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Astroglial responses against Aβ initially occur in cerebral primary cortical cultures: species differences between rat and cynomolgus monkey

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Abstract

In the present study, we investigated how amyloid beta (Aβ) peptides initially affect neuronal cells in primary cerebral cortical cultures from rat and cynomolgus monkey. In these cultures, complicated interactions between glial and neuronal cells occur; moreover, synaptic interactions similar to those observed in vivo also occur between neuronal cells in these cultures. In this study, we applied low concentrations of Aβ to these well-characterized primary cultures to investigate how Aβ initially affects neurons or astroglial cells. In both rat and monkey cortical cultures, treatment with low concentrations of Aβ failed to drastically change or damage of neurons. Aβ treatment, however, significantly activated astrocytes, resulting in increased apolipoprotein E (ApoE) production. Rat astrocytes were more sensitive to Aβ than monkey astrocytes, and responded to Aβ via a different mechanism. In monkey astrocyte cultures, only direct treatment with Aβ increased ApoE production. In rat astrocyte cultures, however, treatment with conditioned media from cortical cultures grown with Aβ increased ApoE production, indicating that some sort of neuron-derived soluble factor(s) was also involved in activating rat astrocytes. These species differences suggest that monkey cortical cultures would be more useful as an in vitro model system to understand the details of how Aβ accumulates in the human brain, since monkeys are phylogenetically more similar to humans.

Keywords: Amyloid beta peptides; Astrocyte; Monkey cortical culture; Rat cortical culture; Species difference

1. Introduction

Amyloid beta (Aβ) peptide consists of 40–43 amino acids and is derived from amyloid precursor protein (APP) (Citron et al., 1997). Aβ is the major protein component of senile plaques (SP), a characteristic feature of Alzheimer's disease (AD) (Glenner, 1988). Since Aβ is toxic to cultured nerve cells, some have argued that Aβ cytotoxicity is the major cause of brain damage observed in AD (Behl et al., 1992; Koh et al., 1990; Mattson et al., 1992; Yankner et al., 1990). Although Aβ toxicity has been thoroughly studied, the manner in which neuronal cells in vivo are initially affected by Aβ accumulation remains unknown.

Many studies also show that astrocytes have an important role in clearing Aβ from the brain (Funato et al., 1998; Matsunaga et al., 2003; Wyss-Coray et al., 2003). We previously showed that both neuronal and glial cells are found in primary cultures prepared from the cerebral cortices of either rat or cynomolgus monkeys (Negishi et al., 2002a,b). In these cultures, complicated interactions between glial and neuronal cells occur; moreover, synaptic interactions similar to those observed in vivo also occur between neuronal cells in these cultures. In the present study, we applied low concentrations of Aβ to these well-characterized primary cultures to investigate how Aβ initially affects neurons or astroglial cells. Since SP are conformed by the aggregation of low concentrations of Aβ with age, Aβ treatment in this study would reflects in vivo event of the early stage of Aβ accumulation. We also investigated how astroglial cells respond to Aβ when conditioned media are applied to the cultures. We were especially interested in determining whether species (rat versus monkey) differences emerged in the responses of neuronal and astroglial cells to Aβ.

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2. Materials and methods

2.1. Animals

Pregnant Sprague-Dawley rats were purchased from SLC Japan (Shizuoka, Japan). The animals were maintained under controlled conditions (temperature, 24 ± 1 °C; humidity,55 ± 5%) in plastic cages with sterilized wood shavings for bedding. They were fed a commercially available diet (CMF; Oriental Yeast, Tokyo, Japan) and had ad libitum access to food and tap water.

Six cynomolgus monkey (*Macaca fascicularis*) fetuses (80 days gestation) were used in this study. Four were purchased from Shin Nippon Biomedical Laboratories (Kagoshima, Japan), and two were obtained from the Tsukuba Primate Center, National Institute of Infectious Diseases, Japan. This experiment was conducted according to the guidelines of the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo.

2.2. Rat primary cerebral cortical cultures

Rat fetuses were removed on gestational day 18 by axillary exsanguination, and their brains were removed then transferred into ice-cold isolation medium (IM) consisting of equal volumes of Ca^{2+}-free phosphate-buffered saline (PBS), Mg^{2+}-free PBS, and Dulbecco's Modified Eagle's Medium containing 1.2 mg/ml $NaHCO_3$, 110 μg/ml pyruvic acid, 25 μg/ml streptomycin, and 50 U/ml penicillin (mDMEM). After bisecting the brains into cerebral hemispheres, the meninges, hippocampi, and other subcortical structures were carefully removed, and the cerebral cortices were rinsed in culture medium (CM: mDMEM with 5% fetal calf serum) and minced into small pieces (<1 mm^3) in CM. The tissue pieces were digested at 32 °C for 30 min in PBS containing 1.5 U/ml papain (Worthington Biochemical Corporation, Lakewood, NJ, USA), 0.1 mg/ml DNase I (Roche Diagnostics, Japan), 0.2 mg/ml cysteine, 0.2 mg/ml albumin, and 5 mg/ml glucose. Cells were dissociated gently by passing the mixture several times through a disposable pipette, and then the mixture was centrifuged three times in CM at 800 rpm for 5 min at 32 °C. For TUNEL staining, cells were plated at 2.5×10^5 cells/cm^2 in CM onto a LAB-TEK chamber slide (Nalge Nunc, Tokyo, Japan) coated with 0.125% polyethylenimine. For other experiments, cells were plated at 4.2×10^5 cells/cm^2 onto culture dishes coated with 0.125% polyethylenimine. All cultures were maintained at 37 °C in a humidified chamber containing 95% air and 5% CO_2. Half the volume of culture supernatant was replaced with pre-warmed CM once per week.

These primary rat cerebral cortical cultures consist mainly of neurons (more than 90%) with some astrocytes. We previously showed that these cultured neuronal cells have complicated interactions with glia and other neurons and make synaptic connections with other neurons similar to those in vivo (Negishi et al., 2002a). After 3 days in vitro, the total proteins of these cultures almost unchanged regardless of time course (data not shown).

2.3. Cynomolgus monkey primary cerebral cortical cultures

Monkey fetuses were removed on gestational day 80, and then digested and dissociated in the identical manner as for the rat cultures (above). Plating onto slides and culture dishes for TUNEL staining and other experiments was carried out in exactly the same way as for the rat cultures. As with the rat cultures, half the volume of culture supernatant was replaced with pre-warmed CM once per week.

As with the rat cultures, these monkey primary cerebral cortical cultures consisted mainly of neurons (more than 90%) with some astrocytes. We previously showed that these cultures also exhibit complicated interactions similar to those observed under in vivo conditions (Negishi et al., 2002b). After 3 days in vitro, the total proteins of these cultures almost unchanged regardless of time course such as rat (data not shown).

2.4. Rat and cynomolgus monkey astrocyte cultures

After 14 days of culturing, cerebral cortical cells were dissociated with 0.025% trypsin (Invitrogen, UK) and washed several times in CM. Proliferating type-1 astrocytes were quickly selected from this suspension. After one subculturing, cells were plated at 4.2×10^5 cells/cm^2 in CM onto uncoated culture dishes. Half the volume of culture supernatant was replaced with pre-warmed CM once per week (Negishi et al., 2003).

2.5. Amyloid beta treatment

Aβ peptides, Aβ1-40 (Aβ40) and Aβ1-42 (Aβ42) (Bachem, Torrance, CA, USA), were dissolved in 100% DMSO, then diluted in CM (0.45% DMSO final concentration). These Aβ40 and Aβ42 (i.e., no pre-aggregating) were ultimately added to primary cortical cultures and astrocyte cultures. After 3 days of culturing, CM containing Aβ peptides was added into the rat or monkey cortical primary cultures at a concentration of 2 μM or 5 μM. These cultures were maintained for 1, 3, 7, or 14 days. Aβ peptides (5 μM) were also added to confluent rat or monkey astrocyte cultures, and these were maintained for 3 days. The CM of control cultures contained the same concentration of DMSO (0.45%).

2.6. Treatment with conditioned media

To examine whether soluble factors produced by neurons affect astrocyte responses to Aβ peptides, the conditioned medium from the primary cortical cultures was collected and used to treat the astrocyte cultures. CM supernatant from the rat and monkey primary cultures was collected after 1, 3,

and 7 days of Aβ treatment (5 μM). Astrocyte cultures (see above) from rat or monkey were maintained in the respective supernatant for 3 days.

2.7. Antibodies

For Western blotting, the following antibodies were used: rabbit polyclonal anti-Caspase-3 (H277; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-Synaptophysin (SY38; DAKO, Denmark), rabbit polyclonal anti-APP (β-APP_{695}; Zymed Laboratories, San Francisco, CA, USA), mouse monoclonal anti-GSK3β (GSK; Transduction Laboratories, Lexington, KY, USA), rabbit polyclonal anti-phospho-GSK3β (S9; Cell Signaling Technology, Beverly, MA, USA), mouse monoclonal anti-Glial fibrillary acidic protein (6F2; DAKO, Denmark), and goat polyclonal anti-ApoE (APO-E; Chemicon). H277 reacts not only with p11, p17, and p20 subunits but also with the full-length precursor of caspase-3. β-APP_{695} reacts with all three forms of β-APP (β-APP_{695}, β-APP_{751}, β-APP_{770}) and recognizes the APP C-terminal fragment (βCTF) that results from the cleavage of APP by β-secretase. S9 recognizes the Ser-9-phosphorylated, inactive form of GSK3β.

2.8. Western blot analyses

To extract total cellular proteins from the cultured cells, the cells were bathed in a solution containing 9.85 mg/ml Tris–HCl, 0.774 mg/ml ethylenediaminetetraacetic acid (EDTA), 0.348 mg/ml ammonium persulfate, 0.5% (v/v) TritonX-100, and 2.3% (w/v) SDS in PBS. Total proteins were isolated by centrifugation, adjusted to 30 μg, then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE with 12.5% acrylamide gel). Separated proteins were blotted onto polyvinylidene fluoride membranes (Immobilon P, Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat dried milk in 20 mM PBS (pH 7.0) and 0.1% Tween-20 overnight at 4 °C, then incubated with primary antibodies (H277, 1:2000; Syn, 1:5000; β-APP_{695}, 1:2000; GSK, 1:10,000; S9, 1:1000; GFAP, 1:10,000; APO-E, 1:3000) for 1 h at room temperature. They were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG, mouse anti-rabbit IgG, or rabbit anti-goat IgG (1:6000, Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. Immunoreactive elements were visualized using enhanced chemiluminescence (ECLplus, Amersham, UK).

