol 18:399–404.
3. Bjorklund LM, R Sanchez-Pernaute, S Chung, T Andersson, IY Chen, KS McNaught, AL Brownell, BG Jenkins, C Wahlestedt, KS Kim and O Isacson. (2002). Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. Proc Natl Acad Sci USA 99:2344–2349.
4. Asano T, N Ageyama, K Takeuchi, M Momoeda, Y Kitano, K Sasaki, Y Ueda, Y Suzuki, Y Kondo, R Torii, M Hasegawa, S Ookawara, K Harii, K Terao, K Ozawa and Y Hanazono. (2003). Engraftment and tumor formation after allogeneic in utero transplantation of primate embryonic stem cells. Transplantation 76:1061–1067.
5. Auchincloss H, Jr. and DH Sachs. (1998). Xenogeneic transplantation. Annu Rev Immunol 16:433–470.
6. Sablinski T, DW Emery, R Monroy, RJ Hawley, Y Xu, P Gianello, T Lorf, T Kozlowski, M Bailin, DK Cooper, AB Cosimi and DH Sachs. (1999). Long-term discordant xenogeneic (porcine-to-primate) bone marrow engraftment in a monkey treated with porcine-specific growth factors. Transplantation 67:972–977.
7. Gritsch HA, RM Glaser, DW Emery, LA Lee, CV Smith, T Sablinski, JS Arn, DH Sachs and M Sykes. (1994). The importance of nonimmune factors in reconstitution by discordant xenogeneic hematopoietic cells. Transplantation 57:906–917.
8. Kehat I, L Khimovich, O Caspi, A Gepstein, R Shofti, G Arbel, I Huber, J Satin, J Itskovitz-Eldor and L Gepstein. (2004). Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. Nature Biotechnol 22:1282–1289.
9. Silverstein AM, RA Prendergast and KL Kraner. (1964). Fetal Response to Antigenic Stimulus. Iv. Rejection of Skin Homografts by the Fetal Lamb. J Exp Med 119:955–964.
10. Flake AW, MR Harrison, NS Adzick and ED Zanjani. (1986). Transplantation of fetal hematopoietic stem cells in utero: the creation of hematopoietic chimeras. Science 233:776–778.
11. Zanjani ED, MG Pallavicini, JL Ascensao, AW Flake, RG Langlois, M Reitsma, FR MacKintosh, D Stutes, MR Harrison and M Tavassoli. (1992). Engraftment and long-term expression of human fetal hemopoietic stem cells in sheep following transplantation in utero. J Clin Invest 89:1178–1188.
12. Civin CI, G Almeida-Porada, MJ Lee, J Olweus, LW Terstappen and ED Zanjani. (1996). Sustained, retransplantable, multilineage engraftment of highly purified adult human bone marrow stem cells in vivo. Blood 88:4102–4109.
13. Flake AW and ED Zanjani. (1999). In utero hematopoietic stem cell transplantation: ontogenic opportunities and biologic barriers. Blood 94:2179–2191.
14. Fujiki Y, K Fukawa, K Kameyama, O Kudo, M Onodera, Y Nakamura, K Yagami, Y Shiina, H Hamada, A Shibuya and H Nakauchi. (2003). Successful multilineage engraftment of human cord blood cells in pigs after in utero transplantation. Transplantation 75:916–922.
15. Narayan AD, JL Chase, RL Lewis, X Tian, DS Kaufman, JA Thomson and ED Zanjani. (2006). Human embryonic stem cell-derived hematopoietic cells are capable of engrafting primary as well as secondary fetal sheep recipients. Blood 107:2180–2183.
16. Sasaki K, Y Nagao, Y Kitano, H Hasegawa, H Shibata, M Takatoku, S Hayashi, K Ozawa and Y Hanazono. (2005). Hematopoietic microchimerism in sheep after in utero transplantation of cultured cynomolgus embryonic stem cells. Transplantation 79:32–37.
17. Menard C, AA Hagege, O Agbulut, M Barro, MC Morichetti, C Brasselet, A Bel, E Messas, A Bissery, P Bruneval, M Desnos, M Puceat and P Menasche. (2005). Transplantation of cardiac-committed mouse embryonic

stem cells to infarcted sheep myocardium: a preclinical study. Lancet 366:1005–1012.

18. Bonnevie L, A Bel, L Sabbah, N Al Attar, P Pradeau, B Weill, F Le Deist, V Bellamy, S Peyrard, C Menard, M Desnos, P Bruneval, P Binder, AA Hagege, M Puceat and P Menasche. (2007). Is xenotransplantation of embryonic stem cells a realistic option? Transplantation 83:333–335.
19. Symonds ME, A Mostyn and T Stephenson. (2001). Cytokines and cytokine receptors in fetal growth and development. Biochem Soc Trans 29:33–37.
20. Takada T, Y Suzuki, Y Kondo, N Kadota, K Kobayashi, S Nito, H Kimura and R Torii. (2002). Monkey embryonic stem cell lines expressing green fluorescent protein. Cell Transplant 11:631–635.
21. Suemori H, T Tada, R Torii, Y Hosoi, K Kobayashi, H Imahie, Y Kondo, A Iritani and N Nakatsuji. (2001). Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. Dev Dyn 222:273–279.
22. Fujimoto Y, K Hasegawa, H Suemori, J Ito and N Nakatsuji. (2006). Molecular cloning and function of Oct-3 isoforms in cynomolgus monkey embryonic stem cells. Stem Cells Dev 15:566–574.
23. Andreoletti O, P Berthon, E Levavasseur, D Marc, F Lantier, E Monks, JM Elsen and F Schelcher. (2002). Phenotyping of protein-prion (PrPsc)-accumulating cells in lymphoid and neural tissues of naturally scrapie-affected sheep by double-labeling immunohistochemistry. J Histochem Cytochem 50:1357–1370.
24. Scanlan CM, ed. (1998). *Introduction to Veterinary Bacteriology*. Iowa State University Press, Ames, Iowa.
25. Kaufman DS and JA Thomson. (2002). Human ES cells—haematopoiesis and transplantation strategies. J Anat 200: 243–248.
26. Hasegawa K, T Fujioka, Y Nakamura, N Nakatsuji and H Suemori. (2006). A method for the selection of human embryonic stem cell sublines with high replating efficiency after single-cell dissociation. Stem Cells 24:2649–2660.
27. Okamura K, K Asahina, H Fujimori, R Ozeki, K Shimizu-Saito, Y Tanaka, K Teramoto, S Arii, K Takase, M Kataoka, Y Soeno, C Tateno, K Yoshizato and H Teraoka. (2006). Generation of hybrid hepatocytes by cell fusion from monkey embryoid body cells in the injured mouse liver. Histochem Cell Biol 125:247–257.
28. Nussbaum J, E Minami, MA Laflamme, JA Virag, CB Ware, A Masino, V Muskheli, L Pabon, H Reinecke and CE Murry. (2007). Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. FASEB J 21:1345–1357.
29. Nichols J, B Zevnik, K Anastassiadis, H Niwa, D Klewe-Nebenius, I Chambers, H Scholer and A Smith. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95:379–391.
30. Niwa H, J Miyazaki and AG Smith. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nature Genet 24:372–376.
31. Cooke MJ, M Stojkovic and SA Przyborski. (2006). Growth of teratomas derived from human pluripotent stem cells is influenced by the graft site. Stem Cells Dev 15: 254–259.
32. Sawyer M, J Moe and BI Osburn. (1978). Ontogeny of immunity and leukocytes in the ovine fetus and elevation of immunoglobulins related to congenital infection. Am J Vet Res 39:643–648.
33. Miyasaka M and B Morris. (1988). The ontogeny of the lymphoid system and immune responsiveness in sheep. Prog Vet Microbiol Immunol 4:21–55.
34. Drukker M, H Katchman, G Katz, S Even-Tov Friedman, E Shezen, E Hornstein, O Mandelboim, Y Reisner and N Benvenisty. (2006). Human embryonic stem cells and their differentiated derivatives are less susceptible to immune rejection than adult cells. Stem Cells 24:221–229.
35. Fox A, J Mountford, A Braakhuis and LC Harrison. (2001). Innate and adaptive immune responses to nonvascular xenografts: evidence that macrophages are direct effectors of xenograft rejection. J Immunol 166:2133–2140.
36. Li L, ML Baroja, A Majumdar, K Chadwick, A Rouleau, L Gallacher, I Ferber, J Lebkowski, T Martin, J Madrenas and M Bhatia. (2004). Human embryonic stem cells possess immune-privileged properties. Stem Cells 22:448–456.
37. Juretic E, A Juretic, B Uzarevic and M Petrovecki. (2001). Alterations in lymphocyte phenotype of infected preterm newborns. Biol Neonate 80:223–227.
38. Thornton CA, JW Upham, ME Wikstrom, BJ Holt, GP White, MJ Sharp, PD Sly and PG Holt. (2004). Functional maturation of $CD4^+CD25^+CTLA4^+CD45RA^+$ T regulatory cells in human neonatal T cell responses to environmental antigens/allergens. J Immunol 173:3084–3092.
39. Chong AS, J Shen, J Tao, D Yin, A Kuznetsov, M Hara and LH Philipson. (2006). Reversal of diabetes in non-obese diabetic mice without spleen cell-derived beta cell regeneration. Science 311:1774–1775.
40. Hori S, T Nomura and S Sakaguchi. (2003). Control of regulatory T cell development by the transcription factor Foxp3. Science 299:1057–1061.
41. Yagi H, T Nomura, K Nakamura, S Yamazaki, T Kitawaki, S Hori, M Maeda, M Onodera, T Uchiyama, S Fujii and S Sakaguchi. (2004). Crucial role of FOXP3 in the development and function of human $CD25^+CD4^+$ regulatory T cells. Int Immunol 16:1643–1656.
42. Condomines M, P Quittet, ZY Lu, L Nadal, P Latry, E Lopez, M Baudard, G Requirand, C Duperray, JF Schved, JF Rossi, K Tarte and B Klein. (2006). Functional regulatory T cells are collected in stem cell autografts by mobilization with high-dose cyclophosphamide and granulocyte colony-stimulating factor. J Immunol 176:6631–6639.
43. Cooper DK, B Gollackner and DH Sachs. (2002). Will the pig solve the transplantation backlog? Annu Rev Med 53:133–147.
44. Sachs DH. (1994). The pig as a potential xenograft donor. Vet Immunol Immunopathol 43:185–191.
45. Minanov OP, S Itescu, FA Neethling, AS Morgenthau, P Kwiatkowski, DK Cooper and RE Michler. (1997). Anti-GaL IgG antibodies in sera of newborn humans and baboons and its significance in pig xenotransplantation. Transplantation 63:182–186.

