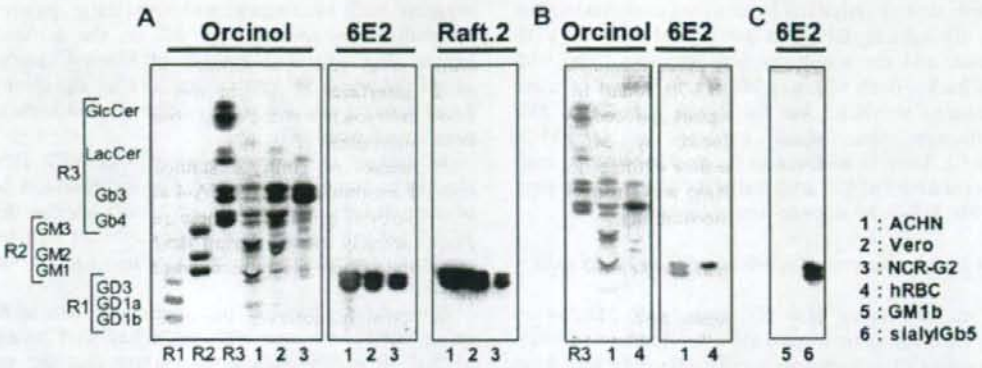

Fig. 1. TLC immunostaining of GSLs prepared from cultured cells and hRBCs. GSLs extracted from cultured cells and hRBCs or purified GSLs were separated by TLC in a solvent system of chloroform/methanol/water containing 0.2% $CaCl_2$ (5:4:1, v/v/v). Plates were chemically stained with orcinol-sulfurnic acid or were immunostained with 6E2 and Raft.2. Lane 1, ACHN; Lane 2, Vero; Lane 3, NCR-G2; Lane 4, hRBCs; Lane 5, GM1b; Lane 6, sialylGb5. Reference markers used were disialosyl gangliosides of GD3, GD1a, and GD1b (R1), monosialosyl gangliosides of GM3, GM2, and GM1 (R2), and neutral GSLs of GlcCer, LacCer, Gb3, and Gb4 (R3). The nomenclature for GSLs follows the recommendations [11] of the IUB, and the ganglioside nomenclature of Svennerholm [12] was used.

Mab. The 80 kDa protein might be associated with sialylGb5 in NCR-G3 cells and thus co-immunoprecipitated by 6E2.

Comparison of reactivity to sialyl Gb5 between 6E2 and MC813-70

MC813-70 established by immunizing with human EC cell lines has been most widely used as an anti-SSEA-4 antibody (mouse IgG_3, κ) [14]. Therefore we compared the reactivities of the Mabs 6E2 and MC813-70 by flow cytometry and dot-blot immunostaining. The fluorescence intensity obtained with 6E2 was stronger than that with MC813-70 in each cell line and hRBCs (Fig. 2A). A recent flow cytometric study showed that MC813-70 strongly stains hRBCs, but other anti-sialyGb5 Mabs do not [15]. However, our data indicate that 6E2 is more reactive than MC813-70. Next we compared the reactivity of the two

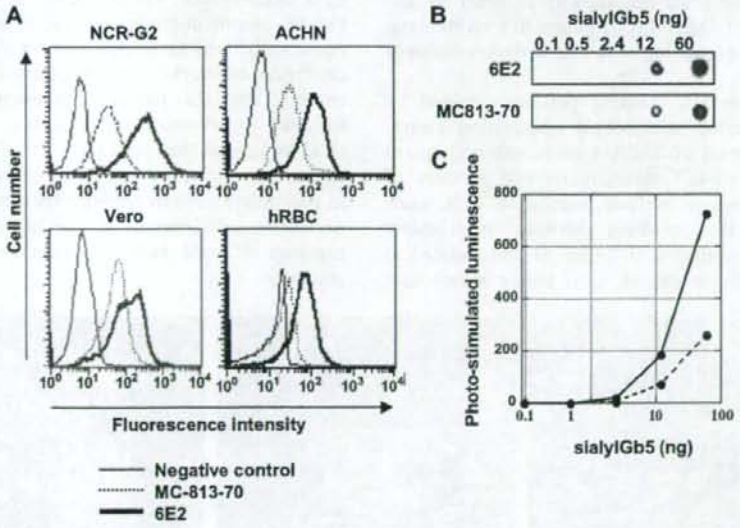

Fig. 2. Reactivity of 6E2 and MC813-70 with sialylGb5. (A) Flow cytometric analysis of SSEA-4-positive cells with 6E2. NCR-G2 cells, ACHN cells, Vero cells, and hRBCs were stained with 6E2 (bold line) or MC813-70 (dotted line) and with a FITC-conjugated secondary antibody and analyzed by flow cytometry. (B) An image of the dot-blot immunostaining of sialylGb5 obtained with a LAS-1000 luminescent imaging analyzer. (C) Measurement of antibodies bound (6E2: solid line, MC813-70: broken line).

Mabs with that of sialylGb5 by dot-blot immunostaining. Serially diluted sialylGb5 was dot-blotted onto a PVDF membrane, and the membrane was immunostained with the two Mabs. Both 6E2 and MC813-70 bound to more than 12 ng of sialylGb5, but the signals induced by 6E2 were stronger than those induced by MC813-70 (Fig. 2B,C). Thus, in addition to the flow cytometric analysis, the reactivity of 6E2 with sialylGb5 was stronger than that of MC813-70 by dot-blot immunostaining.