2.9. Data analyses

The effects of Aβ treatment on the expression of synaptophysin, APP-βCTF, GSK3β, capsase-3, GFAP, and ApoE were confirmed by quantifying the immunoreactive bands (obtained from the Western blots) with commercially available software (Quantity One, PDI, Inc, NY, USA). Data are shown as means ± S.D. For statistical analyses, one-way ANOVAs were performed followed by the Bonferroni/Dunn *post hoc* test.

3. Results

3.1. Western blot analyses of neuron- and astrocyte-related protein expression following aβ treatment

Western blot analyses were performed to determine whether Aβ treatment affected the expression of various neuron- and glia-related proteins. In rat cortical cultures, treatment with either Aβ40 or Aβ42, even at a concentration of 5 μM, did not significantly influence the expression of synaptophysin, APP-βCTF, or GSK3β (Figs. 1A and 2A–C). Aβ40 and Aβ42 also did not affect the expression of the full-length precursor of caspase-3 in these cells; moreover, expression of all caspase-3 subunits was absent, regardless of the duration of Aβ treatment (data not shown). Synaptophysin expression did not decrease even after 14 days of Aβ treatment; rather, synaptophysin expression

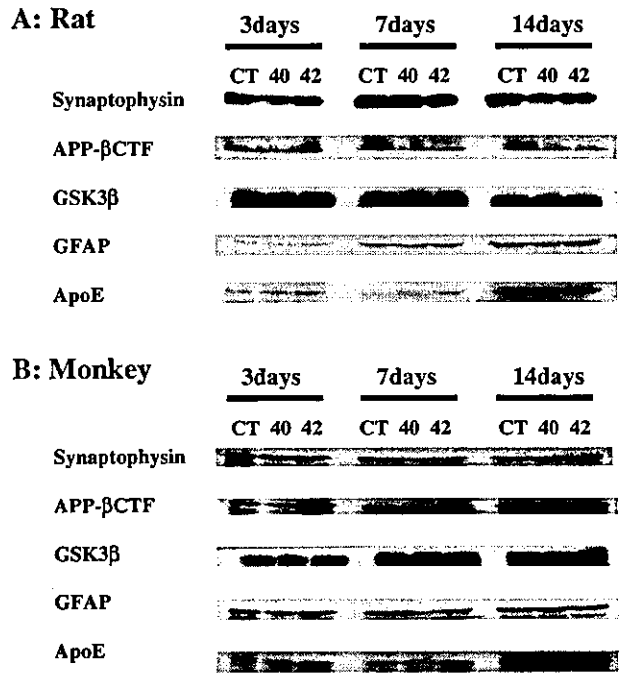

Fig. 1. Western blots showing the expression of neuron-related proteins (synaptophysin, APP-βCTF, and GSK3β) and astrocyte-related proteins (GFAP and ApoE) in rat primary cerebral cortical cultures (A) and monkey primary cerebral cortical cultures (B) following treatment with either 5 μM Aβ40 or 5 μM Aβ42. SY38 immunostained a 38 kDa band representing synaptophysin, β-APP_{695} immunostained a ~15 kDa band representing APP-βCTF, and GSK immunostained a 46 kDa band representing GSK3β. 6F2 immunostained a 52 kDa band representing GFAP, and APO-E immunostained a ~34 kDa band representing ApoE. CT—controls consisted of extracts from cultures grown in standard culture medium with DMSO; 40—extracts from cultures treated with Aβ40; 42—extracts from cultures treated with Aβ42; 3 days—after 3 days of Aβ treatment; 7 days—after 7 days of Aβ treatment; 14 days—after 14 days of Aβ treatment.

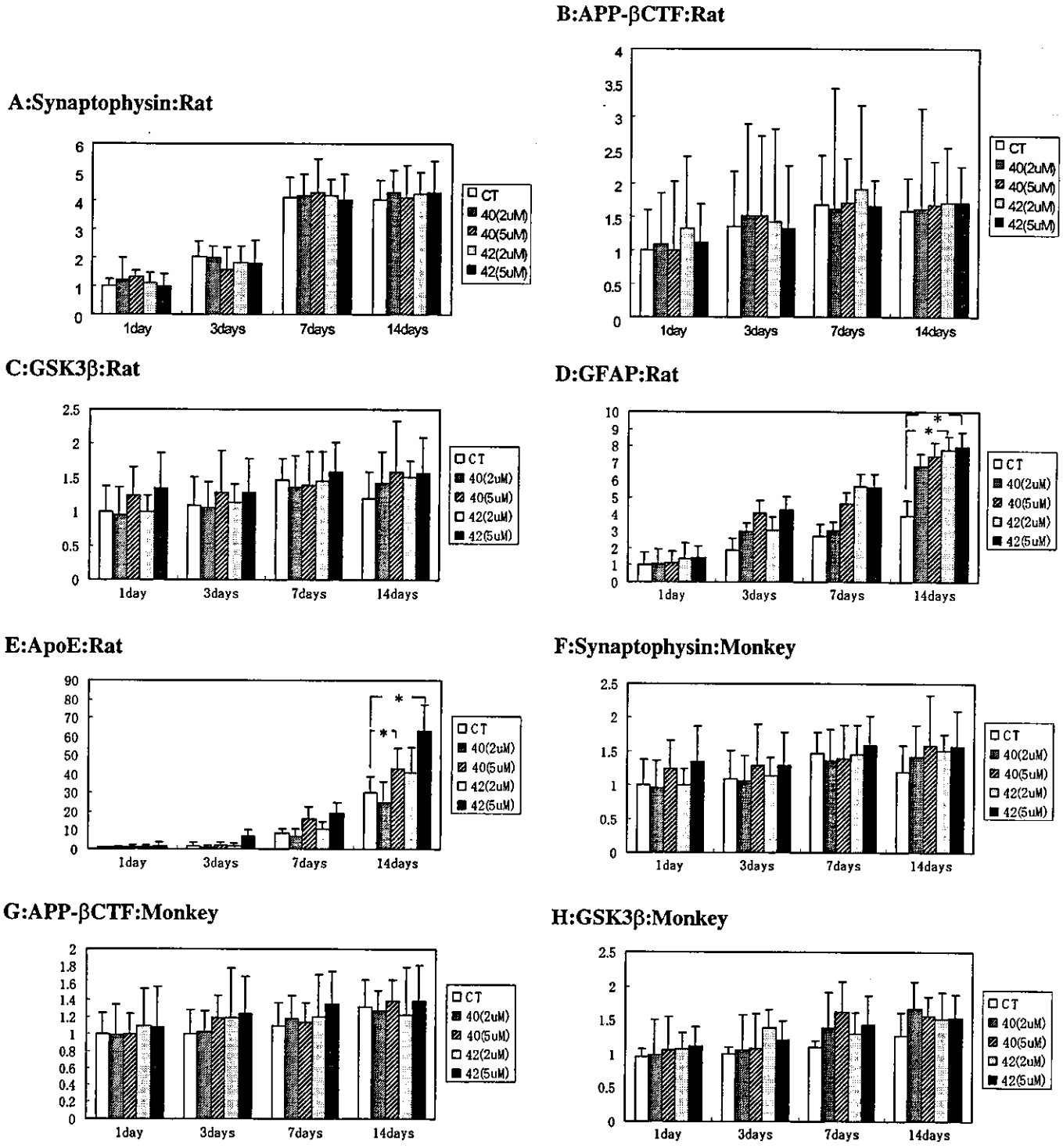

Fig. 2. Immunoreactive bands were quantified, and the resulting density data were compared to those measured from blots containing extracts from control cultures grown for 1 day (*$P < 0.02$). In rat cortical cultures ($N = 10$) (A–E): Aβ treatment did not significantly affect the expression of synaptophysin (A), APP-βCTF (B), and GSK3β (C). Interestingly, synaptophysin expression measured 7 and 14 days after Aβ treatment was slightly elevated and greater than that measured after 1 and 3 day(s) of Aβ treatment. In contrast to the neuron-related proteins, Aβ treatment induced increases in expression of astrocyte-related proteins. GFAP expression was significantly elevated in cultures treated with either 2 μM or 5 μM Aβ42 for 14 days (D). ApoE expression was significantly elevated in cultures treated with either 5 μM Aβ40 or 5 μM Aβ42 treatments (E). In monkey cortical cultures ($N = 6$) (F–J), Aβ treatment did not affect the expression of synaptophysin (F), APP-βCTF (G), and GSK3β (H). Although GFAP levels did not markedly increase in cultures treated with Aβ, even after 14 days of Aβ treatment (I), ApoE expression significantly increased in cultures treated with 5 μM Aβ42 for 14 days (J). Data are means and error bars are S.D.s.

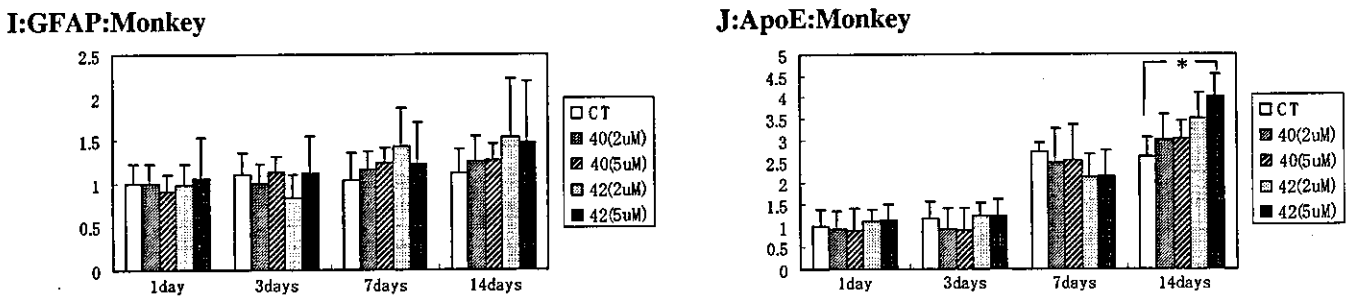

Fig. 2. (*Continued*).

was greater 14 days after treatment compared to that 3 days after treatment (Fig. 2A). APP-βCTF expression remained unchanged throughout the course of Aβ treatment, even after 14 days, indicating that Aβ treatment did not induce endogenous Aβ production from APP (Figs. 1A and 2B). GSK3β expression slightly increased in cultures treated with Aβ (Figs. 1A and 2C). However, levels of Ser-9-phosphorylated GSK3β, the inactive form of GSK3β, remained unchanged regardless of Aβ treatment length or concentration of Aβ (data not shown). Similarly, the expression of neuron-related proteins in monkey cortical cultures did not significantly decrease or increase during Aβ treatment (Figs. 1B and 2F–H).