46. Galili U, SB Shohet, E Kobrin, CL Stults and BA Macher. (1988). Man, apes, and Old World monkeys differ from other mammals in the expression of alpha-galactosyl epitopes on nucleated cells. J Biol Chem 263:17755–17762.
47. Galili U, MR Clark, SB Shohet, J Buehler and BA Macher. (1987). Evolutionary relationship between the natural anti-Gal antibody and the Gal alpha 1-3Gal epitope in primates. Proc Natl Acad Sci USA 84:1369–1373.
48. Abe M, J Qi, M Sykes and YG Yang. (2002). Mixed chimerism induces donor-specific T-cell tolerance across a highly disparate xenogeneic barrier. Blood 99:3823–3829.
49. Lan P, L Wang, B Diouf, H Eguchi, H Su, R Bronson, DH Sachs, M Sykes and YG Yang. (2004). Induction of human T-cell tolerance to porcine xenoantigens through mixed hematopoietic chimerism. Blood 103:3964–3969.
50. Lan P, N Tonomura, A Shimizu, S Wang and YG Yang. (2006). Reconstitution of a functional human immune system in immunodeficient mice through combined human fetal thymus/liver and $CD34^+$ cell transplantation. Blood 108:487–492.
51. Bonde S and N Zavazava. (2006). Immunogenicity and engraftment of mouse embryonic stem cells in allogeneic recipients. Stem Cells 24:2192–2201.
52. Wood KJ and S Sakaguchi. (2003). Regulatory T cells in transplantation tolerance. Nature Rev Immunol 3:199–210.
53. Graca L, SP Cobbold and H Waldmann. (2002). Identification of regulatory T cells in tolerated allografts. J Exp Med 195:1641–1646.
54. Lee MK 4th, DJ Moore, BP Jarrett, MM Lian, S Deng, X Huang, JW Markmann, M Chiaccio, CF Barker, AJ Caton and JF Markmann. (2004). Promotion of allograft survival by $CD4^+CD25^+$ regulatory T cells: evidence for in vivo inhibition of effector cell proliferation. J Immunol 172: 6539–6544.
55. Sun Z, L Zhao, H Wang, L Sun, H Yi and Y Zhao. (2006). Presence of functional mouse regulatory $CD4^+CD25^+$T cells in xenogeneic neonatal porcine thymus-grafted athymic mice. Am J Transplant 6:2841–2850.
56. Takahata Y, A Nomura, H Takada, S Ohga, K Furuno, S Hikino, H Nakayama, S Sakaguchi and T Hara. (2004). $CD25^+CD4^+$ T cells in human cord blood: an immunoregulatory subset with naive phenotype and specific expression of forkhead box p3 (Foxp3) gene. Exp Hematol 32: 622–629.
57. Michaelsson J, JE Mold, JM McCune and DF Nixon. (2006). Regulation of T cell responses in the developing human fetus. J Immunol 176:5741–5748.
58. Gertow K, S Wolbank, B Rozell, R Sugars, M Andang, CL Parish, MP Imreh, M Wendel and L Ahrlund-Richter. (2004). Organized development from human embryonic stem cells after injection into immunodeficient mice. Stem Cells Dev 13:421–435.
59. Kyba M, RC Perlingeiro and GQ Daley. (2002). HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. Cell 109:29–37.
60. Lu SJ, Q Feng, S Caballero, Y Chen, MA Moore, MB Grant and R Lanza. (2007). Generation of functional hemangioblasts from human embryonic stem cells. Nature Methods 4:501–509.
61. Fujikawa T, SH Oh, L Pi, HM Hatch, T Shupe and BE Petersen. (2005). Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. Am J Pathol 166:1781–1791.
62. Shibata H, N Ageyama, Y Tanaka, Y Kishi, K Sasaki, S Nakamura, S Muramatsu, S Hayashi, Y Kitano, K Terao and Y Hanazono. (2006). Improved safety of hematopoietic transplantation with monkey embryonic stem cells in the allogeneic setting. Stem Cells 24:1450–1457.
63. Wootton SK, CL Halbert and AD Miller. (2005). Sheep retrovirus structural protein induces lung tumours. Nature 434:904–907.
64. Klymiuk N, M Muller, G Brem and B Aigner. (2003). Characterization of endogenous retroviruses in sheep. J Virol 77:11268–11273.

Address reprint requests to:
Dr. Yutaka Hanazono
Professor, Division of Regenerative Medicine
Center for Molecular Medicine
Jichi Medical University
3311-1 Yakushiji, Shimotsuke
Tochigi 329-0498, Japan.

E-mail: hanazono@jichi.ac.jp

Received for publication July 6, 2007; accepted after revision November 19, 2007.

Transduction Properties of Adenovirus Serotype 35 Vectors After Intravenous Administration Into Nonhuman Primates

Fuminori Sakurai[1], Shin-ichiro Nakamura[2,3], Kimiyo Akitomo[1], Hiroaki Shibata[2], Keiji Terao[2], Kenji Kawabata[1], Takao Hayakawa[4] and Hiroyuki Mizuguchi[1,5]

[1]Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Ibaraki City, Osaka, Japan; [2]Tsukuba Primates Research Center, National Institute of Biomedical Innovation, Tsukuba City, Ibaraki, Japan; [3]The Corporation for Production and Research of Laboratory Primates, Tsukuba City, Ibaraki, Japan; [4]Pharmaceuticals and Medical Devices Agency, Chiyoda-Ku, Tokyo, Japan; [5]Graduate School of Pharmaceutical Sciences, Osaka University, Suita City, Osaka, Japan

Adenovirus serotype 35 (Ad35) vectors have shown promise as effective gene delivery vehicles. However, the transduction profiles of Ad35 vectors in conventional mice allow only a limited estimation of transduction properties of these vectors, because the mouse analog of the subgroup B Ad receptor, CD46, is restricted to the testis. In order to assess the transduction properties of Ad35 vectors more completely, we performed transduction experiments using cynomolgus monkeys, which ubiquitously express CD46 in a pattern similar to that in humans. *In vitro* transduction experiments demonstrated that cultured cells from the cynomolgus monkey were efficiently transduced with Ad35 vectors. In contrast, after intravenous administration into live monkeys hardly any evidence of Ad35 vector–mediated transduction was found in any of the organs, although Ad35 vector genomes were detected in various organs. Less severe histopathological abnormalities were found in the Ad35 vector–infused monkeys than in the conventional Ad5 vector–injected monkeys. In the latter, serious tissue damage and inflammatory responses, such as hepatocyte necrosis and lymphatic hyperplasia in the colon, were induced. Both Ad35 and Ad5 vectors caused similar hematological changes (increase in $CD3^+$ cells, and decrease in $CD16^+$ cells and $CD20^+$ cells) in peripheral blood cells. These results should provide valuable information for the clinical application of Ad35 vectors.

Received 15 September 2007; accepted 16 January 2007; published online 11 March 2008. doi:10.1038/mt.2008.19

INTRODUCTION

Human adenoviruses (Ads) are nonenveloped, double-stranded DNA viruses that are composed of 51 serotypes.[1,2] Among the 51 serotypes, the conventional Ad vectors that are most widely used, including for human clinical trials, are constructed based on the subgroup C Ad serotype 5 (Ad5). Ad5 vectors have several advantages as gene delivery vehicles, but clinical and preclinical studies have revealed three major disadvantages of Ad5 vectors. First, target cells that are important for gene therapy, including malignant tumor cells and dendritic cells, express nil or insufficient levels of a cellular receptor for Ad5, the coxsackievirus-adenovirus receptor. The transduction efficiencies of Ad5 vectors depend to a large extent on the expression levels of coxsackievirus-adenovirus receptor, leading to refractoriness of coxsackievirus-adenovirus receptor-negative cells to Ad5 vectors.[3] Second, >50% of adults are seropositive for Ad5 because natural infection with Ad5 is common.[4,5] Pre-existing anti-Ad5 antibodies not only largely inhibit Ad5 vector–mediated transduction, but may also enhance the toxicities induced by Ad5 vectors.[6] Third, inflammatory responses are systemically and rapidly induced by intravascular administration of Ad5 vectors, leading to tissue damage, and this can be fatal to the host.[7–10]

In order to address these problems, we as well as others have developed a replication-incompetent subgroup B Ad serotype 35 (Ad35) vector.[11–15] Ad35 vectors utilize human CD46 as a cellular receptor.[16,17] Human CD46 is ubiquitously expressed on almost all human cells, leading to a wide tropism of Ad35 vectors. In addition, pre-existing anti-Ad5 immunity does not hamper Ad35 vector–mediated transduction, and seroprevalence for Ad35 is much lower than that for Ad5 (refs. 13,14). Ad35 vectors have properties that make them very promising prospects for use as transduction vehicles, but the transduction efficiencies of Ad35 vectors in conventional mice are lower than those of Ad5 vectors.[12,14] Conventional mice seem inappropriate as animal models for Ad35 vectors because mouse CD46 is expressed only in the testis.[18] In addition, there is low homology between human CD46 and mouse CD46. We considered that transduction experiments with Ad35 vectors should be performed using nonhuman primates so as to properly evaluate the transduction properties of Ad35 vectors. The CD46 of nonhuman primates is ubiquitously expressed in a similar pattern to humans, and shows high homology to human CD46.[19]