SSEA-4 Immunostaining of cynomolgus monkey ES cells

To confirm whether Mab 6E2 reacts with SSEA-4 on monkey ES cells, we performed an indirect immunofluorescence staining of cynomolgus monkey ES cells with Mab 6E2 and MC813-70. Mab 6E2 reacted with monkey ES cells (Fig. 3A) as well as MC-813-70 did (Fig. 3B). No difference in staining patterns of SSEA-4 between the two Mabs was observed. Mab 6E2 certainly stained SSEA-4 on monkey ES cells.

SSEA-4 immunostaining of "living" mouse preimplantation embryos without fixation

During early embryogenesis in mice, SSEA-4 had been reported to be expressed in fertilized eggs with levels gradually increasing to the morula stage and then decreasing [5]. Thus we examined the expression and distribution of SSEA-4 in preimplantation mouse embryos by immunostaining with both 6E2 and MC813-70. Both Mabs evenly stained the whole surface membranes of fixed mouse embryos, and no difference in staining pattern between the two was observed (data not shown). In order to perform a time-course of SSEA-4 distribution in a viable state, we performed immunostaining of preimplantation embryos without fixation.

3D-images of the 6E2 staining pattern obtained by confocal laser scanning microscopic observation clearly showed the localization of SSEA-4 on mouse preimplantation embryos. Two-cell embryos showed patches of SSEA-4 over the whole surface membrane with some accumulation at the interface between blastomeres (Fig. 4A). In 8-cell embryos, the amount accumulated at interfaces was further increased, as if planer membranes separate each blastomere, and some large patches were internalized but others were left on the surface membranes (Fig. 4B). The amount of SSEA-4 concentrated at the interfaces in morula was not as significant as in 8-cell embryos but still clearly observed and some patches were internalized (Fig. 4C).

2D-images of embryos stained with 6E2 showed a marked accumulation of SSEA-4 at the interfaces between blastomeres (Fig. 4D–F). These results suggest that sialylGb5 actively moves during development and tends to accumulate where blastomeres come into contact with each other.

Interestingly, however, the staining pattern of SSEA-4 using MC813-70 was different from that using 6E2. MC813-70 evenly stained the surface and the interface between blastomeres of 2-cell embryos with patches (Fig. 4G), and the amount of SSEA-4 at interfaces was not significant (Fig. 4J). In 8-cell embryos, there were patches of SSEA-4 in the central area of the outer surface of each blastomere (Fig. 4H, indicated by arrows), but the 2D-image showed that clustering also occurred at surfaces facing blastocoels (Fig. 4K, indicated by arrowheads). In morula embryos, SSEA-4 was distributed on the surface in patches and was enriched at the boundaries between blastomeres on the outer surface (Fig. 4I,L).

It remains unclear why the pattern of staining of mouse preimplantation embryos differs between 6E2 and MC813-70. The composition of fatty acids in GSLs influences the binding of antibodies [16,17] or bacterial toxins [18]. SialylGb5 recognized by the two MAbs might differ in composition of fatty acids, resulting in different immunostaining patterns. It was reported that the clustering of sialylGb5 by a Mab induces the activation of sialylGb5-associated kinases in raft microdomains of human mammary carcinoma cells, leading to downstream signaling [19,20]. The clustering of sialyGb5 by 6E2 on preimplantation mouse embryos may also induce the activation of some kinases, followed by downstream signaling. Recently, Comisky et al. suggested that lipid rafts and their associated molecules are spatiotemporally positioned to play a critical role in preimplantation developmental events [21]. The patches or clusters of sialylGb5 shown in our study suggest the presence of lipid rafts containing sialylGb5 on mouse embryos.

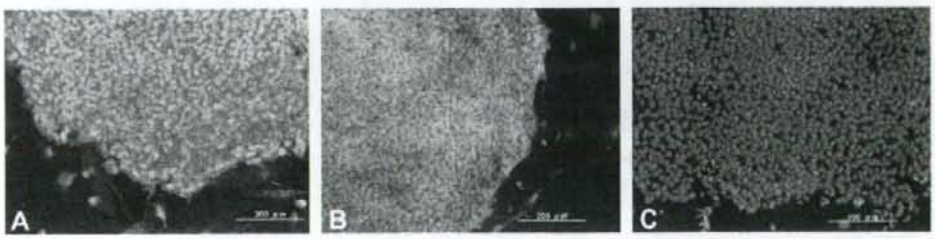

Fig. 3. Indirect immunostaining of cynomolgus monkey ES cell line CMK-6 with 6E2 and MC813-70. The CMK-6 cells were stained with 6E2 (A), MC813-70 (B), or isotype-matched mouse IgG (C), and visualized with secondary antibodies (green), followed by counterstaining of nuclei with DAPI (blue). Scale bars = 200 μm.