In stark contrast to neuron-related proteins, we found that Aβ treatment profoundly affected astrocyte-related proteins in both rat and monkey cortical cultures (Figs. 1 and 2D,E,I,J), and the effects were different for the two species.

In rat cortical cultures, 14 days of Aβ42 treatment, even at a low concentration of 2 μM, increased GFAP expression significantly (Figs. 1A and 2D). Aβ40 treatment also increased GFAP expression, but not significantly (Fig. 2D). In addition, both Aβ40 and Aβ42 treatment (5 μM) significantly increased ApoE expression (Figs. 1A and 2E). On the other hand, in monkey cortical cultures, 14 days of Aβ42 treatment only slightly increased GFAP expression (Figs. 1B and 2I). Furthermore, only Aβ42 treatment (5 μM for 14 days) significantly increased ApoE expression; the magnitude of this increase was less than that observed in correspondingly treated rat cortical cultures (Fig. 2E,J).

3.2. Western blot analyses of astrocyte-related protein expression following treatment with conditioned media

To examine whether some kind of neuron-derived soluble factor(s) influences astrocytic responses to the different Aβ peptides in cortical cultures, we treated rat and monkey astrocyte cultures with either conditioned media from primary cultures treated with Aβ or media containing Aβ (i.e., direct Aβ treatment), then we compared the changes in the expression levels of astrocyte-related proteins (GFAP and ApoE). In rat astrocyte cultures, direct Aβ42 treatment significantly increased GFAP expression (Figs. 3A and 4A). On the other hand, conditioned-media treatment did not affect GFAP expression in astrocyte cultures, even when the conditioned medium was derived from cortical cultures treated with Aβ for 7 days (Figs. 3A and 4A). In monkey astrocyte cultures, direct Aβ treatment also increased GFAP expression, although not significantly (Figs. 3B and 4C). Similar to rat astrocyte cultures, conditioned-media treatment did not affect GFAP expression in monkey astrocyte cultures. Although direct Aβ treatment induced expression of GFAP and conditioned-media treatment had little effect in both rat and monkey astrocyte cultures, the opposite occurred for ApoE (Figs. 3 and 4B,D). In rat astrocyte cultures, direct Aβ treatment induced ApoE expression, and furthermore, treatment with conditioned medium from cortical cultures treated with Aβ42 for either 3 or 7 days also increased ApoE expression levels significantly (Figs. 3A and 4B). Treatment with conditioned media from cortical cultures treated with Aβ40 also induced ApoE expression, but not significantly (Fig. 4B). The degree of ApoE increase depended on the source of the conditioned medium (i.e., media collected from cortical cultures treated with Aβ for 1, 3, or 7 days) (Fig. 4B). In contrast to rat astrocyte cultures, in monkey astrocyte cultures, only direct treatment with Aβ42 significantly increased ApoE expression levels (Figs. 3B and 4D). Direct Aβ40 treatment, as well as any

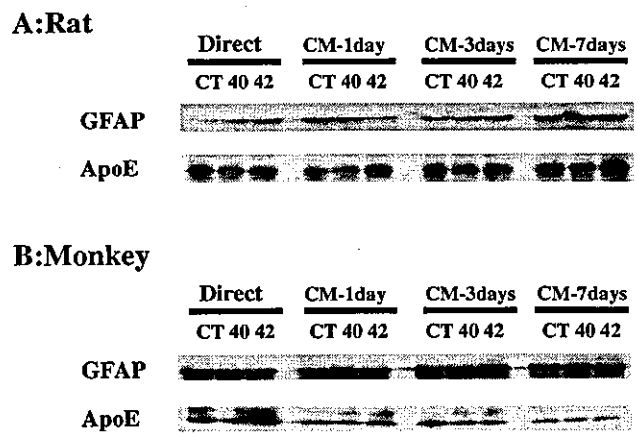

Fig. 3. Western blots showing the expression of GFAP and ApoE in extracts from rat astrocyte cultures (A) and monkey astrocyte cultures (B) following direct treatment with Aβ or treatment with conditioned media from cortical cultures treated with Aβ for 3 days. Direct—direct Aβ treatment; CM—treatment of astrocyte cultures with conditioned medium collected from cortical cultures exposed to Aβ for 1, 3 or 7 days; CT—control; 40—Aβ40 treatment; 42—Aβ42 treatment.

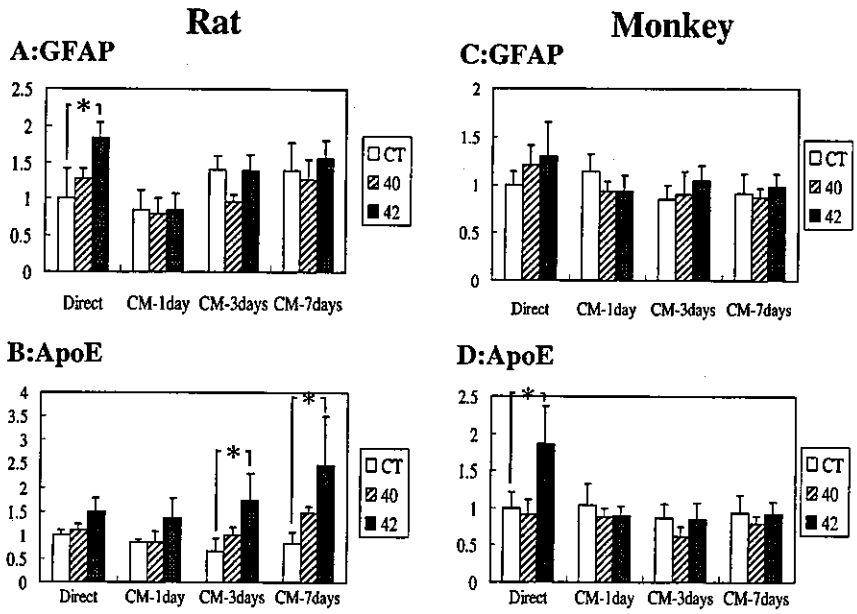

Fig. 4. Immunoreactive bands shown in Fig. 3 were quantified, and the resulting density data were compared to those measured from blots containing extracts from control cultures ($*P < 0.02$). In rat astrocyte cultures ($N = 6$) (A, B), direct treatment with Aβ42 significantly increased GFAP expression levels (A). Direct treatment with Aβ42 increased ApoE expression levels, and furthermore, treatment with conditioned media from cortical cultures treated with Aβ42 for either 3 or 7 days significantly increased ApoE expression (B). The degree of ApoE increase was directly correlated with the number of days the cortical cultures were treated with Aβ. In monkey astrocyte cultures ($N = 6$) (C, D), direct treatment with Aβ42 slightly increased GFAP expression (C). Direct treatment with Aβ42 significantly increased ApoE expression (D). Labeling conventions as in Fig. 3.

of the conditioned-media treatments (i.e., Aβ40, Aβ42, or duration of Aβ exposure), failed to affect ApoE expression levels in monkey astrocyte cultures (Fig. 4D).

4. Discussion

Aβ was previously shown to induce neuronal apoptosis, activate GSK3β, and lead to the accumulation of newly synthesized intracellular Aβ in APP-transfected cells (Cardoso et al., 2002; Ivins et al., 1999; Loo et al., 1993; Mattson et al., 1998; Morishima et al., 2001; Troy et al., 2000; Takashima et al., 1998; Yang et al., 1999). Although these studies shed much light on the various phenomena induced by Aβ, the initial effects of or disturbances caused by Aβ accumulation in the brain remain unknown; astroglial responses primarily occur, or neuronal injuries primarily happen. In the brain, small quantities of Aβ typically accumulate with age; and in some cases, this accumulated Aβ aggregates, causing Aβ-associated pathologies such as AD (Selkoe, 1991). In the present study, we sought to clarify events that may occur early on during the initial stages of Aβ accumulation and aggregation. This was achieved by assessing cultured neuronal cells for signs of apoptosis and altered expression of certain proteins following long-term treatment (14 days) with relatively low concentrations of "no pre-aggregating" forms of Aβ.

In both rat and monkey cortical cultures, Aβ did not significantly decrease or increase expression of caspase-3 or the neuron-related proteins synaptophysin, APP-βCTF, and GSK3β (Figs. 1, 2A–C,F–H, data not shown for caspase-3). These results indicate that Aβ had no effect on neurons during the initial stages of Aβ accumulation and aggregation, indicating that Aβ and its subsequent accumulation and aggregation did not appear to disrupt the formation of synapses.

On the other hand, Aβ treatment increased the expression of the astrocyte-related proteins GFAP and ApoE in both rat and monkey cortical cultures (Fig. 1D,E,I,J), indicating that astroglia, rather than neurons, may be the first neural cells affected by the initial accumulation and aggregation of Aβ in the brain. During the initial stages of Aβ accumulation, therefore, one would intuitively expect to detect astroglial responses (e.g., changes in the expression of astroglia-related proteins) before neuronal responses (e.g., neuronal injury or apoptosis).

The astroglial responses to Aβ differed somewhat in rat and monkey cortical cultures, suggesting possible species differences in how astrocytes react to the accumulation and aggregation of Aβ. In rat cortical cultures, Aβ treatment tended to increase GFAP and ApoE expression levels to a greater degree than in monkey cortical cultures (Figs. 1 and 2D,E,I,J). This finding suggests that rat neuronal cells may be more sensitive to Aβ (thus requiring a greater astroglial response), whereas monkey neuronal cells may be more resistant to Aβ (thus requiring a lesser astroglial response). Cynomolgus monkeys are old world monkeys classified as nonhuman primates and are more similar to humans than rats. Extrapolation of these results, therefore, to humans leads to the prediction that neurons in the human brain may be more resilient than previously thought and may resist

damage during the early stages of Aβ accumulation, thereby allowing astrocytes more time to react and remove excess Aβ from the brain before neuronal injury can occur.

The astroglial responses to different forms of Aβ also differed. In both rat and monkey cortical cultures, Aβ42 treatment increased GFAP and ApoE levels to a greater extent than in cultures receiving Aβ40 treatment (Figs. 1 and 2). Several studies have shown that Aβ42 is more closely associated with AD pathogenesis than Aβ40 (Burdick et al., 1992; Jarrett et al., 1993; Suzuki et al., 1994; Younkin, 1994). These studies are consistent with our results that Aβ42 induced a much stronger astroglial response than did Aβ40 (Figs. 1 and 2).