In this study, we examined the transduction profiles of Ad35 vectors after intravenous administration into nonhuman primates, *i.e.*, cynomolgus monkeys. Ad35 vector–induced immune responses and the blood concentrations of Ad35 vectors were

Correspondence: Hiroyuki Mizuguchi, Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, 7-6-8 Asagi, Saito, Ibaraki City, Osaka 567-0085, Japan. E-mail: mizuguch@nibio.go.jp

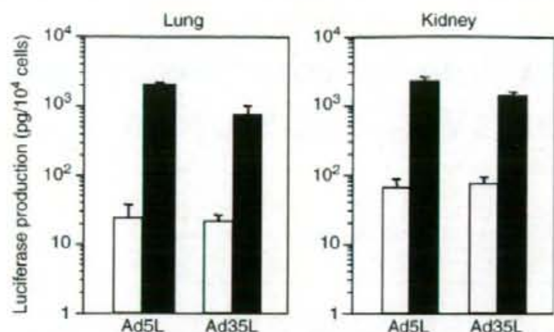

Figure 1 *In vitro* transduction efficiencies of Ad35 and Ad5 vectors in cultured cells of cynomolgus monkey. Luciferase production in primary lung and kidney cells following Ad vector transduction. Primary lung and kidney cells isolated from cynomolgus macaque embryos were transduced with Ad35L or Ad5L at 300 (open bar) and 3,000 vector particles/cell (closed bar) for 1.5 hours. After a 48-hour culture, luciferase production in the cells was measured by luminescence assay. The data are expressed as the mean values ± SD ($n = 4$). Luciferase expression in the mock-infected cells was less than the detectable level. Ad, adenovirus.

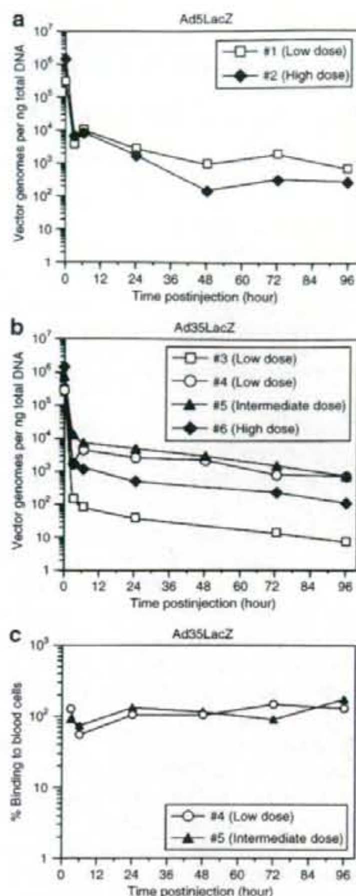

Figure 2 Persistence of adenoviral (Ad) vectors in the blood of cynomolgus monkeys following systemic administration. (**a**) Ad vector DNA concentrations in the blood after intravenous administration. Cynomolgus monkeys were intravenously infused with Ad35LacZ or Ad5LacZ at low [0.4×10^{12} vector particles (VP)/kg], intermediate (1.0×10^{12}VP/kg), or high (2×10^{12}VP/kg) doses. Blood was collected at the indicated time points after injection (3, 6, 24, 48, 72, and 96 hours after injection). Total DNA, including Ad vector DNA, was isolated from the blood, and the Ad vector DNA contents were measured using quantitative TaqMan polymerase chain reaction (PCR) assay. The concentrations of the Ad vectors in the blood at the zero time point were calculated based on the total number of Ad vector particles infused and the estimated circulating blood volume (65 ml/kg). Ad vector DNA was not detected in the blood before injection. (**b**) Percentages of blood cell–associated Ad35lacZ remaining in the blood after systemic administration in cynomolgus monkeys. After isolating the blood as described, blood cells were washed twice with phosphate-buffered saline buffer and the amounts of Ad35LacZ associated with blood cells were evaluated using TaqMan PCR as described earlier. The percentages were calculated as follows: 100 × (the amounts of Ad35 vector DNA associated with blood cells)/(the amounts of Ad35 vector DNA recovered from whole blood).

analyzed for 4 days after the injection. Necropsy was performed 4 days after the injection to examine the transduction efficiencies, tissue accumulations of Ad35 vectors, and histopathological changes in the organs after injection.

RESULTS

In vitro transduction in cultured cynomolgus monkey cells

First, to examine whether cynomolgus monkey cells were susceptible to Ad35 vectors, primary lung and kidney cells isolated from embryonic cynomolgus monkeys were transduced with a firefly luciferase-expressing Ad35 vector (Ad35L) and a conventional Ad5 vector (Ad5L). Both Ad35L and Ad5L vectors were shown to mediate efficient transduction in the cells from both organs (**Figure 1**). Ad35 vectors also efficiently transduced the cynomolgus monkey T-cell line HSC-F (**Supplementary Figure S1**). These results indicate that cynomolgus monkey cells are susceptible to Ad35 vectors. However, peripheral blood mononuclear cells of cynomolgus monkeys were almost refractory to Ad35 vectors (data not shown).

Blood clearance of Ad vectors

Next, the six cynomolgus monkeys (designated #1–#6) were administered either a β-galactosidase-expressing Ad35 vector (Ad35LacZ) or an Ad5 vector (Ad5LacZ) through the femoral vein (**Supplementary Table S1**). The blood clearances of the Ad vectors were examined using a quantitative real-time polymerase chain reaction. Both Ad35LacZ and Ad5LacZ vectors were rapidly cleared from the blood circulation within 24 hours after the injection (**Figure 2a** and **b**). We did not find any apparent differences between the blood-clearance kinetics of Ad35LacZ and Ad5LacZ. Assuming that the entire Ad vector DNA in the blood was completely recovered from the blood samples, there would remain 0.12% and 0.09% of the injected Ad35LacZ in the blood of monkey #6 at 3 and 6 hours after injection, respectively. The lower levels of Ad35LacZ remaining in the blood of monkeys #3 and #6 than those in monkeys #4 and #5 might have been partly because of the low infectious titer-to-particle ratio of the vector batch of Ad35LacZ injected into monkeys #3 and #6. The infectious titer-to-particle

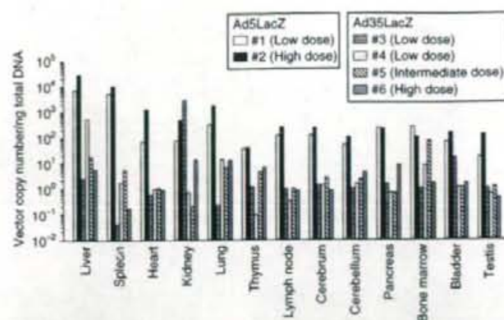

Figure 3 *In vivo* tissue distributions of adenoviral (Ad) vector DNA in cynomolgus monkeys after systemic administration. Ad35LacZ or Ad5LacZ was intravenously administered into cynomolgus monkeys as described for **Figure 2**. Four days after the injection, necropsy was performed, and Ad vector DNA contents were measured using quantitative TaqMan polymerase chain reaction analysis. The Ad vector DNA was not detected in the organs of mock-infected animals.

ratio of the Ad35LacZ used in monkeys #3 and #6 was lower than that used in monkeys #4 and #5 (data not shown). Noninfectious Ad particles might be more easily degraded in the blood or taken up by phagocytic cells.

Further, we examined whether the Ad35 vectors were associated with blood cells in the blood stream after the injection. The majority of Ad35LacZ remaining in the blood was associated with blood cells at all the time points (**Figure 2c**). Similarly, assuming the complete recovery of the Ad vector DNA as described earlier, 1.5% of the injected Ad35LacZ would be associated with blood cells in monkey #5 at 3 hours after the injection. The levels of Ad35LacZ associated with blood cells remained constant during the study. These results suggest that Ad35 vectors may bind to blood cells, or be taken up by blood cells after the injection.

Tissue distribution of Ad vectors

In order to examine the biodistribution of Ad35 and Ad5 vectors in cynomolgus monkeys after intravenous administration, Ad DNA contents in the organs were assessed (**Figure 3**). The Ad35 vector DNA was mainly found in the liver, lung, and kidney; however, the levels of Ad35 vector DNA were one to five orders of magnitude lower in almost all organs than the levels of Ad5 vector DNA, which was found mainly in the liver and spleen. Ad35LacZ was also less efficiently accumulated in the organs that exhibited low levels of Ad5LacZ accumulation, such as the thymus and testis.

Ad vector–mediated transgene expression in organs

In order to evaluate the *in vivo* transduction efficiencies of Ad35 and Ad5 vectors, β-galactosidase expression in the organs was examined. Ad5LacZ efficiently transduced the organs (**Figure 4a**). The highest level of β-galactosidase production was found in the liver, followed by the spleen. Liver parenchymal cells and spleen marginal zone cells were mainly transduced by Ad5LacZ in these organs (**Figure 4b**). On the other hand, Ad35 vector–mediated β-galactosidase expression in the organs at all doses was approximately equal to, or slightly above, the levels in mock-infected animals. X-gal-positive cells were not found in the tissue sections of the liver or spleen of the Ad35LacZ-infused monkeys. These results indicate that Ad35 vectors show much lower transduction activity than Ad5 vectors after systemic delivery in cynomolgus monkeys.