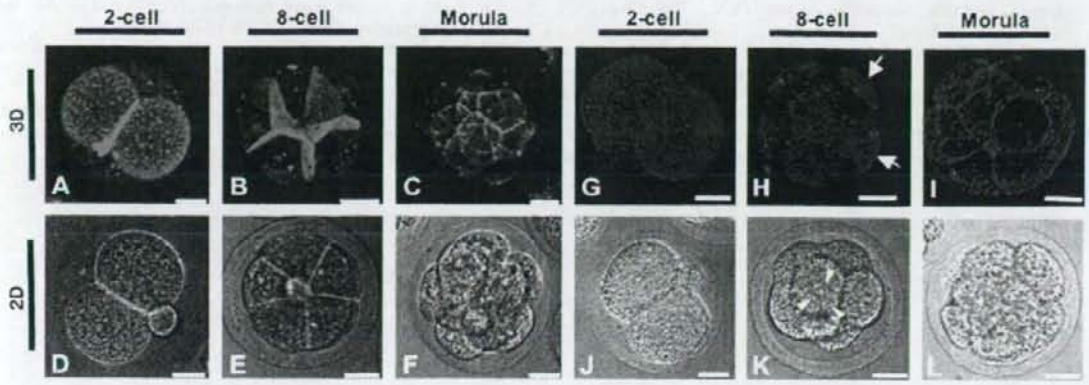

Fig. 4. Immunostaining of SSEA-4 on mouse preimplantation embryos with 6E2 and MC813-70. The embryos at the 2-cell (A, D, G, J), the 8-cell (B, E, H, K), and the morula (C, F, I, L) stages were stained with 6E2 (green) or MC813-70 (red). The panels designated 3D (A, B, C, G, H, I) are three-dimensional images reconstructed by stacking optical slice images using LSM software and the panels designated 2D (D, E, F, J, K, L) are an overlay of a fluorescent image and a differential interference contrast micrograph. Scale bars = 20 μm.

6E2 has high affinity for sialylGb5 and can be effectively conjugated with fluorescence reagents, leading to excellent staining of SSEA-4 in the surface membrane of "living" mouse preimplantation embryos. 6E2 should be of use for research into lipid rafts in early development and of great advantage for the characterization of ES cells and EC cells.

Acknowledgments

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Preferential localization of SSEA-4 in interfaces between blastomeres of mouse preimplantaion embryos

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Abstract

The monoclonal antibody 6E2 raised against the embryonal carcinoma cell line NCR-G3 had been shown to also react with human germ cells. Thin-layer chromatography (TLC) immunostaining revealed that 6E2 specifically reacts with sialosylglobopentaosylceramide (sialylGb5), which carries an epitope of stage-specific embryonic antigen-4 (SSEA-4), known as an important cell surface marker of embryogenesis. The immunostaining of mouse preimplantation embryos without fixation showed that the binding of 6E2 caused the clustering and consequent accumulation of sialylGb5 at the interface between blastomeres. These results suggest that SSEA-4 actively moves on the cell surface and readily accumulates between blastomeres after binding of 6E2.

Keywords: SialylGb5; SSEA-4; Embryonic stem cells; Embryonal carcinoma cells; Preimplantation embryo; Immunostaining

Embryonal carcinoma (EC) cells isolated from teratocarcinomas have been shown to possess pluri- or multipotency in both mouse and human systems [1–3]. In mice, certain EC cells as well as embryonic stem (ES) cells have been considered to be developmentally equivalent to the inner cell mass of blastocysts [1]. These EC cells are useful for clarifying the molecular characteristics of early embryonic cells and thus many efforts have been made to establish EC cell lines and monoclonal antibodies (Mabs) that detect differentiation-related molecules on EC cells. As a consequence, a number of stage-specific markers for embryogenesis have been identified. Notably, it is important that this molecular information is adapted to research on ES cells or mouse preimplantation embryos. Stage-specific embryonic antigen (SSEA) -1, -3, and -4, as well as tumor rejection antigen (TRA) -1-60 and -1-81 [4], have been used as stage-specific markers for embryogenesis, though their functional significance in early development remains unclear. Interestingly, however, most of these antigens are carbohydrates themselves or closely related to the carbohydrates carried on glycosphingolipids (GSLs) and glycoproteins [5].

6E2 is a Mab established by immunizing with NCR-G3 cells, a previously established multipotent human EC cell

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line capable of differentiating into trophoblastic cell lineages other than somatic cells [3]. It has been revealed that 6E2 reacts with not only human ECs, including NCR-G2 and 3 cells, but also other germ cell tumors, as well as normal human germ cells such as spermatogonia and oocytes [6]. Although a previous study reported that 6E2 immunoprecipitates a cell surface protein having a molecular weight of approximately 80 kDa from ^{125}I-labeled NCR-G3 cells, the specific antigen recognized by 6E2 still remains unknown. To characterize the antigen specificity of 6E2, we examined the reactivity of the Mab with other cell lines using several distinct methods. In this paper, we present evidence that 6E2 recognizes SSEA-4 carried by sialylGb5. Using 6E2, we determined the localization of SSEA-4 in "living" mouse preimplantation embryos and observed its preferential localization in interface between blastomeres.

Materials and methods

Cells, antibodies, and animals. The human renal carcinoma cell line ACHN was purchased from American Type Culture Collection. The African green monkey kidney cell line Vero was a gift from Dr. T. Takeda of Department of Infectious Diseases Research, National Children's Medical Research Center, Tokyo, Japan. Cells were maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM) (Sigma Chem., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS). The human EC cell line NCR-G2 [3] was cultured in a 1:1 mixture of DMEM and Ham's F12 medium (DMEM/F12) (Invitrogen Gibco, Carlsbad, CA) supplemented with 10% FBS(JRH Bioscience), non-essential amino acid solution (NEAA) (Invitrogen Gibco), and Insulin-Transferin-Sodium Selenite media (Invitrogen Gibco). The cynomolgus monkey ES cell line CMK-6 [7] were provided by Dr. Yasushi Kondo of Mitsubishi Tanabe Pharma Corporation. ES cells were grown on mouse embryonic fibroblast feeder cells that were inactivated by gamma-irradiation in DMEM/F12 supplemented with 20% Knockout™ Serum Replacement, 2 mM Glutamax-I, 1% NEAA, 50 units/ml penicillin, 50 μg/ml streptomycin, 0.1 mM 2-mercaptoethanol, 1% sodium pyruvate, and 5 ng/ml bFGF (all from Invitrogen GIBCO). The cultures were performed at 37 °C in a 5% CO_2 incubator. The human venous blood from a healthy consenting volunteer was drawn in a heparin-coated syringe. The blood was spun at 3000 rpm for 15 min and human red blood cells (hRBCs) were washed three times in phosphate buffered saline (PBS).