We also investigated whether neuron-derived soluble factor(s) may influence astrocytic responses to Aβ. This was addressed by treating astrocyte cultures with conditioned media from neuronal cultures previously treated with Aβ (see Section 2 for details). In rat astrocyte cultures, direct Aβ treatment increased GFAP expression significantly, and ApoE expression also increased with direct Aβ treatment (Figs. 3A and 4); ApoE expression, however, not significant. Furthermore, ApoE expression increased significantly in response to treatment with conditioned medium from cortical cultures exposed to Aβ for either 3 or 7 days (Figs. 3A and 4B). These results indicate that rat astrocytes themselves can be directly activated by Aβ and then increase ApoE expression, but expression of ApoE may be also influenced by an as of yet unidentified soluble factor(s) from neurons treated with Aβ. In monkey astrocyte cultures, only direct Aβ treatment increased both GFAP and ApoE expression, although the increase in GFAP was not significant (Figs. 3B and 4C,D). Similar to the case with rat astrocytes, monkey astrocytes can also be directly activated by Aβ. Unlike with rat astrocytes, ApoE expression in monkey astrocytes is only induced directly by Aβ. Thus, in the monkey brain, astrocytes may remove Aβ without requiring induction by neuronal signals, suggesting that possible species differences exist in the astrocytic responses to Aβ and in the mechanisms underlying these responses. From the study of cortical cultures, it was suggested that rat neuronal cells would be more sensitive to Aβ so that they may require a greater astroglial responses (Figs. 1 and 2D,E,I,J). ApoE is known to play a key role in lipid transport and metabolism, and is also important for neuronal repair (Poirier, 1994). Then rat astrocytes would be stimulated not only directly with Aβ but also by some soluble factors produced by neurons for much ApoE expression. On the other hand, it was also suggested that monkey neuronal cells would be more resistant to Aβ (Figs. 1 and 2D,E,I,J). Then monkey astrocytes would not need to express much ApoE such as rat. This may similarly occur in the human brain in which astrocytes may also be directly activated by Aβ. From these results, we concluded that astrocytes would be activated by Aβ in the early stages of Aβ accumulation and aggregation in the brain, during which time they remove excess Aβ before neurons can be damaged. Our conclusion is consistent with the findings that astrocytes are activated by Aβ, then take up Aβ for degradation (Funato et al., 1998; Matsunaga et al., 2003; Wyss-Coray et al., 2003), and that Aβ also induces astrocytes to produce ApoE and chemokines (Deb et al., 2003; LaDu et al., 2001; Smits et al., 2002). Taken together, these findings show that astroglia are in a pivotal position to reduce Aβ pathogenesis at an early stage, and thus may be a therapeutic target of great interest. Hence, additional studies will be required to clarify astroglial responses to Aβ.

In the present study, we also found species differences in the way astroglia respond to Aβ. Rat astrocytes were more sensitive to Aβ, and as such, may be a useful model to further investigate astroglial responses induced by Aβ. However, since the mechanism underlying the astroglial responses to Aβ was different in monkeys, and monkeys are phylogenetically more similar to humans, monkey cortical and astroglial cultures would be the models of choice to study potential therapeutic applications for humans.

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ARTICLE

High-Level *in Vivo* Gene Marking after Gene-Modified Autologous Hematopoietic Stem Cell Transplantation without Marrow Conditioning in Nonhuman Primates

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The successful engraftment of genetically modified hematopoietic stem cells (HSCs) without toxic conditioning is a desired goal for HSC gene therapy. To this end, we have examined the combination of intrabone marrow transplantation (iBMT) and *in vivo* expansion by a selective amplifier gene (SAG) in a nonhuman primate model. The SAG is a chimeric gene consisting of the erythropoietin (EPO) receptor gene (as a molecular switch) and c-Mpl gene (as a signal generator). Cynomolgus CD34$^+$ cells were retrovirally transduced with or without SAG and returned into the femur and humerus following irrigation with saline without prior conditioning. After iBMT without SAG, 2–30% of colony-forming cells were gene marked over 1 year. The marking levels in the peripheral blood, however, remained low (<0.1%). These results indicate that transplanted cells can engraft without conditioning after iBMT, but *in vivo* expansion is limited. On the other hand, after iBMT with SAG, the peripheral marking levels increased more than 20-fold (up to 8–9%) in response to EPO even at 1 year posttransplant. The increase was EPO-dependent, multilineage, polyclonal, and repeatable. Our results suggest that the combination of iBMT and SAG allows efficient *in vivo* gene transduction without marrow conditioning.

Key Words: gene therapy, hematopoietic stem cell, intrabone marrow transplantation, nonconditioning, *in vivo* expansion, selective amplifier gene, nonhuman primate

INTRODUCTION

The ability to expand selectively cells containing potentially therapeutic genes *in vivo* would represent an important tool for the clinical application of hematopoietic stem cell (HSC)-based gene transfer. This would circumvent low gene transfer efficiency into HSCs, which is one of the current limitations of this promising technology. Furthermore, the ability to expand genetically modified cells *in vivo* would circumvent another major problem of HSC gene therapy; myeloablative conditioning is necessary unless gene-modified cells have clear growth advantage [1]. Current myeloablative conditioning regimens are associated with high systemic toxicity and potential damage to marrow stroma, possibly resulting in impaired engraftment [2]. With the *in vivo* selection method using a drug-resistance gene, engraftment of transduced cells at low levels may allow successful expansion to clinically relevant levels even without marrow conditioning, although the administration of cytotoxic agents is required for the selection [3]. It has recently been reported that bone marrow cells can efficiently engraft mice without marrow conditioning when implanted directly into the

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bone marrow cavity (intrabone marrow transplantation, iBMT) [4,5]. Using the iBMT method, human cord blood cells are also able to engraft efficiently in bone marrow of sublethally irradiated immunodeficient mice [6–8]. Although the iBMT method has been successful in mice, the efficacy in primates remains to be examined.

We have previously developed a selective amplifier gene (SAG) consisting of a chimeric gene encoding the granulocyte colony-stimulating factor (G-CSF) receptor (as a growth-signal generator) and the hormone-binding domain of the steroid receptor (as a molecular switch) [9]. Hematopoietic cells genetically engineered to express this SAG can be expanded in a steroid-dependent manner *in vitro* and *in vivo* in mice and nonhuman primates [10,11]. Here we have examined such expansion in the setting of nonhuman primate iBMT without marrow conditioning using a new SAG encoding the erythropoietin (EPO) receptor (as a molecular switch) and thrombopoietin receptor (c-Mpl; as a signal generator) [12].

Results

Engraftment after iBMT

First, we examined whether gene-marked CD34$^+$ cells engraft after iBMT using two cynomolgus macaques. Cynomolgus CD34$^+$ cells were transduced with the nonexpression retroviral vector PLI (which contains untranslated sequence) [13]. The transduction results are summarized in Table 1. We injected the transduced CD34$^+$ cells directly into the bone marrow cavity of four proximal limb bones (the femurs and humeri) after gently irrigating the cavity with saline (Fig. 1). This transplant procedure was safely performed without pulmonary embolism or infection of bone marrow. Conditioning treatment such as irradiation was not conducted prior to transplantation. In addition, we returned the transduced CD34$^+$ cells into two monkeys by the conventional transplantation method without prior conditioning.

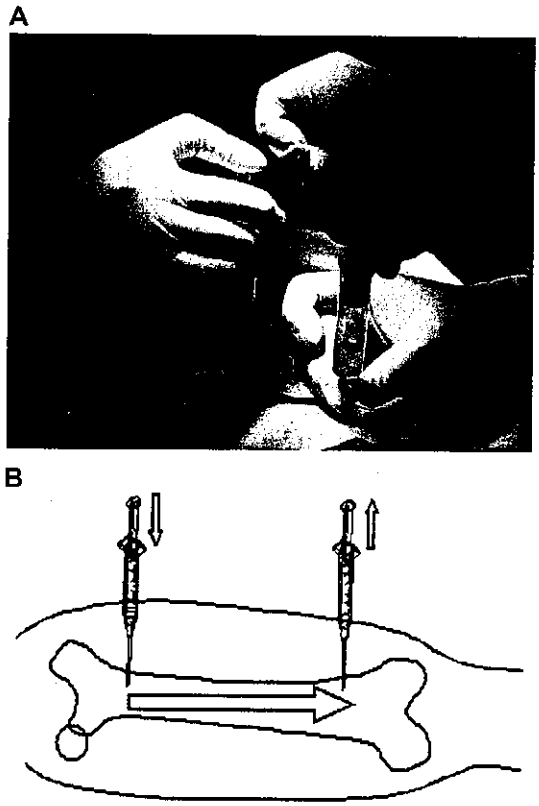

FIG. 1. The iBMT method. We inserted needles at both ends of limb bones (femurs and humeri) and irrigated the bone marrow cavity gently with saline without inflicting extra pressure (A, photo; B, schematic diagram). Gene-modified CD34$^+$ cells were then injected directly into the bone marrow through the needle on one side.

After iBMT, we plated cells from the nonimplanted iliac marrow in methylcellulose medium. We examined the resulting colonies (colony-forming units, CFU) for the provirus by PCR (Fig. 2A and 2B). Two to 30% of colonies (overall 14.2% (74/522)) were positive for the

TABLE 1: *Ex vivo* transduction

Animal	Target cell source	Vector	No. of infused CD34$^+$ cells/kg	Fraction of provirus-positive CFUs in infused CD34$^+$ cells
		Intrabone marrow transplantation		
IB3048	Bone marrow	PLI	4.5×10^7	34/46 (73.9%)
IB3053	Peripheral blood	PLI	8.1×10^6	49/78 (62.8%)
S9042	Peripheral blood	SAG	2.6×10^7	20/35 (57.1%)
S3047	Peripheral blood	SAG	8.1×10^6	11/21 (52.4%)
D8058	Peripheral blood	SAG	7.8×10^5	11/43 (25.6%)
		PLI	5.7×10^5	9/42 (21.4%)
		Intravenous transplantation		
V0065	Peripheral blood	PLI	1.2×10^7	3/45 (6.7%)
V1007	Peripheral blood	PLI	1.5×10^6	14/41 (34.1%)

PLI, nonexpression vector; SAG, selective amplifier gene vector.