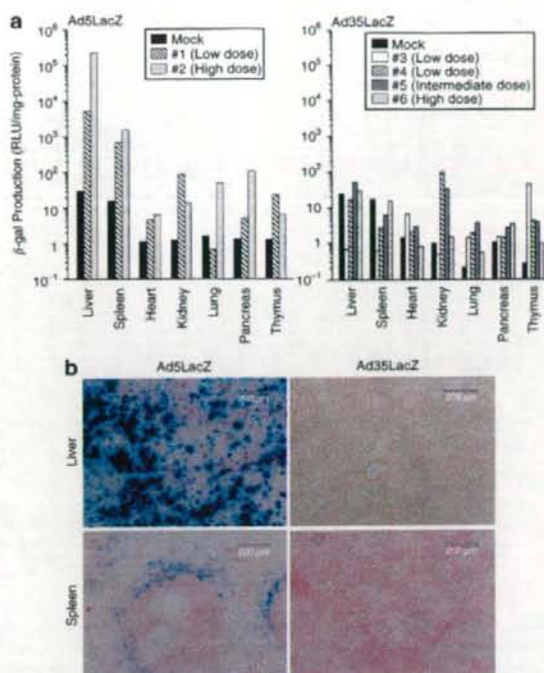

Figure 4 Adenoviral (Ad) vector-mediated transgene expression in cynomolgus monkeys after systemic administration. (**a**) Chemiluminescence analysis of β-galactosidase production in cynomolgus monkeys after systemic administration of Ad35LacZ or Ad5LacZ. Ad35LacZ or Ad5LacZ was intravenously injected into cynomolgus monkeys as described for **Figure 2**. Four days after injection, the organs were collected, and β-galactosidase production in the organs was assessed using a chemiluminescence assay. (**b**) X-gal staining of tissue sections of cynomolgus monkeys receiving Ad5LacZ or Ad35LacZ. Four days after intravenous administration of Ad35LacZ or Ad5LacZ at a high dose (2×10^{12} vector particles/kg), tissues were collected, and X-gal staining was performed as described in Materials and Methods. RLU, relative light units.

Serum chemistry profiles

Next, we measured the levels of serum biochemical markers to assess Ad vector–induced tissue/organ damage. Almost all the markers were increased following Ad vector injection; however, overall, the markers examined appeared to be more elevated in the monkeys receiving Ad5LacZ than in those receiving Ad35LacZ (**Figure 5a**). Aspartate aminotransferase (AST) levels were elevated as early as 3 hours after the injection, and peaked at 24 hours in most cases. The peak levels of AST in Ad35LacZ-injected monkeys #3, #4, #5, and #6 were 6.1-, 4.8-, 8.2-, and 3.8-fold higher than the preinjection levels, respectively. By contrast, Ad5LacZ-infused monkeys (#1 and #2) showed 4.9- and 27.5-fold increases in AST at the peak points, respectively. Significant elevations in alanine

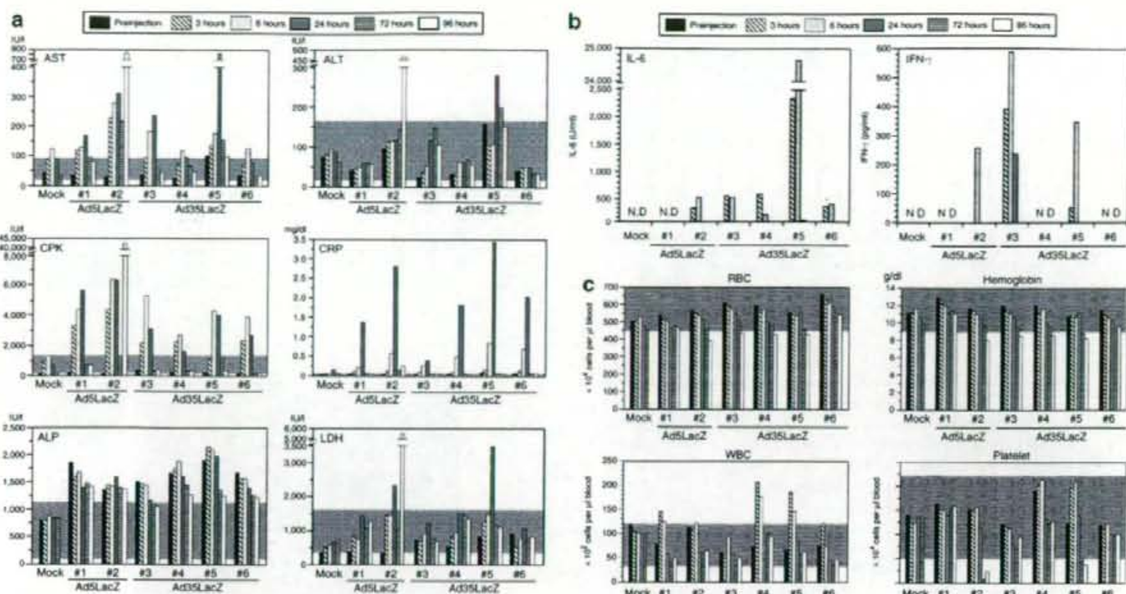

Figure 5 Blood analysis after adenoviral (Ad) vector administration to cynomolgus monkeys. **(a)** Serum marker levels, **(b)** inflammatory cytokine productions, and **(c)** blood cell counts in the peripheral blood after Ad vector administration. The gray area in the graphs of serum markers and blood cell counts indicates the normal range for adult cynomolgus monkeys. Ad35LacZ or Ad5LacZ was intravenously injected into cynomolgus monkeys and blood was collected as described for **Figure 2**. Serum marker levels and blood cell counts were measured using routine methods. Inflammatory cytokine levels were examined using enzyme-linked immunosorbent assay. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CPK, creatine phosphokinase; CRP, C-reactive protein; IFN-γ, interferon-γ; IL-6, interleukin-6; LDH, lactate dehydrogenase; ND, not detected (under the limit of detection); RBC, red blood cell; WBC, white blood cell.

aminotransferase were also found in several of the monkeys, but the alanine aminotransferase levels were within the normal range at almost all the time points. Creatine phosphokinase (CPK) levels sharply rose to a peak 6 or 24 hours after injection. CPK in the Ad35 vector–injected monkeys #3, #4, #5, and #6 showed 14.2-, 9.7-, 16.3-, and 17.7-fold increases at the peak points. On the other hand, the Ad5 vector–injected monkeys #1 and #2 exhibited 16.6- and 40.9-fold elevations in CPK at 6 hours after the injection. Dramatic increases in AST, alanine aminotransferase, and CPK levels in monkey #2 at 96 hours after injection was possibly caused by a slight expression of Ad5 E2 and/or E4 proteins. E4 protein was expressed in the liver 4 days after injection of conventional Ad vectors in mice, leading to liver damage.[20] Levels of C-reactive protein were also sharply increased in all the Ad vector–injected animals. A high dose of Ad35LacZ and Ad5LacZ caused 29-fold (#6) and 56.2-fold (#2) increases in C-reactive protein levels 24 hours after injection, respectively. Alkaline phosphatase levels gradually decreased over the first 96 hours after injection. Alkaline phosphatase levels at preinjection were higher than the normal range in the monkeys. This is because young cynomolgus monkeys (<4 years of age) often have alkaline phosphatase levels >1,000 IU/L. Apparent increases in lactate dehydrogenase were found in monkeys #2 and #5. The lactate dehydrogenase levels in the other animals were within the normal range. There were no abnormalities in the other parameters, including serum albumin, glucose, cholesterol, calcium, sodium, potassium, and chloride (data not shown).

Inflammatory cytokine induction

In order to examine the innate immune responses after Ad vector injection, inflammatory cytokine levels in the serum were measured (**Figure 5b**). Interleukin-6 (IL-6) was rapidly induced with a peak at 3 or 6 hours after the injection in all the animals except in monkey #1. There were no apparent differences in IL-6 levels between Ad35LacZ-treated and Ad5LacZ-treated animals, except that monkey #5 produced an extremely high level of IL-6. The levels of interferon-γ were also elevated and reached a peak at 6 hours after the injection in monkeys #2, #3, and #5. Tumor necrosis factor-α was not detected in any of the animals (data not shown).

Hematological profiles

In order to evaluate the influence of Ad vector injection on the hematological profiles, we examined the changes in peripheral blood cell counts (**Figure 5c**). The changes in the levels of red blood cells and hemoglobin were marginal, but the levels gradually decreased after injection in all the monkeys, including a mock-infected animal, probably because of the collection of large volumes of blood samples (>5 ml/time point) every day. Ad35LacZ-injected monkeys #3, #4, and #5, and Ad5LacZ-injected monkey #2 showed a rapid decline in platelet levels beginning at 24 hours after the injection. A transient increase in the platelet levels was found 3 and 6 hours after the injection in monkey #5. It remains unclear why the platelet levels increased in monkey #5; however, the previous study also reported an initial increase in the platelet levels after Ad5 vector injection in nonhuman primates.[21] A rapid

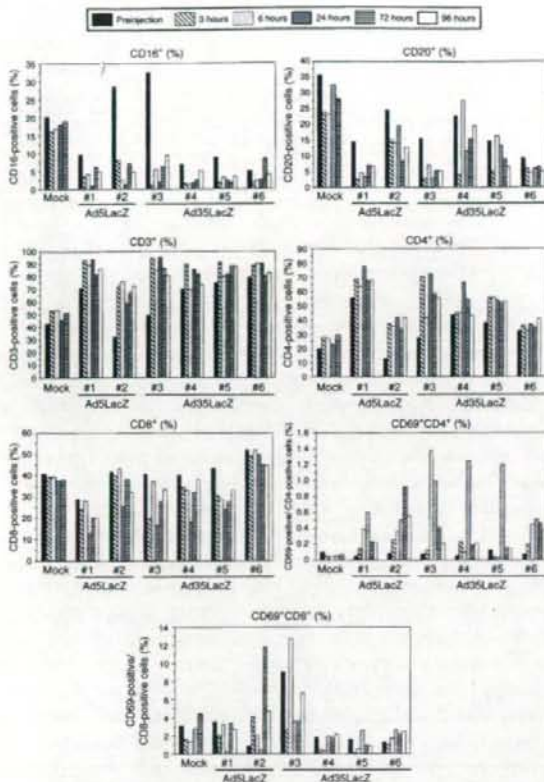

Figure 6 Profiles of peripheral blood lymphocyte subsets after systemic administration of adenoviral (Ad) vectors in cynomolgus monkeys. Ad35LacZ or Ad5LacZ was intravenously injected into cynomolgus monkeys and blood was collected as described for **Figure 2**. Peripheral blood mononuclear cells were stained with monoclonal antibodies following hemolysis, and fluorescence-activated cell sorting analysis was performed for evaluation of profiles of lymphocyte subsets.

elevation in the white blood cells was observed in the Ad vector–injected monkeys. The elevated white blood cells level returned to normal at 24 hours after the injection.