The conjugation of affinity-purified 6E2 (mouse IgG_3, κ) [6] to the fluorescence reagent was performed with an Alexa Fluor® 488 monoclonal antibody labeling kit (Molecular Probes, Eugene, OR.) according to the manufacturer's instructions. The anti-SSEA-4 Mabs used in this study were Raft.2 [8] and MC813-70 (R&D Systems, Inc Minneapolis, MN). Alexa Fluor® 488 goat anti-mouse IgG and Streptavidin Alexa Fluor® 568 were purchased from Molecular probes.

BDF_1 mice were purchased from Clea Japan (Tokyo, Japan).

TLC immunostaining of GSLs. TLC immunostaining of GSLs from cultured cells and hRBCs was performed as previously described [9]. Reference GSLs were purchased from Matlayer, Inc. (Pleasant Gap, PA). SialylGb5 was purified from ACHN cells by preparative TLC. Purified GM1 b was kindly provided by Dr. Nakamura of RIKEN, Saitama, Japan [10].

Flow cytometry. Cells were harvested and incubated with a primary antibody (1 μg/ml) for 1 h on ice, followed by treatment with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) at a dilution of 1:50 and analyzed with an EPICS-XL flow cytometer (Beckman Coulter, Inc, Miami, FL).

Dot blot analysis. Purified sialylGb5 was serially diluted (0.1–60 ng) and vacuum blotted onto a PVDF membrane by using a 96-well format dot blot apparatus (Bio-Rad Laboratories, Richmond, CA). The membrane was immunostained with the Mab 6E2 or MC813-70 (0.5 μg/ml) according to a previously described procedure [9]. The antibodies that bound to the membranes were visualized with ECL-plus Western Blotting Detection Reagents (GE Healthcare UK Ltd, Buckinghamshire, UK) and scanned with a LAS-1000 luminescent imaging analyzer (Fujifilm, Tokyo, Japan). Scanned images were analyzed using the software Image Gauge with which the LAS-1000 was equipped.

Indirect immunostaining of cynomolgus monkey ES cells. Cells were grown on a glass-bottomed dish (IWAKI) for 3 days and then these cells were fixed for 30 min with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-200 in PBS for 20 min. Subsequently, the cells were washed three times with PBS for 5 min and blocked with 5% normal goat serum in PBS for 30 min. The fixed cells were incubated with anti-SSEA-4 antibodies or isotype-matched mouse IgG at a dilution of 1:300 for 2 h, followed by incubation with Alexa Fluor® 488-conjugated goat anti-mouse IgG at a dilution of 1:300 for 30 min. DAPI was used for counter staining of nuclei.

Immunostaining of mouse preimplantation embryos. Mouse preimplantation embryos were collected from superovulated mice. Seven-week-old BDF1 female mice were induced to superovulate with intraperitoneal injections of pregnant mare's serum gonadotropin (ASKA Pharmaceutical co., Ltd., Tokyo, Japan) (5 IU) and human chorionic gonadotropin (hCG) (ASKA Pharmaceutical co) (5 IU) 48 h apart and mated with individual BDF1 male mice after the hCG injection. The 2-cell, the 8-cell, and the morula stage embryos were flushed out from oviducts at 36, 60, and 72 h after the hCG injection, respectively. Animals were treated according to the institutional animal care and use guidelines of National Research Institute for Child Health and Development.

Embryos immediately after being collected and those prefixed with 2% paraformaldehyde in Hepes buffered saline were incubated in 30 μl drops of M16 medium containing 0.45 μg of Alexa Fluor® 488-conjugated 6E2 for 1 h or biotinylated MC813-70 for 1 h, treated with streptavidin Alexa Fluor® 568 diluted 1:300, and then they were washed three times in 30 μl drops of M16 medium. All staining steps were carried out at 37 °C in a CO_2 incubator for fresh embryos and at 4 °C for fixed embryos. The stained embryos were placed in drop of a M16 medium on glass-bottomed dishes (IWAKI, Tokyo, Japan), and were observed with a LSM510 Zeiss Confocal laser-scanning microscope (Carl Zeiss, Thornwood, NY) to obtain a field of view of the embryo only with a 40× objective lens.