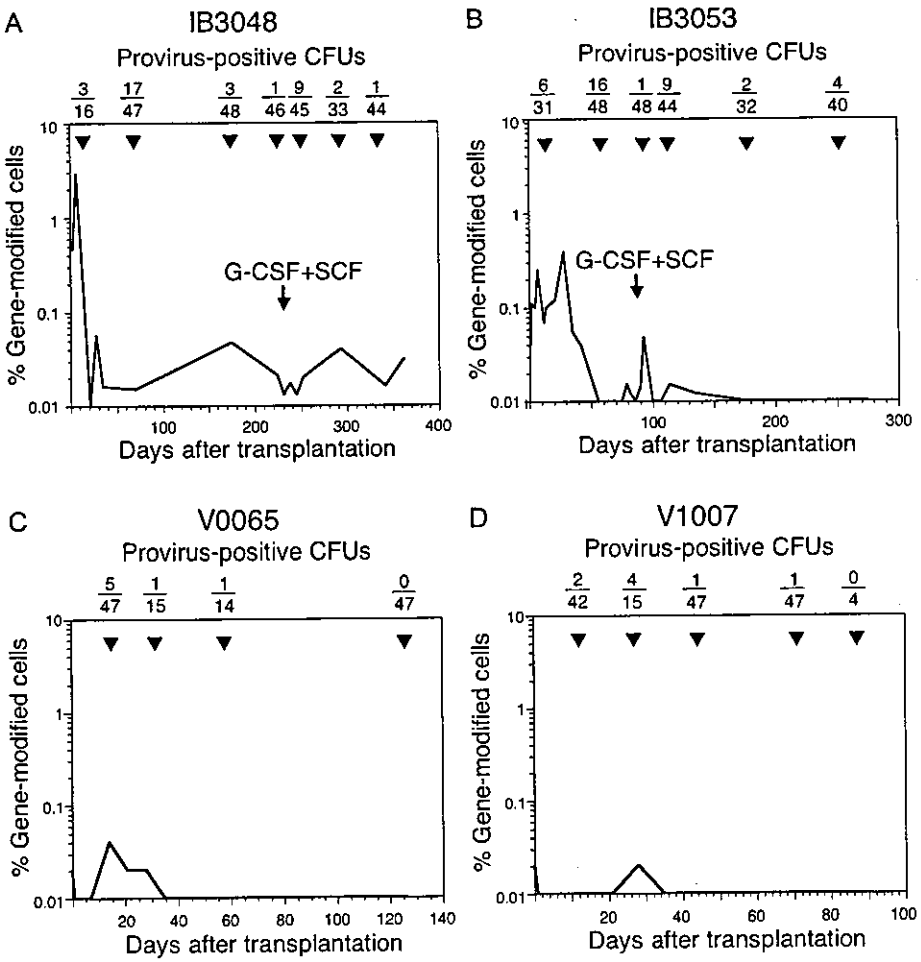

FIG. 2. *In vivo* marking after iBMT and intravenous transplantation without marrow conditioning. CD34$^+$ cells were transduced with nonexpression retroviral vector PLI and returned by iBMT (A, IB3048, and B, IB3053) or by intravenous transplantation (C, V0065, and D, V1007) without conditioning. The upper row shows ratios of provirus-positive CFUs to β-actin-positive CFUs taken from the nonimplanted marrow at time points indicated by arrows. Overall number of provirus-positive CFUs versus overall number of β-actin-positive CFUs was 74/522 (14.2%) for iBMT (A and B) and 15/274 (5.5%) for the intravenous transplantation (C and D). The lower diagram shows percentages of gene-modified cells in the peripheral blood as assessed by quantitative PCR.

provirus and this high marking level persisted for over 1 year posttransplantation. On the other hand, after the conventional intravenous transplantation, generally fewer CFU contained the provirus (overall 5.5% (15/274)) in the bone marrow (Fig. 2C and 2D). Interestingly, the provirus in CFU from the nonimplanted marrow was detectable within 2 weeks after iBMT. Thus, transplanted cells relocated from an implanted bone to another at early time points. A similarly early translocation posttransplantation has also been reported in mouse syngeneic iBMT and human-mouse xeno-iBMT models [4,6–8]. We also examined peripheral blood cells for the provirus by quantitative PCR (Fig. 2A and 2B). The marking levels were, however, very low (<0.1%) in the peripheral blood.

Taken together, these results suggest that transplanted cells can engraft nonconditioned recipients after iBMT but their contribution to the peripheral blood is minimal compared to myeloablated recipients. The cells stay at a resting state in bone marrow without proliferation. In an attempt to proliferate and mobilize iBMT-engrafted resting progenitor cells, we administered G-CSF and stem cell factor (SCF) for 5 consecutive days [14]; however, no obvious increase in the vector-containing cells was observed in the peripheral blood (Fig. 2A and 2B).

EPO-dependent expansion with SAG

We constructed a retroviral vector expressing an SAG that is a chimeric gene of the human EPO receptor gene (extra-plus transmembrane region as a molecular switch) and the human c-Mpl gene (cytoplasmic region as a signal generator) [12]. Cells genetically engineered to express this SAG will proliferate in an EPO-dependent manner. We transduced cynomolgus CD34$^+$ cells with the SAG retroviral vector and introduced them into nonconditioned autologous recipients by iBMT (Table 1). *In vivo* results after transplantation are summarized in Table 2.

In one animal (Fig. 3A), EPO administration triggered a striking elevation in marking levels (7.4% at day 105 posttransplantation) in the peripheral blood. The level of marking in the periphery stayed high for the duration of EPO administration. After cessation of EPO, the level fell to <0.1%. Resumption of EPO administration produced a

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TABLE 2: *In vivo* expansion with SAG after iBMT

Animal	EPO treatment: Treatment course	EPO treatment: Period (days posttransplant)	EPO treatment: Dosage	Marked leukocytes (%)[a]: Basal marking before treatment	Marked leukocytes (%)[a]: Peak marking after treatment (day posttransplant)
S9042	1	1–40	200 IU/kg once daily	NA	7.36% (day 105)
		41–100	200 IU/kg twice daily	NA	7.36% (day 105)
	2	132–210	200 IU/kg twice daily	0.02%	7.72% (day 188)
	3	246–367	200 IU/kg twice daily	0.41%	8.90% (day 348)
S3047	1	75–134	200 IU/kg once daily	0.01%	0.23% (day 145)
		135–166	200 IU/kg twice daily	0.01%	0.23% (day 145)
	2	210–289	200 IU/kg twice daily	0.02%	0.00% (day 289)
D8058	1	1–86	200 IU/kg twice daily	NA	2.30% (day 14)

[a]As assessed by quantitative PCR (see Materials and Methods). NA, not applicable.

similar elevation in the marking levels. The third EPO administration again resulted in the increased marking levels to 8.9% at day 348 posttransplantation. EPO administration was associated with a mild increase in hematocrit (up to 63.5%), which was manageable by occasional phlebotomy. No other adverse effects were observed.

In another animal (Fig. 3B), the SAG-transduced cells increased following transplantation even without exogenous EPO administration. The increase may have been due to increased endogenous EPO elevation resulting from anemia present in the second animal. Overall marking fell with resolution of the anemia. Following resolution, EPO was administered, resulting in an increase in marking levels by more than 20-fold. Marking levels declined to the basal level after discontinuation of EPO. A second attempt to increase marking levels failed, with clearance of SAG-positive cells from the periphery within a month after the second administration, most likely due to cellular immune responses to the xenogeneic SAG (see below).

Multilineage and Polyclonal Expansion

In situ PCR for the proviral sequence showed many transduced cells in the peripheral blood taken from animal S9042 receiving EPO at day 89 posttransplantation (Fig. 4A). We subjected granulocytes and T and B lymphocytes sorted from the peripheral blood of this animal at day 91 posttransplantation to semiquantitative PCR for the provirus. The provirus-containing fraction in granulocytes was 6% and that in B and T lymphocytes was 2% (Fig. 4B), thus indicating that multilineage expansion had occurred. The persistence of marked, short-lived granulocytes for the long term is also another evidence of the successful engraftment of gene-modified HSCs after iBMT. The integration site analysis using the linear amplification-mediated (LAM) PCR method [15]

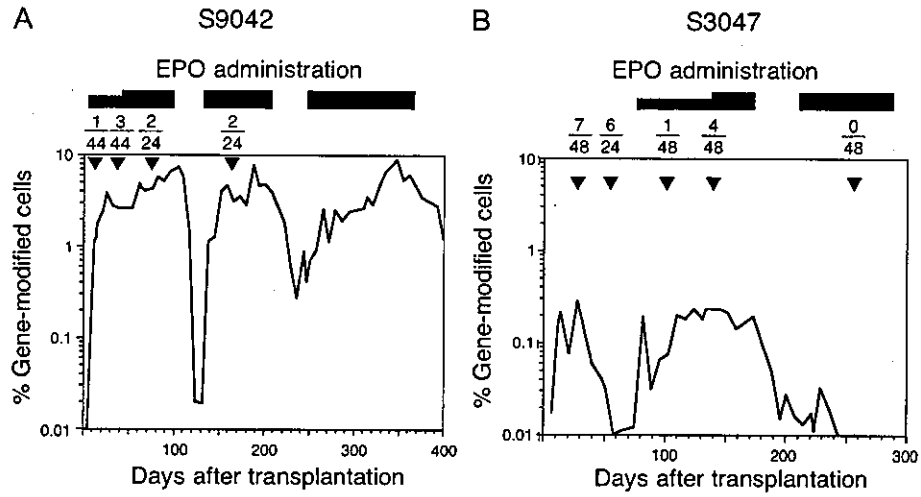

FIG. 3. Expansion of SAG-transduced cells by treatment with EPO after iBMT. $CD34^+$ cells transduced with SAG were returned to each animal by iBMT without conditioning. The animals (A) S9042 and (B) S3047 received EPO at 200 IU/kg once or twice daily (indicated by closed bars). The upper row shows ratios of provirus-positive CFUs to β-actin-positive CFUs taken from the nonimplanted marrow at time points indicated by arrows. The lower diagram shows percentages of gene-modified cells in the peripheral blood as assessed by quantitative PCR.