Next, we examined which types of blood cells were increased or decreased after Ad vector injection (**Figure 6**). The Ad vector injection induced a rapid decline in the percentages of $CD16^+$ cells (natural killer cells, granulocytes, and monocytes). Monkeys #2 and #3 showed sharp decreases of 71 and 97% of $CD16^+$ cells, respectively, at 3 hours after the injection. The percentages of $CD20^+$ cells (B cells) quickly dropped in all the monkeys, including a mock-infected monkey. In contrast, the $CD3^+$ cell (T-cell) levels were sharply elevated in the animals receiving the Ad vectors. We found a 1.1- to 2.3-fold increase in $CD3^+$ cell levels at 3 hours after the injection. $CD8^+$ cells did not increase, but rather decreased after the injection; however, increases in $CD4^+$ cells were found in the Ad vector–injected monkeys. The $CD4^+$ cell levels were 1.1- to 3.4-fold elevated compared with the preinjection levels, with a peak at 24 hours after the injection, in most of the animals. The administration of Ad vectors also increased

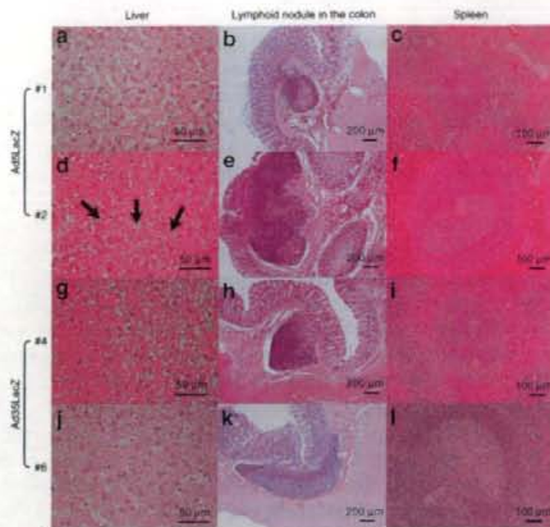

Figure 7 Histopathological analysis of liver, lymphoid nodules in the colon, and spleen. Representative histological sections of the liver (**a**, **d**, **g**, **j**), lymphoid nodules in the colon (**b**, **e**, **h**, **k**), and spleen (**c**, **f**, **i**, **l**) from animals killed 4 days after systemic injection of a low or high dose of Ad35LacZ (monkeys #4 and #6) or Ad5LacZ (#1 and #2). The arrows indicate necrosis of hepatocytes.

$CD69^+CD4^+$ cells (activated $CD4^+$ cells) more predominantly than $CD69^+CD8^+$ cells. Both $CD29^+CD4^+$ cells (memory helper T cells) and $CD29^-CD4^+$ cells (naive helper T cells) increased in the Ad vector–injected animals (data not shown). These results indicate that, overall, both Ad35 and Ad5 vectors cause similar changes in hematological profiles after systemic infusion.

Clinical observation and histopathological examinations

In order to perform a safety assessment of the Ad vectors, the health condition of the animals was monitored until necropsy. None of the Ad vector–infused monkeys showed any apparent abnormalities in appetite, body weight, body temperature, or heart rate. However, the low dose of Ad35LacZ (#3) induced vomiting 3 hours after the injection, and a skin rash was observed in monkey #2 on day 2.

In order to further evaluate the safety profiles of Ad vectors, organ histopathology was examined during necropsy. There were no obvious changes in the spleens of monkeys #1 and #3–#6, or in the livers in any of the animals. However, splenomegaly was found in monkey #2. The whitish nodules at the cut surface of the spleen in monkey #2 were the largest among those of all the monkeys examined. Marked swelling of the lymph nodules, especially in the colon and mesentery, was also found in monkey #2.

Microscopic analysis of tissue sections revealed that no apparent damage and inflammation were found in the liver of monkey #1 (**Figure 7a**). Although slight hyperplasia in the spleen white pulp occurred in this monkey (**Figure 7c**), no obvious changes were found in the lymphatic nodules of the colon (**Figure 7b**). In contrast, severe damage and inflammation, including necrosis of hepatocytes (**Figure 7d**, arrows) and infiltration of lymphocytes

into the Glisson's sheath (data not shown) were found in monkey #2. Furthermore, apparent severe hyperplasia in the lymphoid nodules of the colon (**Figure 7e**) and spleen white pulp (**Figure 7f**) had been induced in monkey #2. On the other hand, the livers of Ad35LacZ-treated monkeys exhibited almost no damage or inflammation (**Figure 7g** and **j**). In addition, Ad35LacZ induced hyperplasia in lymphoid nodules of the colon (**Figure 7h** and **k**) was only slightly developed and less serious than that induced by the high dose of Ad5LacZ. These results suggest that Ad5 vectors may cause more severe damage and/or inflammation in the liver and lymphoid nodules of the colon than Ad35 vectors. The spleen white pulp developed only slight hyperplasia in monkey #4 (**Figure 7i**), in contrast, the high dose of Ad35LacZ induced severe hyperplasia in the spleen white pulp (**Figure 7l**). The level of hyperplasia in spleen white pulp of monkey #6 appeared to be slightly more severe than that of monkey #2. The monkeys #3 and #5 did not show apparent abnormalities in the spleen or colon, although slight vacuolation in hepatocytes and infiltration of lymphocytes in the Glisson's sheath was found (data not shown). Hyperplasia in spleen white pulp and lymphatic follicles in the mesenteric, axillary, and inguinal lymph nodes (data not shown) occurred dose-dependently in the Ad35-injected animals as well as in the Ad5-injected ones.

DISCUSSION

In this study, subgroup B Ad35 vectors were intravenously infused into cynomolgus monkeys in order to evaluate the *in vivo* fundamental transduction properties of Ad35 vectors more thoroughly. Cynomolgus monkey CD46 and the CD46 of other nonhuman primates, have significant homology with human CD46 (ref. 19). In particular, short consensus repeats 1 and 2 (which are crucial for Ad35 binding to CD46),[22–24] of the CD46 of the cynomolgus monkey show high homology (85%) with those in human CD46. In addition, we confirmed that the monkey cells used in this study were highly stained with anti-human CD46 monoclonal antibody M177, which is specific for short consensus repeat 2, and that the antibody M177 significantly inhibited Ad35 vector–mediated transduction in the cynomolgus monkey cells (data not shown). The amino acid sequences important for Ad35 binding to CD46 (refs. 23,24) are also well conserved in cynomolgus monkey CD46. These results indicate that cynomolgus monkey CD46 serves as a cellular receptor for Ad35, at least in the context of *in vitro* transduction.

In this study, four and two cynomolgus monkeys were intravenously injected with the Ad35 and Ad5 vectors, respectively. We must exercise caution in interpreting the results because the sample size is small, as is natural in nonhuman primate studies. Overall, there are no dose responses in several transduction profiles of both Ad35 and Ad5 vectors, including blood concentration of Ad vectors and inflammatory cytokine production. The variations in the transduction profiles suggest that these profiles may depend largely on the specific Ad vector batch and on the differences between individuals, such as health conditions and genetic backgrounds, as well as on Ad vector doses. In the clinical trials using Ad vectors, inflammatory responses were dramatically different between patients receiving the same vector dose.[10] Gene therapy studies, both preclinical and clinical, should be performed with considerable caution in view of these individual differences. Further studies, including toxicogeomics, would be necessary in order to clarify which parameters play the most crucial roles in this entire process of transduction. Such studies would enable prediction of profiles of Ad vector–mediated transduction, and associated toxicities.

Although efficient transduction was achieved using Ad35 vectors *in vitro*, transduction of Ad35 vectors in the organs *in vivo* was hardly detectable after systemic infusion (**Figure 4**). In addition, the levels of Ad35 vector genome in the organs were one to five orders lower than those of the Ad5 vector genome (**Figure 3**). Previous studies demonstrated that, after systemic injection, Ad35 vectors were poor at transducing CD46-transgenic (CD46TG) mice, which ubiquitously express human CD46 in all the organs.[25,26] Chimeric Ad5 vectors containing Ad35 fiber protein also mediated much lower transgene expression in baboons than conventional Ad5 vectors did.[27] These results indicate that Ad35 vectors cannot transduce organs efficiently when introduced into the blood stream. There are two possible explanations for the poor transduction activity of Ad35 vectors after systemic administration. First, Ad35 vectors might be more susceptible than Ad5 vectors to degradation in the blood or in intracellular compartments such as endosomes/lysosomes after internalization. Fiber-substituted Ad5 vectors containing a fiber protein of Ad35 remain for a longer time in late endosome/lysosomal compartments after internalization than Ad5 vectors do.[28] Ad35 vectors might exhibit similar intracellular trafficking to the fiber-substituted Ad5 vectors, leading to high susceptibility to intracellular degradation. Second, Ad35 vectors might not be able to gain access to CD46 after systemic injection. CD46 is predominantly expressed on the basolateral sides of cells,[29,30] making it inaccessible to Ad35 vectors. Ad35 vectors which are not able to bind to CD46 on the cell surface would be phagocytosed into phagocytic cells, such as liver Kupffer cells, leading to degradation.

It is well known that erythrocytes of cynomolgus monkeys express CD46 (ref. 19) and that Ad35 causes hemagglutination of monkey erythrocytes.[31] Ad35 vectors might induce hemagglutination in the blood vessels after the injection, and this might lead to hemolysis and a decrease in the transduction efficiencies of Ad35 vectors. A large percentage of the Ad35 vectors recovered from the blood after the injection were associated with blood cells (**Figure 2c**). However, lactate dehydrogenase (a marker of hemolysis) levels in the sera of Ad35LacZ-injected animals at most of the time points were within normal levels and comparable with those in the sera of animals injected with Ad5LacZ, which does not induce hemagglutination of monkey erythrocytes. These results suggest that hemagglutination by Ad35 vectors would have, at most, a minimal influence on the transduction profiles of Ad35 vectors.