Results and discussion

6E2 specifically binds to sialylGb5

In order to examine whether the 80 kDa membrane protein is recognized by 6E2, we performed a Western analysis of the cell lysates or their immunoprecipitates with 6E2. Since no significant signal was detected on the blot (data not shown), we examined TLC immunostaining of GSLs extracted from several 6E2-positive cell lines. ACHN cells showed the expression of comparable amounts of Gb3, Gb4, Gb5, and sialylGb5, whereas Vero cells and NCR-G2 cells expressed predominantly Gb3 (Fig. 1A). TLC immunostaining analysis revealed that 6E2 binds to a major slow-migrating GSL extracted from these three cell lines. The slow-migrating GSL was identified as sialylGb5, defined by the Mab Raft.2. We observed that 6E2 bound to sialylGb5 (LKE-antigen) of hRBCs [13] (Fig. 1B). Finally, we examined the reactivity of 6E2 with purified GSLs and found that the Mab reacts with purified sialylGb5, but not purified GM1 b (Fig. 1C). These results indicate that 6E2 specifically binds to sialylGb5 and thus is an anti-SSEA-4

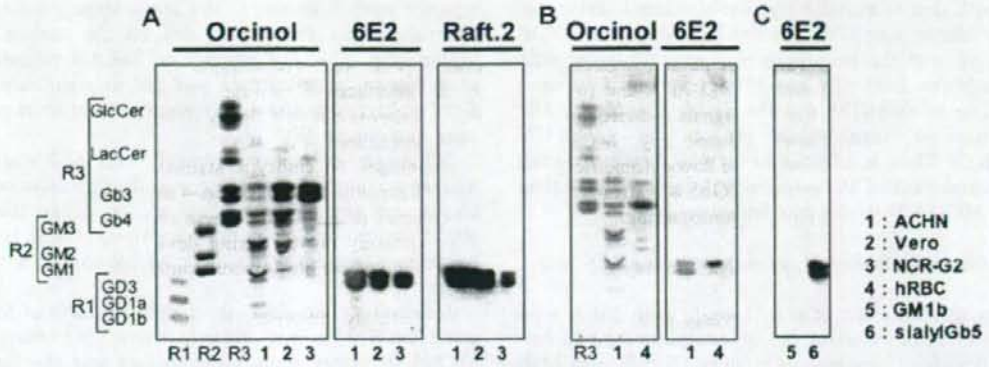

Fig. 1. TLC immunostaining of GSLs prepared from cultured cells and hRBCs. GSLs extracted from cultured cells and hRBCs or purified GSLs were separated by TLC in a solvent system of chloroform/methanol/water containing 0.2% $CaCl_2$ (5:4:1, v/v/v). Plates were chemically stained with orcinol-sulfurnic acid or were immunostained with 6E2 and Raft.2. Lane 1, ACHN; Lane 2, Vero; Lane 3, NCR-G2; Lane 4, hRBCs; Lane 5, GM1b; Lane 6, sialylGb5. Reference markers used were disialosyl gangliosides of GD3, GD1a, and GD1b (R1), monosialosyl gangliosides of GM3, GM2, and GM1 (R2), and neutral GSLs of GlcCer, LacCer, Gb3, and Gb4 (R3). The nomenclature for GSLs follows the recommendations [11] of the IUB, and the ganglioside nomenclature of Svennerholm [12] was used.

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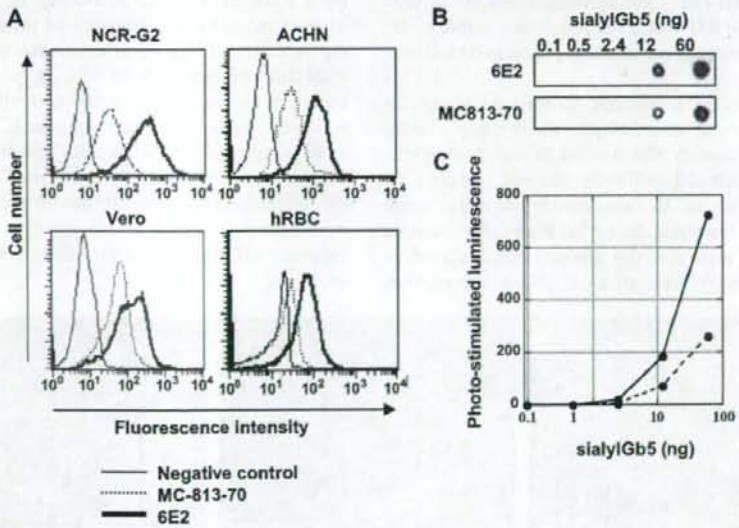

Fig. 2. Reactivity of 6E2 and MC813-70 with sialylGb5. (A) Flow cytometric analysis of SSEA-4-positive cells with 6E2. NCR-G2 cells, ACHN cells, Vero cells, and hRBCs were stained with 6E2 (bold line) or MC813-70 (dotted line) and with a FITC-conjugated secondary antibody and analyzed by flow cytometry. (B) An image of the dot-blot immunostaining of sialylGb5 obtained with a LAS-1000 luminescent imaging analyzer. (C) Measurement of antibodies bound (6E2: solid line, MC813-70: broken line).

Mabs with that of sialylGb5 by dot-blot immunostaining. Serially diluted sialylGb5 was dot-blotted onto a PVDF membrane, and the membrane was immunostained with the two Mabs. Both 6E2 and MC813-70 bound to more than 12 ng of sialylGb5, but the signals induced by 6E2 were stronger than those induced by MC813-70 (Fig. 2B,C). Thus, in addition to the flow cytometric analysis, the reactivity of 6E2 with sialylGb5 was stronger than that of MC813-70 by dot-blot immunostaining.