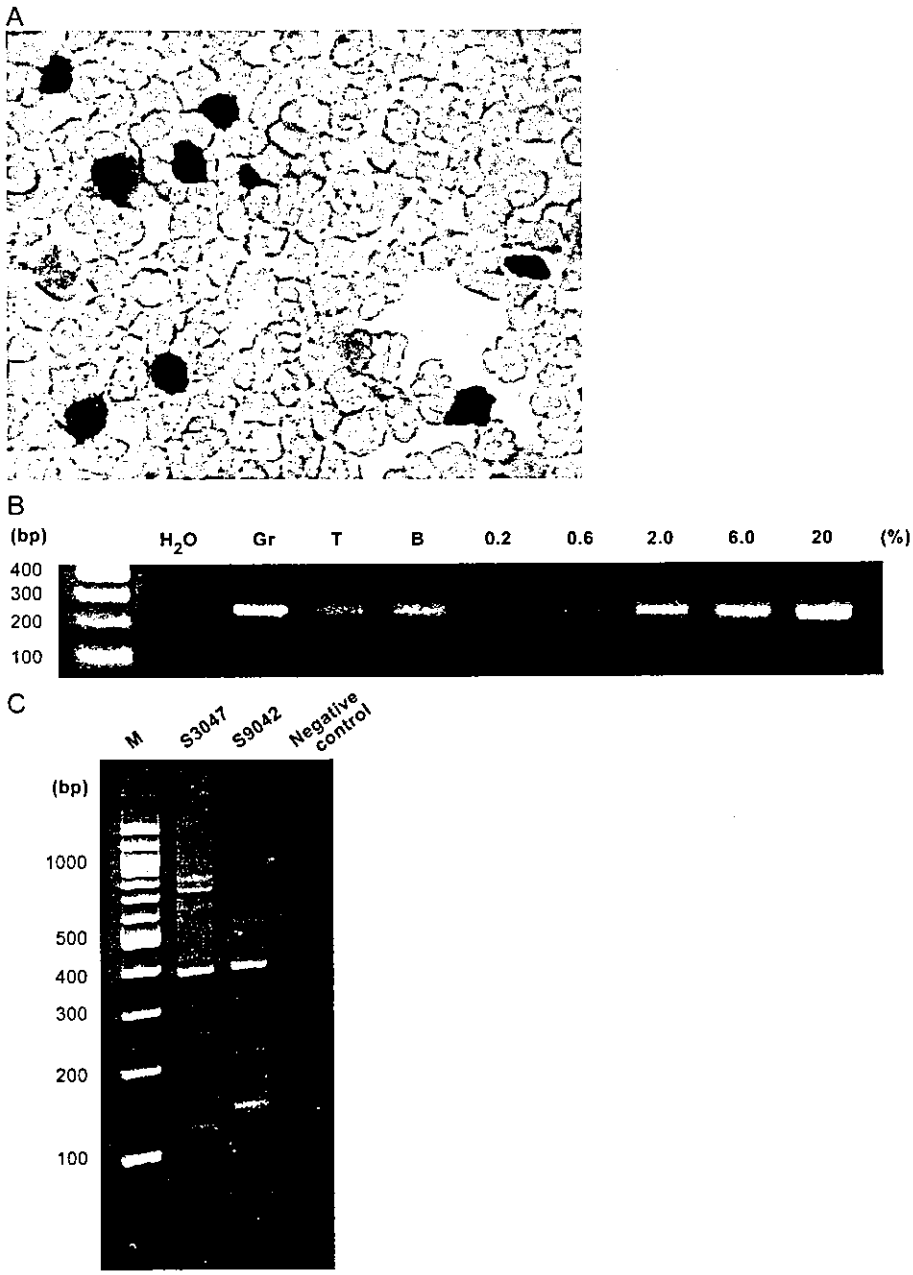

FIG. 4. High-level, multilineage, and polyclonal expansion of gene-modified cells in the peripheral blood after iBMT with SAG in nonconditioned recipients. (A) *In situ* PCR for the provirus. Peripheral blood nucleated cells were collected from animal S9042 receiving EPO at day 89 posttransplantation. Many SAG-transduced cells (stained in black) were detected by *in situ* PCR. (B) Lineage analysis by semiquantitative PCR. DNA from granulocytes (Gr) and T and B lymphocytes sorted from animal S9042 receiving EPO at day 91 posttransplantation was examined for the provirus by semiquantitative PCR. Positive controls corresponding to 0.2, 0.6, 2.0, 6.0, and 20% of transduced cells in peripheral blood were included. (C) Clonal analysis by LAM-PCR. Genomic DNA from peripheral blood of the animals receiving EPO (S9042 at day 90 and S3047 at day 150 posttransplantation) was analyzed by LAM-PCR. Each band indicates different integrants. Negative control was genomic DNA from a naive monkey. M, molecular weight marker.

indicates that the expansion of transduced cells in response to EPO was polyclonal, not mono- or oligoclonal (Fig. 4C).

Dual-Marking Study

We then compared the effects of the SAG vector to a non-SAG vector within, rather than between, individual animals. We harvested cytokine-mobilized peripheral blood $CD34^+$ cells and split them into two equal aliquots. We transduced one aliquot with the SAG vector and the other with the control nonexpression vector (PLI). We mixed both aliquots and returned them by iBMT without marrow conditioning. The animal received EPO from the day after transplantation, and we examined *in vivo* marking levels derived from the two populations by quantitative PCR.

Cells containing the SAG vector increased by 2 logs in the peripheral blood in response to EPO, although cells containing the nonexpression vector remained at low levels (Fig. 5). However, SAG-containing cells were rapidly cleared within 1 month posttransplantation from the periphery and overall SAG-vector marking

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levels became even lower than those from the nonexpression vector-marked fraction. Since cyclosporin A was concomitantly administered to prevent immune responses to human EPO, human EPO concentrations were maintained within an effective range. Thus, it is unlikely that the clearance of xenogeneic human EPO due to immune responses turned off the molecular switch of SAG, resulting in the decrease in SAG-transduced cells.

Immune Responses

The current SAG is a chimeric gene of human origin (the human EPO receptor and human c-Mpl). We collected peripheral lymphocytes from the animal receiving both SAG and nonexpressing PLI (D8058, Fig. 5) at day 169 posttransplantation and examined whether the lymphocytes responded to the xenogeneic SAG *in vitro* (Fig. 6). The response to SAG-transduced target cells was stronger than that to nontransduced target cells ($P = 0.05$), while the response to PLI-transduced target cells did not differ significantly from that to nontransduced target cells ($P = 0.13$). The cellular immune response is, therefore, the most likely reason for the clearance of SAG-transduced cells in this animal. This is not novel, but it has been reported that immune responses against transgene products recognized as foreign can indeed be a major obstacle to long-term persistence of gene-modified cells *in vivo* [13,16,17]. In the human clinical setting, however, immune responses should not occur against SAG, because the SAG is made of human genes.

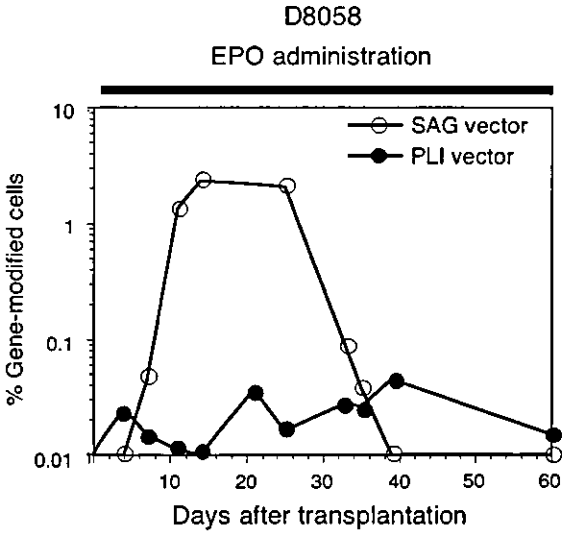

FIG. 5. Dual genetic marking study. CD34$^+$ cells from monkey D8058 were split into two equal aliquots; one aliquot was transduced with SAG vector and the other with nonexpression PLI vector. Both aliquots were together returned to the bone marrow cavity by iBMT without conditioning. EPO (200 IU/kg, twice daily) was administered from the day after transplantation (indicated by a closed bar).

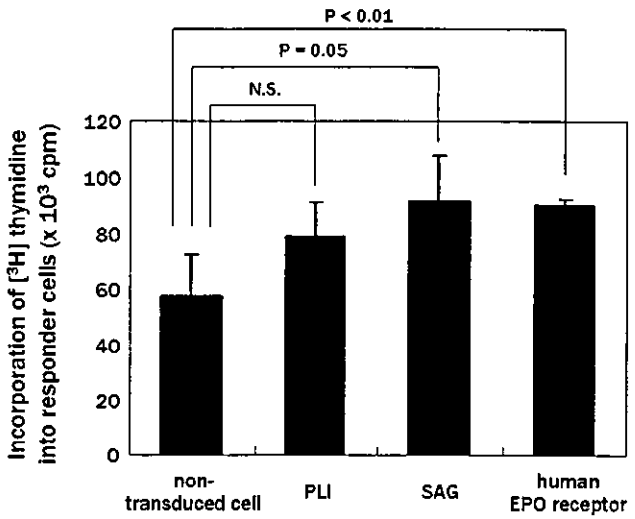

FIG. 6. Positive blastogenic response of lymphocytes to SAG. Peripheral blood mononuclear cells (responder cells) were isolated from monkey D8058 at day 169 posttransplantation (Fig. 5) and cocultured with stimulator cells. The stimulator cells were autologous stromal cells untransduced or transduced retrovirally with PLI, SAG, or human EPO receptor cDNA followed by irradiation with 4000 cGy. After 5 days in culture, the blastogenesis of responder cells was assessed by counting the [^{3}H]thymidine incorporation into responder cells. The averages ± SD of triplicate experiments are shown. N.S., not significant.

Discussion

Previous papers documented that, without marrow conditioning, very low levels (much less than 0.1%) of cells were marked (or corrected) after CD34$^+$ cell gene therapy of chronic granulomatous disease and Gaucher disease [18,19]. This clinical observation has formed the foundation for the contention that myeloablation (or at least conditioning of reduced intensity) is necessary for successful engraftment of transplanted, genetically modified cells. Our results, however, suggest that nonconditioned iBMT results in much higher gene marking levels (up to 8–9%) through the utilization of an SAG. The physical elimination of endogenous marrow with saline before injection might increase gene marking. In the current study, the marrow of four proximal limb bones (femurs and humeri) was replaced with transplanted cells. If other bones such as the iliac bone (which contains more marrow) are similarly used for iBMT, even higher *in vivo* marking levels may be achieved using an SAG.

Expansion of SAG-transduced cells was seen in three lineages: granulocytes, B lymphocytes, and T lymphocytes. The c-Mpl signal generated by the SAG may work even in lymphocytes. In fact, B lymphocytes were shown to be increased by the activated c-Mpl in a canine trans-

plantation model [20]. The expansion was transient, as is the case with other chimeric genes containing c-Mpl as a signal generator [20], although basal marking levels seemed to increase gradually after repeated EPO administration as shown in Fig. 3A. The method largely results in the selection of transduced cells, not at the level of HSCs, but within the differentiated progeny of transduced HSCs.