As mentioned earlier, CD46TG mice as well as cynomolgus monkeys were only poorly transduced with Ad35 vectors after intravenous administration, thereby suggesting that the transduction profiles of Ad35 vectors in CD46TG mice would correspond to those in primates and that CD46TG mice might be suitable as a small animal model for the study of Ad35 vectors. The profiles of inflammatory cytokine production by Ad35 vectors in cynomolgus monkeys were also approximately similar to those in CD46TG mice. Intravenous

infusion of Ad35 vectors resulted in levels of inflammatory cytokine production comparable to those induced by Ad5 vectors in the monkeys (**Figure 5b**) as well as in CD46TG mice.[32]

Histopathological analysis demonstrated that tissue damage and inflammatory responses, including hepatocyte necrosis, were less severe in all the Ad35 vector–infused monkeys than in the Ad5 vector–injected ones (**Figure 7**). Previous studies also demonstrated that Ad35 vectors are less immunogenic than Ad5 vectors in mice,[33,34] and this may result in the higher safety profiles of Ad35 vectors as compared to Ad5 vectors. It remains to be elucidated why Ad35 vectors produce less severe side effects than Ad5 vectors. Ad5 vectors were more widely distributed in most organs than Ad35 vectors, suggesting that Ad5 vectors may cause tissue damage and inflammatory responses throughout the whole body. On the other hand, Ad35LacZ induced much higher levels of IL-6 and interferon-γ in monkeys #5 and #3, respectively, than in the other Ad35LacZ-infused monkeys (**Figure 5b**), although no severe damage or inflammation was observed in these two animals. It remains unclear why such high levels of inflammatory cytokines were induced by Ad35 vectors in these animals; however, previous studies have indicated that the high levels of inflammatory cytokine induction might be involved in tissue damage.[9,35] It is important to pay attention to Ad35 vector–induced innate immune responses.

The poor transduction efficiencies of Ad35 vectors in organs after systemic administration could constitute another potential advantage in their use, namely, that locally administered Ad35 vectors would not cause unwanted side effects in organs other than the targeted organs, when draining from injected sites into the blood stream. This is in contrast to Ad5 vectors which, after injection into local tissues, have been shown to drain into the blood stream in large quantities and cause unwanted side effects in the liver and other organs.[36,37] We previously demonstrated that intramuscular injection of Ad35 vectors led to efficient transduction at the injected sites,[12] and thus local injection of Ad35 vectors would be expected to mediate efficient transduction at the injected sites without side effects in other organs.

In summary, we have demonstrated the transduction properties of Ad35 vectors after intravenous administration in nonhuman primates. Systemic infusion of Ad35 vectors did not result in detectable levels of transgene expression in the organs. Also, the tissue damage was less severe in the animals receiving Ad35 vectors than in those receiving Ad5 vectors, although two monkeys produced marked inflammatory cytokines after receiving Ad35 vectors. Further studies are in progress, focusing on the local injection of Ad35 vectors, and the results of these studies may further clarify the potential utility of Ad35 vectors.

MATERIALS AND METHODS

Ad vectors. An Ad5 vector and an Ad35 vector containing a β-galactosidase expression cassette, Ad5LacZ and Ad35LacZ, respectively, were prepared using an improved *in vitro* ligation method.[38–40] Briefly, for preparation of Ad5LacZ, pHMCMV6-LacZ, which was constructed by insertion of the β-galactosidase gene derived from pCMVβ (Clontech, Palo Alto, CA) into pHMCMV6,[39] was digested with I-*Ceu*I and PI-*Sce*I, and then ligated with I-*Ceu*I- and PI-*Sce*I-digested Ad5 vector plasmid pAdHM4.[39] The resulting plasmid was digested with *Pac*I and transfected into 293 cells with Superfect (Qiagen, Valencia, CA). The vector plasmid for Ad35LacZ was constructed in a similar manner, but using pHMCMV6-LacZ and pAdMS18.[25] The resulting plasmid was digested with *Sbf*I and transfected into 293-E1B cells,[25] which are a 293 transformant stably expressing Ad35 E1B-55K protein. The viruses were prepared using a standard method, and purified by $CsCl_2$ step gradient ultracentrifugation followed by $CsCl_2$ linear gradient ultracentrifugation. Determination of virus particle titers was accomplished spectrophotometrically using the methods of Maizel *et al.*[41] Luciferase-expressing Ad5 and Ad35 vectors, Ad5L and Ad35L, were constructed as explained earlier.[11]

In vitro transduction. Lung and kidney primary cells, isolated from embryonic cynomolgus monkeys and cultured in Roswell Park Memorial Institute-1640 medium supplemented with 10% fetal bovine serum, antibiotics, and L-glutamine, were seeded in a 96-well dish at 1×10^4 cells/well. On the following day, they were transduced with Ad5L or Ad35L at 300 and 3,000 vector particles/cell for 1.5 hours. After a 48-hour culture period, luciferase production in the cells was measured using a luciferase assay system (PicaGene LT2.0; Toyo Inki, Tokyo, Japan).

Animals. Young male cynomolgus monkeys (*Macaca fascicularis*) were housed and handled in accordance with the rules for animal care and management of the Tsukuba Primate Center and the guiding principles for animal experiments using nonhuman primates formulated by the Primate Society of Japan. The animals (~3 years of age, 1.88–2.96 kg) were certified free of intestinal parasites and seronegative for simian type-D retrovirus, herpesvirus B, varicella-zoster-like virus, and measles virus. The protocol of the experimental procedures was approved by the Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation (Osaka, Japan).

In vivo transduction. Cynomolgus monkeys were sedated with ketamine (5–10 mg/kg) and injected with phosphate-buffered saline (mock), or Ad5LacZ or Ad35LacZ at 2×10^{12} vector particles/kg (high dose), 1×10^{12} vector particles/kg (intermediate dose), or 0.4×10^{12} vector particles/kg (low dose) through the saphenous vein at a rate of ~2 ml/minutes. Blood was collected for analysis at 3, 6, 24, 48, 72, and 96 hours after injection. Four days after vector administration, the monkeys were killed and the tissues were collected. Tissue samples were subjected to analysis as described in the later text.

β-Galactosidase assay and X-gal staining. β-Galactosidase activity in the organs was measured using Galacto-Light Systems (Applied Biosystems, Foster City, CA) as earlier described.[42] Protein concentrations were determined with a Bio-Rad assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. X-gal staining of tissue sections was performed as earlier described.[43]

Blood clearance and tissue distribution of Ad vectors. Blood clearance analysis of Ad vectors was performed using a real-time polymerase chain reaction assay, as earlier described.[8,40] Briefly, total DNA, including the Ad vector DNA, was isolated from whole blood samples. After isolation, the total DNA concentrations were determined, and the Ad DNA contents were quantified using a TaqMan fluorogenic detection system (ABI Prism 7700 sequence detector; Perkin-Elmer Applied Biosystems, Foster City, CA).

The association of Ad35 vectors to blood cells circulating in the blood stream was evaluated using a real-time polymerase chain reaction assay. Blood samples collected at the indicated time points were washed two times with phosphate-buffered saline immediately after isolation to remove unbound Ad35 vectors. After washing, total DNA was extracted from blood cells and the Ad35 DNA contents were assessed as described earlier.

The Ad DNA contents in each organ were similarly quantified using a real-time polymerase chain reaction assay, as described earlier, after isolation of the total DNA from each organ using a Automatic Nucleic Acid Isolation System (NA-2000; KURABO, Osaka, Japan).

Histopathology. For routine histopathology, tissues were fixed in 10% formalin at the time of necropsy, and processed for paraffin embedding.

Sections of 4-μm thickness were cut and stained with hematoxylin and eosin. The tissue sections were examined under a microscope.

Analysis of inflammatory cytokines, serum chemistry profiles, and hematology parameters. Blood was drawn from the saphenous veins of all the monkeys prior to vector administration and at 3, 6, 24, 72, and 96 hours after vector administration. Blood samples were collected into separate tubes containing either EDTA or no anticoagulant, for hematology and for determination of inflammatory cytokines and serum chemistry, respectively. Serum samples for analysis of inflammatory cytokines and serum chemistry were separated by centrifugation (4 °C, 2,500 rpm, 15 minutes), stored in a freezer at −80 °C, and thawed at the time of measurement. The levels of inflammatory cytokines (IL-6 and interferon-γ) in serum samples were measured using enzyme-linked immunosorbent assay (BioSource, Camarillo, CA). The serum chemistry parameters, which were measured with an automated chemistry analyzer AU400 (OLYMPUS, Tokyo, Japan), included AST, alanine aminotransferase, CPK, alkaline phosphatase, lactate dehydrogenase, and C-reactive protein. The hematology parameters that were determined included white blood cells, red blood cells, hemoglobin, platelets, $CD3^+$ cells, $CD4^+$ cells, $CD8^+$ cells, $CD16^+$ cells, $CD20^+$ cells, $CD29^+$ cells, and $CD69^+$ cells.

ACKNOWLEDGMENTS

The authors thank Fumiko Ono and Chieko Ohno (The Corporation for Production and Research of Laboratory Primates, Tsukuba City, Ibardki, Japan) for their help. This work was supported by grants from the Ministry of Health, Labour, and Welfare of Japan and a Grant-in-Aid for Scientific Research on Priority Areas of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan.

SUPPLEMENTARY MATERIAL

Figure S1. *In vivo* transduction efficiencies of Ad35 and Ad5 vectors in cultured cynomolgus monkey T-cell line H-SCF.

Table S1. Dosing of cynomolgus macaques with β-galactosidase-expressing Ad vectors in this study.