SSEA-4 Immunostaining of cynomolgus monkey ES cells

To confirm whether Mab 6E2 reacts with SSEA-4 on monkey ES cells, we performed an indirect immunofluorescence staining of cynomolgus monkey ES cells with Mab 6E2 and MC813-70. Mab 6E2 reacted with monkey ES cells (Fig. 3A) as well as MC-813-70 did (Fig. 3B). No difference in staining patterns of SSEA-4 between the two Mabs was observed. Mab 6E2 certainly stained SSEA-4 on monkey ES cells.

SSEA-4 immunostaining of "living" mouse preimplantation embryos without fixation

During early embryogenesis in mice, SSEA-4 had been reported to be expressed in fertilized eggs with levels gradually increasing to the morula stage and then decreasing [5]. Thus we examined the expression and distribution of SSEA-4 in preimplantation mouse embryos by immunostaining with both 6E2 and MC813-70. Both Mabs evenly stained the whole surface membranes of fixed mouse embryos, and no difference in staining pattern between the two was observed (data not shown). In order to perform a time-course of SSEA-4 distribution in a viable state, we performed immunostaining of preimplantation embryos without fixation.

3D-images of the 6E2 staining pattern obtained by confocal laser scanning microscopic observation clearly showed the localization of SSEA-4 on mouse preimplantation embryos. Two-cell embryos showed patches of SSEA-4 over the whole surface membrane with some accumulation at the interface between blastomeres (Fig. 4A). In 8-cell embryos, the amount accumulated at interfaces was further increased, as if planer membranes separate each blastomere, and some large patches were internalized but others were left on the surface membranes (Fig. 4B). The amount of SSEA-4 concentrated at the interfaces in morula was not as significant as in 8-cell embryos but still clearly observed and some patches were internalized (Fig. 4C).

2D-images of embryos stained with 6E2 showed a marked accumulation of SSEA-4 at the interfaces between blastomeres (Fig. 4D–F). These results suggest that sialylGb5 actively moves during development and tends to accumulate where blastomeres come into contact with each other.

Interestingly, however, the staining pattern of SSEA-4 using MC813-70 was different from that using 6E2. MC813-70 evenly stained the surface and the interface between blastomeres of 2-cell embryos with patches (Fig. 4G), and the amount of SSEA-4 at interfaces was not significant (Fig. 4J). In 8-cell embryos, there were patches of SSEA-4 in the central area of the outer surface of each blastomere (Fig. 4H, indicated by arrows), but the 2D-image showed that clustering also occurred at surfaces facing blastocoels (Fig. 4K, indicated by arrowheads). In morula embryos, SSEA-4 was distributed on the surface in patches and was enriched at the boundaries between blastomeres on the outer surface (Fig. 4I,L).

It remains unclear why the pattern of staining of mouse preimplantation embryos differs between 6E2 and MC813-70. The composition of fatty acids in GSLs influences the binding of antibodies [16,17] or bacterial toxins [18]. SialylGb5 recognized by the two MAbs might differ in composition of fatty acids, resulting in different immunostaining patterns. It was reported that the clustering of sialylGb5 by a Mab induces the activation of sialylGb5-associated kinases in raft microdomains of human mammary carcinoma cells, leading to downstream signaling [19,20]. The clustering of sialyGb5 by 6E2 on preimplantation mouse embryos may also induce the activation of some kinases, followed by downstream signaling. Recently, Comisky et al. suggested that lipid rafts and their associated molecules are spatiotemporally positioned to play a critical role in preimplantation developmental events [21]. The patches or clusters of sialylGb5 shown in our study suggest the presence of lipid rafts containing sialylGb5 on mouse embryos.

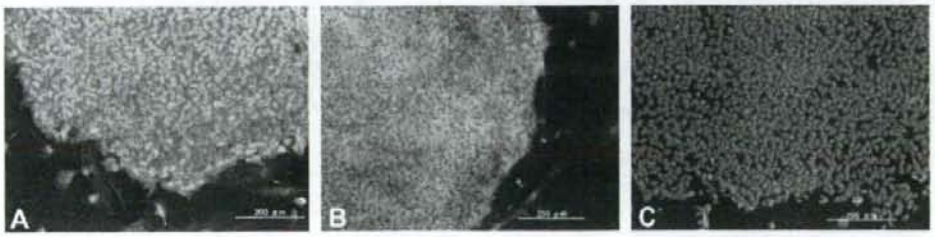

Fig. 3. Indirect immunostaining of cynomolgus monkey ES cell line CMK-6 with 6E2 and MC813-70. The CMK-6 cells were stained with 6E2 (A), MC813-70 (B), or isotype-matched mouse IgG (C), and visualized with secondary antibodies (green), followed by counterstaining of nuclei with DAPI (blue). Scale bars = 200 μm.