In the clinical setting, even if the expansion of gene-modified cells is transient, patients can expect therapeutic effects from EPO administration when used as necessary, such as for infection events in patients with chronic granulomatous disease. EPO is a safe drug and can be administered repeatedly with minimal adverse effects. Polycythemia was the only side effect observed in the present study but was manageable by periodic phlebotomy. Therapeutic effects might also be expected from continuously elevated levels of endogenous EPO, such as in patients with thalassemia. When anemia is ameliorated by the gene therapy and endogenous EPO levels return to physiological levels, then the positive selection system is "automatically" turned off, making this a convenient system in such disorders.

Although this "leave it to patients" system would be convenient, a safety concern may be raised regarding leukemogenesis [21]. The SAG proliferation signal that is persistently turned on *in vivo* by endogenous EPO could trigger a secondary event in addition to possible retroviral insertional mutagenesis, although physiological levels of EPO will not induce a significant proliferative response of SAG [12]. Since a set of EPO-mimetic peptides or a modified EPO such as the erythropoiesis stimulating protein has been developed [22,23], it may be possible to develop an SAG containing a mutant EPO receptor that does not bind to endogenous EPO but binds to such EPO-mimetic peptides or modified EPO.

Materials and Methods

Animals. 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 (2.5–5.6 kg, 3–5 years) 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 experimental procedures was approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases (Tokyo, Japan).

Collection of cynomolgus CD34⁺ cells. Cynomolgus monkeys received recombinant human (rh) SCF (50 μg/kg; Amgen, Thousand Oaks, CA, USA) and rhG-CSF (50 μg/kg; Chugai, Tokyo, Japan) as daily subcutaneous injections for 5 days prior to blood cell collection. Peripheral blood or bone marrow cells were then collected by leukapheresis or by aspiration from iliac bones, respectively. From the harvested cells, the leukocyte cell fraction was obtained after red blood cell lysis with ACK buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA; Wako, Osaka, Japan). Enrichment of CD34⁺ cells was performed using magnet beads conjugated with anti-human CD34 (clone 561; Dynal, Lake Success, NY, USA), which cross-reacts with cynomolgus CD34 [24]. The purity of CD34⁺ cells ranged from 90 to 95% as assessed with another anti-human CD34 (clone 563; PharMingen, San Diego, CA, USA) which cross-reacts with cynomolgus CD34 [24]. Mean CFU enrichment was 48-fold as assessed by colony-forming progenitor assays performed before and after enrichment.

Retroviral transduction. We used a retroviral vector expressing SAG (a chimeric gene of the human EPO receptor extra- plus transmembrane region and c-Mpl cytoplasmic region) [12] and PLI nonexpression retroviral vector containing untranslated *neo*^{R} and β-*gal* sequences [13]. The titers of the viral supernatants used in the present study were both 1×10^6 particles/ml, as assessed by RNA dot blot. CD34⁺ cells were cultured at a starting concentration of $1–5 \times 10^5$ cells/ml in fresh vector supernatant of PLI or SAG with rhSCF (Amgen), rh thrombopoietin (Kirin, Tokyo, Japan), and rh Flt-3 ligand (Research Diagnostics, Flanders, NJ, USA), each at 100 ng/ml in dishes coated with 20 μg/cm^2 of RetroNectin (Takara, Shiga, Japan). Every 24 h, culture medium was replaced with fresh vector supernatant and cytokines. After 96-h transduction, cells were washed and continued in culture (Dulbecco's modified Eagle's medium (Gibco, Rockville, MD, USA) containing 10% fetal calf serum (Gibco) and 100 ng/ml rhSCF alone) for 2 additional days in the same RetroNectin-coated dishes [25].

Intrabone marrow transplantation. Cynomolgus monkeys were anesthetized. Two needles were inserted into both ends of the femur or humerus [26]. A syringe containing 50 ml of heparin-added saline was connected to one needle and an empty syringe was connected to the other. Normal saline was irrigated gently from one syringe to another through the marrow cavity twice (Fig. 1). Gene-modified cells were suspended in 1 ml of phosphate-buffered saline containing 10% autologous serum and then injected into the marrow cavity and the needle holes were sealed with bone wax (Lukens, Reading, PA, USA). We measured the internal pressure in the marrow cavity during the procedure in some animals and carefully performed saline irrigation and iBMT without inflicting extra pressure on the marrow cavity. No animals suffered from neutropenia, thrombocytopenia, infection, or pulmonary embolism and there was no morbidity. After transplantation, rhEPO (Chugai) was administered to some animals at a dose of 200 IU/kg once or twice daily subcutaneously. Administration of cyclosporin A (Novartis, Basel, Switzerland) to animals was started a week prior to the EPO administration to prevent the development of anti-human EPO antibody [27].

Clonogenic hematopoietic progenitor assays. Cells were plated in a 35-mm petri dish in 1 ml of α-minimum essential medium containing 1.2% methylcellulose (Shin-Etsu Chemicals, Tokyo, Japan) supplemented with 100 ng/ml rh interleukin-3 (PeproTech, Rocky Hill, NJ, USA), 100 ng/ml rh interleukin-11 (PeproTech), 100 ng/ml rhSCF (Biosource, Camarillo, CA, USA), 2 U/ml rhEPO (Roche, Basel, Switzerland), 20% fetal calf serum, 1% bovine serum albumin, 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO, USA), and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). RhEPO was not added to the culture for colony formation from SAG-transduced cells, to avoid excess proliferative response of the transduced cells to EPO. After incubation for 14 days at 37°C with 5% CO_2, colonies containing more than 50 cells were counted using an inverted light microscope. Experiments were conducted in triplicate.

Quantitative PCR. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Chatsworth, CA, USA). DNA (250 ng) was amplified in triplicate with *neo*-specific primers for PLI (5′-TCCATCATGGATGCAATGCGGC-3′ and 5′-GATAGAAGGCGATGCGCTGCGAATCG-3′) or with SAG-specific primers (5′-GACGCTCTCCCTCATCCTCGT-3′ and 5′-GAGGACTTGGGGAGGATTTCA-3′). Standards consisted of DNA extracted from an SAG- or PLI-producer cell line (which has a known copy number of the proviral sequence) serially diluted with control cynomolgus genomic DNA. Negative controls consisted of DNA extracted from peripheral blood cells of naive monkeys. A β-actin-specific primer set (5′-

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CCTATCAGAAAGTGGTGGCTGG-3′, 5′-TTGGACAGCAAGAAAGTGAGCTT-3′) was used to certify equal loading of DNA per reaction. Reactions were run using the Qiagen SYBR Green PCR Master Mix (Qiagen) on the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) using the following conditions: 50°C for 2 min and 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 62°C for 30 s, 72°C for 30 s, and 83°C for 15 s. The quantitative PCR was certified each time to yield linear amplifications in the range of the intensity of a positive control series (0.01–100%, correlation coefficient >0.98). For calculating the transduction efficiencies, the C_t value of the vector sequence was normalized based on the C_t value of the internal control β-actin sequence on the same sample as directed in the manufacturer's protocol. Gene marking percentages were calculated given that each provirus-positive cell contains one copy of the vector sequence.

Colony PCR. Well-separated, individual colonies at day 14 were plucked into 50 μl of distilled water, digested with 20 μg/ml proteinase K (Takara) at 55°C for 1 h followed by 99°C for 10 min, and assessed for the SAG or nonexpression PLI vector sequence by nested PCR. The outer primer sets were the same as were used in the quantitative PCR described above. Amplification conditions for the outer PCR were 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min with 20 cycles. The outer PCR products were purified using MicroSpin S-400 HR Columns (Amersham, Piscataway, NJ, USA). The inner primer set for the SAG vector was 5′-CCACCCCTAGCCCTAAATCTTATG-3′ and 5′-GGTGGTTCAGCATCCAATAAGG-3′, and that for the PLI vector was 5′-ATACGCTTGATCCGGCTACCTG-3′ and 5′-GATACCGTAAAGCACGAGGAAG-3′. Amplification conditions for the inner PCR were 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min with 20 cycles. Simultaneous PCR for the β-actin sequence was also performed to certify DNA amplification of the sample in each colony. The primer set for β-actin was the same as was used in the quantitative PCR described above. Amplification conditions for β-actin PCR were 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min with 30 cycles. The final PCR products were separated on 2% agarose gels. The sizes of the products were 206, 483, and 232 bp for SAG, nonexpressing PLI vector, and β-actin sequences, respectively. The transduction efficiency of CFU was calculated by dividing the number of colonies positive for the vector sequence by the number positive for the β-actin sequence. Plucked methylcellulose not containing colonies served as negative controls.

In situ PCR. *In situ* detection of transplanted cell progeny was performed by amplifying the SAG sequence as previously reported [28]. Peripheral blood nucleated cells were spun down to glass slides. The SAG-specific primer sequences were the same as were used for the quantitative PCR described above. The reaction mixture consisted of 420 μM dATP, 420 μM dCTP, 420 μM dGTP, 378 μM dTTP, 42 μM digoxigenin-labeled dUTP (Roche), 0.8 μM each SAG primer, 4.5 mM $MgCl_2$, PCR buffer (Mg^{2+} free), and 4 U Takara *Taq* DNA polymerase (Takara). Slides were covered with the Takara Slide Seal for *in situ* PCR (Takara). PCR was performed using the PTC100 Peltier thermal cycler (MJ Research, Watertown, MA, USA) under the following conditions: 94°C for 1 min and 55°C for 1 min with 15 cycles. The digoxigenin-incorporated DNA fragments were detected using the horseradish peroxidase (HRP)-conjugated rabbit F(ab′) anti-digoxigenin antibody (Dako). Slides were then stained for HRP using the Vector SG Substrate Kit. Finally, slides were counterstained with Kernechtrot dye that stains nucleotides, mounted in glycerol, and examined under a light microscope.