REFERENCES

1. Havenga, MJ, Lemckert, AA, Ophorst, OJ, van Meijer, M, Germeraad, WT, Grimbergen, J *et al.* (2002). Exploiting the natural diversity in adenovirus tropism for therapy and prevention of disease. *J Virol* **76**: 4612–4620.
2. De Jong, JC, Wermenbol, AG, Verweij-Uijterwaal, MW, Slaterus, KW, Wertheim-Van Dillen, P, Van Doornum, GJ *et al.* (1999). Adenoviruses from human immunodeficiency virus-infected individuals, including two strains that represent new candidate serotypes Ad50 and Ad51 of species B1 and D, respectively. *J Clin Microbiol* **37**: 3940–3945.
3. Wickham, TJ (2000). Targeting adenovirus. *Gene Ther* **7**: 110–114.
4. Ophorst, OJ, Radošević, K, Havenga, MJ, Pau, MG, Holterman, L, Berkhout, B *et al.* (2006). Immunogenicity and protection of a recombinant human adenovirus serotype 35-based malaria vaccine against *Plasmodium yoelii* in mice. *Infect Immun* **74**: 313–320.
5. Chirmule, N, Propert, K, Magosin, S, Qian, Y, Qian, R and Wilson, J (1999). Immune responses to adenovirus and adeno-associated virus in humans. *Gene Ther* **6**: 1574–1583.
6. Vlachaki, MT, Hernandez-Garcia, A, Ittmann, M, Chhikara, M, Aguilar, LK, Zhu, X *et al.* (2002). Impact of preimmunization on adenoviral vector expression and toxicity in a subcutaneous mouse cancer model. *Mol Ther* **6**: 342–348.
7. Muruve, DA (2004). The innate immune response to adenovirus vectors. *Hum Gene Ther* **15**: 1157–1166.
8. Koizumi, N, Kawabata, K, Sakurai, F, Watanabe, Y, Hayakawa, T and Mizuguchi, H (2006). Modified adenoviral vectors ablated for coxsackievirus-adenovirus receptor, alphav integrin, and heparan sulfate binding reduce *in vivo* tissue transduction and toxicity. *Hum Gene Ther* **17**: 264–279.
9. Koizumi, N, Yamaguchi, T, Kawabata, K, Sakurai, F, Sasaki, T, Watanabe, Y *et al.* (2007). Fiber-modified adenovirus vectors decrease liver toxicity through reduced IL-6 production. *J Immunol* **178**: 1767–1773.
10. Raper, SE, Chirmule, N, Lee, FS, Wivel, NA, Bagg, A, Gao, GP *et al.* (2003). Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab* **80**: 148–158.
11. Sakurai, F, Mizuguchi, H and Hayakawa, T (2003). Efficient gene transfer into human $CD34^+$ cells by an adenovirus type 35 vector. *Gene Ther* **10**: 1041–1048.
12. Sakurai, F, Mizuguchi, H, Yamaguchi, T and Hayakawa, T (2003). Characterization of *in vitro* and *in vivo* gene transfer properties of adenovirus serotype 35 vector. *Mol Ther* **8**: 813–821.
13. Seshidhar Reddy, P, Ganesh, S, Limbach, MP, Brann, T, Pinkstaff, A, Kaloss, M *et al.* (2003). Development of adenovirus serotype 35 as a gene transfer vector. *Virology* **311**: 384–393.
14. Vogels, R, Zuijdgeest, D, van Rijnsoever, R, Hartkoorn, E, Damen, I, de Béthune, MP *et al.* (2003). Replication-deficient human adenovirus type 35 vectors for gene transfer and vaccination: efficient human cell infection and bypass of preexisting adenovirus immunity. *J Virol* **77**: 8263–8271.
15. Gao, W, Robbins, PD and Gambotto, A (2003). Human adenovirus type 35: nucleotide sequence and vector development. *Gene Ther* **10**: 1941–1949.
16. Gaggar, A, Shayakhmetov, DM and Lieber, A (2003). CD46 is a cellular receptor for group B adenoviruses. *Nat Med* **9**: 1408–1412.
17. Segerman, A, Atkinson, JP, Marttila, M, Dennerquist, V, Wadell, G and Arnberg, N (2003). Adenovirus type 11 uses CD46 as a cellular receptor. *J Virol* **77**: 9183–9191.
18. Tsujimura, A, Shida, K, Kitamura, M, Nomura, M, Takeda, J, Tanaka, H *et al.* (1998). Molecular cloning of a murine homologue of membrane cofactor protein (CD46): preferential expression in testicular germ cells. *Biochem J* **330**: 163–168.
19. Hsu, EC, Sabatinos, S, Hoedemaeker, FJ, Rose, DR and Richardson, CD (1999). Use of site-specific mutagenesis and monoclonal antibodies to map regions of CD46 that interact with measles virus H protein. *Virology* **258**: 314–326.
20. Gao, GP, Yang, Y and Wilson, JM (1996). Biology of adenovirus vectors with E1 and E4 deletions for liver-directed gene therapy. *J Virol* **70**: 8934–8943.
21. Brunetti-Pierri, N, Palmer, DJ, Beaudet, AL, Carey, KD, Finegold, M and Ng, P (2004). Acute toxicity after high-dose systemic injection of helper-dependent adenoviral vectors into nonhuman primates. *Hum Gene Ther* **15**: 35–46.
22. Sakurai, F, Murakami, S, Kawabata, K, Okada, N, Yamamoto, A, Seya, T *et al.* (2006). The short consensus repeats 1 and 2, not the cytoplasmic domain, of human CD46 are crucial for infection of subgroup B adenovirus serotype 35. *J Control Release* **113**: 271–278.
23. Gaggar, A, Shayakhmetov, DM, Liszewski, MK, Atkinson, JP and Lieber, A (2005). Localization of regions in CD46 that interact with adenovirus. *J Virol* **79**: 7503–7513.
24. Fleischli, C, Verhaagh, S, Havenga, M, Sirena, D, Schaffner, W, Cattaneo, R *et al.* (2005). The distal short consensus repeats 1 and 2 of the membrane cofactor protein CD46 and their distance from the cell membrane determine productive entry of species B adenovirus serotype 35. *J Virol* **79**: 10013–10022.
25. Sakurai, F, Kawabata, K, Koizumi, N, Inoue, N, Okabe, M, Yamaguchi, T *et al.* (2006). Adenovirus serotype 35 vector-mediated transduction into human CD46-transgenic mice. *Gene Ther* **13**: 1118–1126.
26. Verhaagh, S, de Jong, E, Goudsmit, J, Lecollinet, S, Gillissen, G, de Vries, M *et al.* (2006). Human CD46-transgenic mice in studies involving replication-incompetent adenoviral type 35 vectors. *J Gen Virol* **87**: 255–265.
27. Ni, S, Bernt, K, Gaggar, A, Li, ZY, Kiem, HP and Lieber, A (2005). Evaluation of biodistribution and safety of adenovirus vectors containing group B fibers after intravenous injection into baboons. *Hum Gene Ther* **16**: 664–677.
28. Shayakhmetov, DM, Li, ZY, Ternovoi, V, Gaggar, A, Gharwan, H and Lieber, A (2003). The interaction between the fiber knob domain and the cellular attachment receptor determines the intracellular trafficking route of adenoviruses. *J Virol* **77**: 3712–3723.
29. Ichida, S, Yuzawa, Y, Okada, H, Yoshioka, K and Matsuo, S (1994). Localization of the complement regulatory proteins in the normal human kidney. *Kidney Int* **46**: 89–96.
30. Maisner, A, Zimmer, G, Liszewski, MK, Lublin, DM, Atkinson, JP and Herrler, G (1997). Membrane cofactor protein (CD46) is a basolateral protein that is not endocytosed. Importance of the tetrapeptide FTSL at the carboxyl terminus. *J Biol Chem* **272**: 20793–20799.
31. Shayakhmetov, DM, Papayannopoulou, T, Stamatoyannopoulos, G and Lieber, A (2000). Efficient gene transfer into human $CD34^+$ cells by a retargeted adenovirus vector. *J Virol* **74**: 2567–2583.
32. Stone, D, Liu, Y, Li, ZY, Tuve, S, Strauss, R and Lieber, A (2007). Comparison of adenoviruses from species B, C, e, and f after intravenous delivery. *Mol Ther* **15**: 2146–2153.
33. Nanda, A, Lynch, DM, Goudsmit, J, Lemckert, AA, Ewald, BA, Sumida, SM *et al.* (2005). Immunogenicity of recombinant fiber-chimeric adenovirus serotype 35 vector-based vaccines in mice and rhesus monkeys. *J Virol* **79**: 14161–14168.
34. Lemckert, AA, Sumida, SM, Holterman, L, Vogels, R, Truitt, DM, Lynch, DM *et al.* (2005). Immunogenicity of heterologous prime-boost regimens involving recombinant adenovirus serotype 11 (Ad11) and Ad35 vaccine vectors in the presence of anti-Ad5 immunity. *J Virol* **79**: 9694–9701.
35. Nazir, SA and Metcalf, JP (2005). Innate immune response to adenovirus. *J Investig Med* **53**: 292–304.
36. Mizuguchi, H and Hayakawa, T (2002). Enhanced antitumor effect and reduced vector dissemination with fiber-modified adenovirus vectors expressing herpes simplex virus thymidine kinase. *Cancer Gene Ther* **9**: 236–242.
37. Okada, Y, Okada, N, Mizuguchi, H, Hayakawa, T, Mayumi, T and Mizuno, N (2003). An investigation of adverse effects caused by the injection of high-dose TNFα-expressing adenovirus vector into established murine melanoma. *Gene Ther* **10**: 700–705.
38. Mizuguchi, H and Kay, MA (1998). Efficient construction of a recombinant adenovirus vector by an improved *in vitro* ligation method. *Hum Gene Ther* **9**: 2577–2583.
39. Mizuguchi, H and Kay, MA (1999). A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. *Hum Gene Ther* **10**: 2013–2017.
40. Sakurai, F, Kawabata, K, Yamaguchi, T, Hayakawa, T and Mizuguchi, H (2005). Optimization of adenovirus serotype 35 vectors for efficient transduction in human hematopoietic progenitors: comparison of promoter activities. *Gene Ther* **12**: 1424–1433.
41. Maizel, JV, Jr, White, DO and Scharff, MD (1968). The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* **36**: 115–125.
42. Nakamura, T, Sato, K and Hamada, H (2003). Reduction of natural adenovirus tropism to the liver by both ablation of fiber-coxsackievirus and adenovirus receptor interaction and use of replaceable short fiber. *J Virol* **77**: 2512–2521.
43. Sakurai, F, Nishioka, T, Saito, H, Baba, T, Okuda, A, Matsumoto, O *et al.* (2001). Interaction between DNA-cationic liposome complexes and erythrocytes is an important factor in systemic gene transfer via the intravenous route in mice: the role of the neutral helper lipid. *Gene Ther* **8**: 677–686.