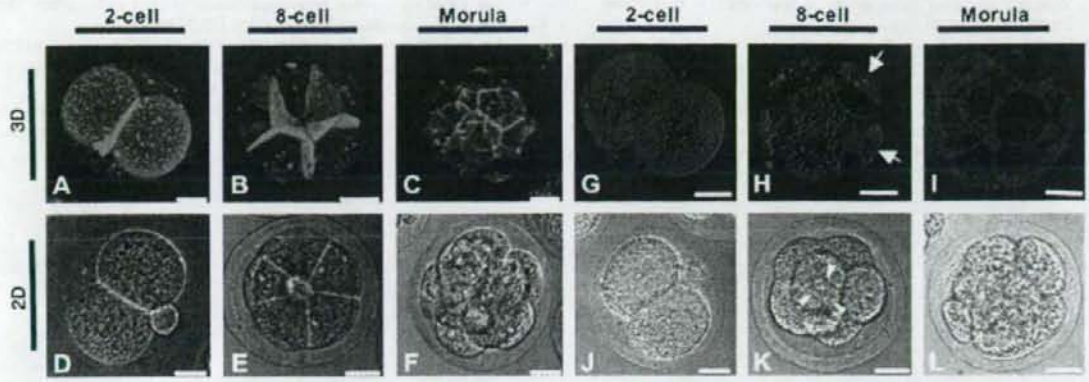

Fig. 4. Immunostaining of SSEA-4 on mouse preimplantation embryos with 6E2 and MC813-70. The embryos at the 2-cell (A, D, G, J), the 8-cell (B, E, H, K), and the morula (C, F, I, L) stages were stained with 6E2 (green) or MC813-70 (red). The panels designated 3D (A, B, C, G, H, I) are three-dimensional images reconstructed by stacking optical slice images using LSM software and the panels designated 2D (D, E, F, J, K, L) are an overlay of a fluorescent image and a differential interference contrast micrograph. Scale bars = 20 μm.

6E2 has high affinity for sialylGb5 and can be effectively conjugated with fluorescence reagents, leading to excellent staining of SSEA-4 in the surface membrane of "living" mouse preimplantation embryos. 6E2 should be of use for research into lipid rafts in early development and of great advantage for the characterization of ES cells and EC cells.

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SHORT COMMUNICATION

The detergent-insoluble microdomains, rafts, can be used as an effective immunogen

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Abstract Detergent-insoluble microdomains, or rafts, act as a platform to transduce signals from the extracellular space into the cytoplasm. In the process of developing monoclonal antibodies against raft molecules for the purpose of studying the molecular mechanism of raft-mediated signaling, we observed the uniqueness and certain advantages of immunization with rafts. Simple subcutaneous injection of mice with a phosphate-buffered saline (PBS) suspension of rafts without mixing with Freund's adjuvant made it possible to increase the titer of antiserum reacting with raft components. Interestingly, injection of rafts prepared from certain specific cell lines induced monoglycolipid-specific antibodies. Furthermore, antibodies were produced by raft-immunization of even syngeneic mice. Our findings suggest that this phenomenon does not represent a breakdown of immunological self-tolerance, but typical immune reactions accompanying the class switch from IgM antibodies to IgG antibodies.

Keywords Raft · Antibody · Immune Response · Monoglycolipid-specific · Syngeneic antigen

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Introduction

There is evidence that detergent-insoluble microdomains, or rafts, are important in signal transduction, because a variety of signaling molecules, such as Src-family kinases, heterotrimeric G proteins, and GPI-anchored proteins, are concentrated in rafts. We have previously shown that the binding of Shiga-toxin (Stx) to the globotriaosylceramide (Gb3[1]) in rafts temporally activates the Src-family kinase Yes in human renal cancer cell line ACHN [1]. In order to study the downstream signaling mechanism after Stx binding to Gb3, we attempted to develop monoclonal antibodies against components of rafts prepared from ACHN cells and established several clones [2].

In the process we observed the uniqueness of immunization using raft suspensions. Before immunizing animals in an attempt to induce antibody production, antigen solutions or cell suspensions are generally mixed with Freund's adjuvant to obtain an oil emulsion, whereas we succeeded in raising antibody titer by the raft immunization method without mixing them with adjuvants. Interestingly, two thirds of the clones obtained reacted with lipid components of the raft, and further analysis showed that all of the lipid-reactive clones recognized monosialosylgalactosylgloboside (sialylGb5).

To ascertain whether raft immunization always induces monospecific antibodies that recognize a certain glycolipid, we immunized mice with rafts prepared from several cell lines and examined the glycolipid antigens recognized by the antibodies induced. In this paper we report that injection with

[1] Glycosphingolipids are abbreviated according to the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature. http://www.chem.qmul.ac.uk/iupac/misc/glylp.html.

rafts prepared from certain specific cell lines can induce the production of monoglycolipid-specific antibodies and that raft immunization can induce antibody production even in syngeneic mice.

Materials and methods

Cell culture and antibodies Human renal cancer cell line ACHN, human T-cell leukemia cell lines Jurkat and MOLT-4, and mouse myeloma cell line P3U1 were purchased from the American Type Culture Collection, and the African green monkey kidney cell line Vero was a gift of Dr. T. Takeda of the Department of Infectious Diseases Research, National Children's Medical Research Center, Tokyo, Japan. Anaplastic large cell lymphoma Karpas 299 cells [3] were gifted by Dr. K. Kikuchi of Sapporo Medical University, School of Medicine, Sapporo, Japan. Human pre-B ALL cell line NALM-6, mouse T lymphoma cell line EL4, mouse melanoma cell line B16F1, and mouse leukemia cell line RL2 were obtained from the Institute of Development, Aging and Cancer of Tohoku University, Sendai, Japan. The ACHN cells, Vero cells, and B16F1 cells were cultured in Dulbecco's modified Eagle's medium (Sigma Chem., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Cansera International Inc., CCT, Canada). All other cell lines were cultured in RPMI 1640 supplemented with 10% FBS. The NZB/WF1 serum was a kind gift of Dr. S. Kon of the Institute of Genetic Medicine, Hokkaido University, Sapporo.