LAM-PCR. The LAM-PCR was performed as previously described [15]. The genomic-proviral junction sequence was preamplified by repeated primer extension using 0.25 pmol of vector-specific, 5′-biotinylated primer LTR1 (5′-AGCTGTTCCATCTGTTCTTGGCCCT-3′) with *Taq* polymerase (2.5 U; Qiagen) from 100 ng of each sample DNA. One hundred cycles of amplification were performed with the addition of fresh *Taq* polymerase (2.5 U) after 50 cycles. Biotinylated extension products were selected with 200 μg of magnetic beads (Dynabeads Kilobase BINDER Kit; Dynal). The samples were incubated with Klenow polymerase (2 U; Roche), dNTPs (300 μM; Pharmacia, Uppsala, Sweden), and a random hexanucleotide mixture (Roche) in a volume of 20 μl for 1 h at 37°C. Samples were washed on the magnetic particle concentrator (Dynal) and incubated with *TasI* (Fermentas, Hanover, MD, USA) to cut the 5′ long terminal repeat-flanking genomic DNA for 1 h at 65°C. After an additional wash step, 100 pmol of a double-stranded asymmetric linker cassette and T4 DNA ligase (6 U; New England Biolabs, Beverly, MA, USA) was incubated with the beads in a volume of 10 μl at 16°C overnight. Denaturing was performed with 5 μl of 0.1 N NaOH for 10 min at room temperature. Each ligation product was amplified with *Taq* polymerase (5 U; Qiagen), 25 pmol of vector-specific primer LTR2a (5′-AACCTTGATCTGAACTTCTC-3′), and linker cassette primer LC1 (5′-GACCCGGGAGATCTGAATTC-3′) by 35 cycles of PCR (denaturation at 95°C for 60 s, annealing at 60°C for 45 s, and extension at 72°C for 60 s). Of each PCR product, 0.2% served as a template for a second, nested PCR with internal primers LTR3 (5′-TCCATGCCTTGCAAAATGGC-3′) and LC2 (5′-GATCTGAATTCAGTGGCACAG-3′) under identical conditions. Final products were separated on a 2% agarose gel.

Flow-cytometric sorting. We used the FSC/SSC profile (forward and side scatter) to sort granulocytes (purity 95%). Anti-CD3 and anti-CD20 were used to sort T lymphocytes (purity 99%) and B lymphocytes (purity 95%), respectively. Cells were sorted using an EPICS Elite cell sorter equipped with an argon-ion laser (Beckman Coulter, Fullerton, CA, USA). Data acquisition and analysis were performed using the EXPO2 software (Beckman Coulter).

Cellular immune response assay. Peripheral blood mononuclear cells and bone marrow stromal cells were isolated from monkey D8058. The stromal cells were transduced with a retroviral vector carrying the PLI, SAG, or human EPO receptor cDNA. The transduced stromal cells were irradiated with 4000 cGy and used as stimulator cells. Untransduced stromal cells irradiated with 4000 cGy served as a control. The peripheral blood mononuclear cells (responder cells, 2×10^5/well) were cocultured with the stimulator or control cells (5×10^4/well) in 96-well, flat-bottom plates with RPMI 1640 medium (Sigma) containing 10% fetal calf serum and 20 IU/ml rh interleukin-2 (Shionogi, Osaka, Japan). After 5 days in culture, the blastogenesis of responder cells was assessed. Briefly, the cells were labeled with 1 μCi/well of [*methyl*-^{3}H]thymidine (Amersham) for 16 h and harvested with an automated cell harvester (Laboratory Science, Tokyo, Japan) onto glass-fiber filters (Molecular Devices, Sunnyvale, CA, USA). The incorporation of [*methyl*-^{3}H]thymidine into responder cells was quantified in a liquid scintillation counter (Aloka, Tokyo, Japan). All experiments were performed in triplicate.

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ORIGINAL PAPER

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Detection of 14 alleles derived from the MHC class I *A* locus in cynomolgus monkeys

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Abstract A basic understanding of the major histocompatibility complex (MHC) class I, which, together with T-cell receptors, is a key player in antigen recognition by cytotoxic T lymphocytes, is necessary to study the cellular immune response to intracellular pathogens. The MHC has hardly been reported in cynomolgus monkeys (*Macaca facicularis*), although cynomolgus monkeys have been frequently used as the surrogate animal model. We attempted to determine the nucleotide sequences of the MHC class I *A* locus of cynomolgus monkeys (*Mafa-A*) and eventually 34 independent sequences of *Mafa-A* were obtained from 29 cynomolgus monkeys. These 34 sequences were classified into 14 *Mafa-A* alleles according to the results of phylogenetic analyses using the neighbor-joining method. One to three *Mafa-A* alleles were obtained from a single animal. We also tried to establish a multiplex PCR-SSP method for convenient typing of *Mafa-A* alleles. cDNA from a family of cynomolgus monkeys, which is composed of four sirs and four dams, were examined by multiplex PCR-SSP. The result of multiplex PCR-SSP showed that an individual cynomolgus monkey had two or three *Mafa-A* alleles, suggesting that the *A* locus of cynomolgus monkeys might be duplicated.

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Introduction

The major histocompatibility complex (MHC) class I consists of heavy chain, β_2-microglobulin (β_2m), and antigen peptide (Hennecke et al. 2001). Human cells are known to express three highly polymorphic MHC heavy chains (*HLA-A*, *-B*, and *-C*) and three conserved MHC heavy chains (*HLA-E*, *-F*, and *-G*). *HLA-A*, *-B*, and *-C* present antigen peptides to cytotoxic T lymphocytes (CTL) and the CTL are then activated (Flynn et al. 1992; Hou et al. 1992; York and Rock 1996). These classical molecules, especially *HLA-C*, also provide both stimulatory and inhibitory signals to natural killer (NK) cells through killer cell immunoglobulin-like receptors (KIR) (Valiante et al. 1997).

The gene encoding the class I heavy chain is composed of eight exons. Exon 1 encodes the signal peptide, exons 2–4 specify the extracellular domains α_1–α_3, exon 5 codes for the transmembrane domain, and exons 6–8 code for the cytoplasmic domain. The α_1 and α_2 domains are the most polymorphic, while the α_3 domain contributes to the β_2m association (Hebert et al. 2001) and interaction with the CD8 molecule (Salter et al. 1990).

The rhesus MHC has been extensively studied among non-human primates because rhesus monkeys are most frequently used as the surrogate animal model (Allen et al. 2001; Horton et al. 2001; Mothe et al. 2002) for HIV infection in human. Rhesus MHC (*Mamu*) class I *A* (Boyson et al. 1996b; Miller et al.1991; Urvater et al. 2000a; Voss and Letvin 1996; Watanabe et al. 1994), *B* (Boyson et al. 1996b; Voss and Letvin 1996; Yasutomi et al. 1995), *E* (Boyson et al. 1995), *F* (Otting and Bontrop 1993), *G* (Boyson et al. 1996a), *AG* (Slukvin et al. 1999), and *I* (Urvater et al. 2000b) have already been reported. Rhesus monkeys were shown to carry at least one *A* and two *B* loci, because three *Mamu-A* and five *Mamu-B* alleles have been identified in a single animal (Boyson et

al. 1996b). *HLA-C* homologues have been identified in the common chimpanzee, bonobo, gorilla, and orangutan (Adams et al. 1999, 2000; Cooper et al. 1998; de Groot et al. 2000; Lawlor et al. 1990, 1991), while no evidence of an *HLA-C* homologue was observed in old and new world monkeys (Adams and Parham 2001). Although SIV infection in cynomolgus monkeys is also used as the animal model for human HIV infection (McClure et al. 1990; Putkonen et al. 1992), there are few reports about cynomolgus MHC (*Mafa*) except for class II loci (Gaur and Nepom 1996; Kriener et al. 2000; Otting et al. 1992), class I *E* (Alvarez et al. 1997; Boyson et al. 1995), and *I* loci (Urvater et al. 2000b).

In this study, we have determined the nucleotide sequences of the genes coding for the cynomolgus MHC class I *A* molecules and found 14 *Mafa-A* alleles. In addition, we established a convenient method to detect the *Mafa-A* alleles.

Materials and methods

Animals

All the cynomolgus monkeys were raised and reared in the Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases. Both genders were involved and the cynomolgus monkeys were between 2 and 23 years old. This study was conducted in accordance with the Guide for Animal Experiments Performed at the National Institute of Infectious Diseases.

RT-PCR and nucleotide sequencing

Peripheral blood mononuclear cells (PBMC) were isolated from the fresh blood of 29 cynomolgus monkeys by a standard Ficoll-Hypaque gradient method. PBMC were washed twice with PBS and suspended in 5 ml of RPMI-1640 (Sigma, St. Louis, Mo.) containing 100 U/ml penicillin (Meiji Seika Kaisha, Tokyo, Japan), 10% FCS (GIBCO-BRL, Grand Island, N.Y.), and 5 μg/ml concanavalin A (ConA; Pharmacia, Cleveland, Ohio) at a concentration of 10^5 cells/ml. PBMC were cultured at 37 °C for 3–4 days. Messenger RNA extracted from the cultured PBMC (2–

Table 1 Primers used for the amplification and sequencing of MHC class I cDNAs from cynomolgus monkeys

Primer	Sequence	Concentration (pmol/sample)	Annealing temperature (°C)
Primers used for RT-PCR			60
Mafa-A-s	5′-GCAGGATCCGAATCTCCCCAGACGCGCA-3′	10	
Mafa-A-a	5′-GCTCTAGACCTCACAAGGCAGCTGTC-3′	10	
Mafa-A13-s	5′-CGAACCCTCCTCCTGG-3′	10	
Mafa-A1013-a	5′-CTGAGAGTAGCTCCCTCCTTTTCTAT-3′	10	
Primers used for multiplex PCR			
Primer set 1			72
IA01-s	5′-GCAGCGGGATGGAGAGGAA-3′	20	
IA02-s	5′-GCTGTGGTTGTGCCTTCTGGAAAA-3′	10	
IA03-s	5′-ACGCTGCAGCGCGCA-3′	2	
IA04-s	5′-GCGGCGGATGTGGCGGAGAG-3′	2	
IA05-s	5′-CTGCGACCTGGGGCCG-3′	2	
IA-a	5′-CCTGGGCACTGTCACTGCTT-3′	20	
Primer set 2			72
IA06-s	5′-GGGCCTGTGCGTGGAGTCCCTG-3′	10	
IA07-s	5′-CACACTGACCTGGCAGCGT-3′	10	
IA08-s	5′-CTGCGACCTGGGGCCA-3′	10	
IA09-s	5′-CTACAACCAGAGCGAGGCCA-3′	10	
IA10-s	5′-GCAGCCCCGCTTCATCT-3′	10	
IA-a	5′-CCTGGGCACTGTCACTGCTT-3′	20	
Primer set 3			70
IA11-s	5′-ACACATGTGACCCATCACCCT-3′	5	
IA12-s	5′-GCCGGAGTATTGGGACCA-3′	20	
IA13-s	5′-GGCCTGCAGGAGATGGAAA-3′	20	
IA14-s	5′-CGGACCTGGGGGCTCAA-3′	15	
IA-a	5′-CCTGGGCACTGTCACTGCTT-3′	20	
Primers used for sequencing			
T7 primer	5′-TAATACGACTCACTATAGGG-3′	3	55
SP6 primer	5′-ATTTAGGTGACACTATAG-3′	3	55
Ia698	5′-TAGAAGCCCAGGGCCCAGGC-3′	3	55
Is437	5′-ATTACATCGCCCTGAACGAG-3′	3	55