Cell Transplantation, Vol. 17, pp. 1095–1102, 2008
Printed in the USA. All rights reserved.
Copyright © 2008 Cognizant Comm. Corp.

0963-6897/08 $90.00 + .00
E-ISSN 1555-3892
www.cognizantcommunication.com

Variation in the Incidence of Teratomas After the Transplantation of Nonhuman Primate ES Cells Into Immunodeficient Mice

Yukiko Kishi,* Yujiro Tanaka,* Hiroaki Shibata,*§ Shinichiro Nakamura,¶ Koichi Takeuchi,‡ Shigeo Masuda,* Tamako Ikeda,* Shin-ichi Muramatsu,† and Yutaka Hanazono*

*Division of Regenerative Medicine, Center for Molecular Medicine, Jichi Medical University, Tochigi 329-0498, Japan
†Division of Neurology, Department of Internal Medicine, Jichi Medical University, Tochigi 329-0498, Japan
‡Department of Anatomy, Jichi Medical University, Tochigi 329-0498, Japan
§Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Ibaraki 305-0843, Japan
¶The Corporation for Production and Research of Laboratory Primates, Ibaraki 300-2658, Japan

Embryonic stem (ES) cells have the ability to generate teratomas when transplanted into immunodeficient mice, but conditions affecting the generation remain to be elucidated. Nonhuman primate cynomolgus ES cells were transplanted into immunodeficient mice under different conditions; the number of transplanted cells, physical state (clumps or single dissociated cells), transplant site, differentiation state, and immunological state of recipient mice were all varied. The tumorigenicity was then evaluated. When cynomolgus ES cells were transplanted as clumps into the lower limb muscle in either nonobese diabetic/severe combined immunodeficiency (NOD/SCID) or NOD/SCID/γc^{null} (NOG) mice, teratomas developed in all the animals transplanted with 1×10^5 or more cells, but were not observed in any mouse transplanted with 1×10^3 cells. However, when the cells were transplanted as dissociated cells, the number of cells necessary for teratomas to form in all mice increased to 5×10^5. When the clump cells were injected subcutaneously (instead of intramuscularly), the number also increased to 5×10^5. When cynomolgus ES cell-derived progenitor cells (1×10^6), which included residual pluripotent cells, were transplanted into the lower limb muscle of NOG or NOD/SCID mice, the incidence of teratomas differed between the strains; teratomas developed in five of five NOG mice but in only two of five NOD/SCID mice. The incidence of teratomas varied substantially depending on the transplanted cells and recipient mice. Thus, considerable care must be taken as to tumorigenicity.

Key words: Nonhuman primate embryonic stem cells; NOD/SCID mouse; NOG mouse; Teratoma

INTRODUCTION

Human embryonic stem (ES) cells are established by propagating cell clumps from the inner cell mass of blastocysts developed from fertilized ova (18,24). Because they are pluripotent and can proliferate indefinitely, their application to regenerative medicine is anticipated (6, 10). When ES cells are transplanted into immunodeficient hosts, they form teratomas that contain three germ layer cells. This teratoma-forming ability is a reflection of the pluripotency of ES cells.

However, the development of teratomas posttransplant poses a serious obstacle to the clinical application of ES cells. The safety of human ES cell-derived progenitor preparations is usually assessed with an in vivo assay to see if tumors form after the injection of cells into immunodeficient mice. However, innate immunity is present even in so-called immunodeficient mice, and innate immune responses against ES cell-derived tumors might be more rigorous in such a xenogeneic (human-to-mouse) setting than in allotransplanted animals, resulting in a failure to detect tumorigenesis in xenotransplantation models.

Indeed, we have shown that the incidence with which tumors were formed by cynomolgus ES cell-derived progenitor cells was much higher in the allogeneic (cynomolgus-to-cynomolgus) setting than in the xenogeneic (cynomolgus-to-immunodeficient mice and cynomolgus-to-fetal sheep) setting (2,19,20,23). Therefore, conventional teratoma-forming assays using mice or other xenogeneic animals might underestimate the tumorigenicity of human ES cell-derived progenitor preparations.

Received December 14, 2007; final acceptance May 5, 2008.
Address correspondence to Yutaka Hanazono, M.D., Ph.D., Professor, Division of Regenerative Medicine, Center for Molecular Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan. Tel: +81-285-58-7451; Fax: +81-285-44-5205; E-mail: hanazono@jichi.ac.jp

However, a systematic study regarding human or nonhuman primate ES cell-derived teratomas in mice has not been performed. Here we show that the incidence of teratomas varied substantially and was affected by factors relating to both the transplanted cells and recipient mice.

MATERIALS AND METHODS

Animals

Six- to 8-week-old nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice were purchased from Clea Japan (Tokyo, Japan) and 6- to 8-week-old NOD/SCID/γc^{null} (NOG) mice were purchased from the Central Institute for Experimental Animals (Kawasaki, Japan). All mice were housed at no more than five per cage with 12:12-h light/dark cycles and free access to standard rodent chow and water. All procedures of animal experiments were approved by the Animal Care and Use Committee of Jichi Medical University.

ES Cell Culture and Transplantation

A cynomolgus macaque ES cell line (CMK6) and a subline expressing GFP (CMK6G) were maintained on a feeder layer of mitomycin C (Kyowa, Tokyo, Japan)-treated mouse (BALB/c, Clea) embryonic fibroblasts, as described previously (21,22). The mouse bone marrow stromal cell line OP9 was maintained in α-minimum essential medium (Invitrogen, Rockville, MD, USA) supplemented with 20% fetal bovine serum, as previously described (13).

Cultured ES cells were treated with 0.1% collagenase type IV (Invitrogen) for 10 min at 37°C. The resultant cell clumps were further incubated in 2.5% trypsin for 5 min at 37°C and then subjected to pipetting to become single cells. Clumps of cells or single dissociated cells (10^3 to 10^6 cells per site) were injected into the indicated sites of NOD/SCID or NOG mice using 23-gauge needles.

ES Cell-Derived Progenitor Preparation

To prepare cynomolgus ES cell-derived progenitors, ES cells were induced to differentiate into hematopoietic (20) or neural precursor cells (14). For hematopoietic differentiation, ES cells were seeded onto mitomycin C-treated confluent OP9 cell layers in culture dishes in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 16% fetal bovine serum, 5×10^{-6} M hydrocortisone (Sigma, St. Louis, MO, USA), and 20 ng/ml recombinant human vascular endothelial growth factor (R&D, Minneapolis, MN, USA). During the differentiation, media were changed every 2–3 days. After 6 days of culture, cells were collected with a micropipetter, washed with phosphate-buffered saline (PBS, Sigma), resuspended in 1×10^3 to 1×10^6/100 μl of 0.1% bovine serum albumin/PBS, and used for transplantation.

For neural differentiation, clusters of undifferentiated cynomolgus ES cells were transferred to nonadhesive bacteriological dishes in astrocyte-conditioned medium (ACM) under free-floating conditions (14) supplemented with 20 ng/ml recombinant human fibroblast growth factor-2 (FGF-2) and 20 ng/ml recombinant epidermal growth factor (EGF) (both from R&D). The clusters were cultured for 10 days, giving rise to floating neural stem spheres (NSSs), composed of plenty of neural stem cells (NSCs). Then, the NSSs were plated onto Matrigel-coated dishes (BD Biosciences, Bedford, MA, USA) and cultivated for up to 10 days in Neurobasal medium supplemented with 2% B-27 (both from Invitrogen), 20 ng/ml FGF-2, and 20 ng/ml EGF. Subsequent culture of NSSs formed circular clusters of cells, from which many nestin-positive NSCs migrated, and these cells were used for transplantation.

Flow Cytometry

The expression of stage-specific embryonic antigen 4 (SSEA-4), an undifferentiated marker of cynomolgus ES cells (21), was analyzed on a FACS Calibur (BD Pharmingen, CA, USA) using CellQuest software (BD Pharmingen). For staining SSEA-4, ES cells were incubated with an anti-SSEA-4 antibody (MC-813-70; Chemicon, Temecula, CA, USA) conjugated with Alexa Fluor 647 monoclonal antibody (Invitrogen). Cocultured BALB/c feeder cells could be distinguished from cynomolgus ES cells by using phycoerythin (PE)-conjugated anti-mouse H-$2K^d$ (SF1-1.1; BD Pharmingen), which does not react to cynomolgus cells but does react to BALB/c cells.

Immunohistochemistry

All mice were euthanized at 12 weeks posttransplant unless otherwise indicated. Tumors and transplant sites were fixed in 4% paraformaldehyde for 1 day at 4°C. Tissues were embedded in OCT compound (Sakura, CA, USA) and sectioned at 10 μm on a cryostat. Sections were blocked and incubated with rat anti-mouse CD45R/B220 (CL8990AP; Cedarlane, Ontario, Canada), rat anti-mouse F4/80 (BM8; BMA, Augst, Switzerland), or rat anti-mouse Ly-6G and Ly-6C (Gr-1; BD Pharmingen) for 1 h at room temperature. In NOD/SCID and NOG mice, B cells are absent and thus CD45R/B220-positive cells should be plasmacytoid dendritic cells (5) or active NK cells (3). To detect primary antibodies, slides were incubated with Alexa Fluor 555 goat anti-rat IgG (Invitrogen) for 45 min at room temperature. Stained sections were analyzed with a confocal laser scanning microscope (Nikon, Tokyo, Japan).

Cytotoxicity Assay

The cytotoxicity assay of mouse spleen cells was performed as described previously (27). Briefly, spleen