Raft preparation Rafts were prepared as described previously [2]. Briefly, packed cells were homogenized in 1% Triton lysis buffer (1% Triton X-100, 25 mM Tris–HCl buffer, pH 7.5, 0.15 M NaCl) by 20 strokes with a hand-driven Teflon glass homogenizer. Cell lysates, sucrose concentration of which was adjusted to 40% with 85% sucrose solution, were placed on the bottom of an ultracentrifuge tube, and a 5/30% discontinuous sucrose gradient was formed over the sample. After centrifugation at 39,000 rpm for 18 h at 4°C in a Beckman SW 40Ti rotor, rafts were recovered as visible bands at the interface between 5 and 30% sucrose solution. After several washes with PBS, raft suspensions in PBS were stored at −30°C until used.

Immunization of mice Rafts prepared from 1.2×10^6–1.5×10^8 cells or 10^7 cells irradiated at 10 Gy were suspended in 100 μl of PBS. They were subcutaneously injected into mice in triplicate, followed by three booster shots at 1-week intervals. Five days after the final injection, a peripheral blood specimen was collected from the mice, and the level of antibodies against rafts was evaluated.

TLC immunostaining Lipids were prepared from packed cells as previously described [4] and separated on a Silica gel 60-precoated HPTLC aluminium sheet (Merck, Darmstadt, Germany) with a solvent system consisting of chloroform/methanol/water containing 0.2% $CaCl_2$ (5:4:1, *v/v*). After drying, the TLC plates were coated with 0.1% polyisobutylmethacrylate (Sigma-Aldrich, Milwaukee, WI) in cyclohexane and blocked with 1% bovine serum albumin (BSA) in PBS. The plates were probed with anti-sera (diluted to 1:500 in 1% BSA in PBS) for 1 h at room temperature. After three washes with PBS for 5 min each, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulins G+M (DAKO, A/S, Denmark) at a 1:2,000 dilution ratio were used as the second antibody. The antibodies that bound to the plates were visualized with enhanced chemiluminescence reagent Super Signal (Pierce, Rockford, IL) and detected with a luminescent imaging analyzer, LAS-1000 (Fuji Film, Tokyo, Japan). To compare the amounts of antibodies in the sera, the intensity of chemiluminescence was measured with Image Gauge analysis software equipped to LAS-1000 and shown as Photo Stimulated Luminescence (PSL).

Dot-blot immunostaining assay The ACHN rafts were dot-blotted on a PVDF membrane (Millipore Corp., Bedford, MA) and immunostained as described previously [2] with a slight modification. The dots were probed with antisera (diluted to 1 in 500 with 1% BSA in PBS) for 1 h at room temperature. After four washes with PBS containing 0.025% Tween 20 (PBS-Tween), the membranes were treated with HRP-conjugated rabbit anti-mouse IgG antibodies specific to Fcγ fragment and HRP-conjugated goat anti-mouse IgM antibodies specific to μ chain (Jackson Immuno Research Laboratories, West Grove, PA) to detect IgG and IgM, respectively. The antibodies that bound to the membrane were visualized with enhanced chemiluminescence (ECL Western blotting system; Amersham Pharmacia Biotech. UK Ltd., Buckinghamshire) and detected by a luminescent imaging analyzer as mentioned above.

Flow cytometry Cells were harvested from culture plates, and after incubating with the antisera (diluted to 1:100 in RPMI medium containing 5% FBS and 0.1% NaN_3) for 1 h on ice, they were treated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (Jackson Immuno Research Laboratories) at a 1:50 dilution ratio and analyzed by flow cytometry (EPICS-XL, Beckman-Coulter, Fullerton, CA).

Measurement of anti-ss and -dsDNA antibodies in sera by ELISA The ELISA was performed as described by Iizuka *et al.* [5] with a slight modification by using calf thymus ssDNA (Sigma) and salmon sperm dsDNA (Sigma). For

the substrate solution, 120 µl of 0.1 mg/ml 3,3′,5,5′-tetramethylbenzidine (Dojindo, Kumamoto, Japan) solution in *N,N*,dimethylformamide and 1.3 µl of 30% H_2O_2 was mixed with 7.88 ml of 0.1 M sodium acetate buffer, pH 5.5, immediately before use. A 0.5 µg amount of ssDNA or dsDNA dissolved in 50 µl of PBS were allowed to dry in a flat 96-well NUNC-IMMUNO Plate (Nunc, Roskilde, Denmark). Wells were blocked with 3% BSA in PBS and washed with PBS-Tween. A 50 µl of the serum (diluted to 1:100) was added to a well in triplicate, and allowed to stand at room temperature for 2 h. After five washes with PBS-Tween, HRP-conjugated donkey anti-mouse µ chain antibodies or rabbit anti-mouse γ chain antibodies (Jackson Laboratory; diluted to 1:2,000) in 1% BSA in PBS was added to each well and incubated for 1 h at room temperature. After three washes with PBS-Tween, 50 µl of substrate solution was added, and the plates were incubated at room temperature until the solution turned yellow. The reaction was stopped by adding 50 µl of 2 M H_2SO_4, and absorbance at 450 nm was measured with a microplate reader (Model 550 Bio-Rad, Richmond, CA).

Results and discussion

The antisera obtained from Balb/c mice in response to subcutaneous injection of rafts prepared from a variety of cell lines were examined by TLC-immunostaining to analyze the reactivity of the antibodies against glycolipids. Both ACHN cells and Vero cells are derived from kidney and express globoseries glycosphingolipids whereas Karpas cells predominantly express LacCer, and EL4 cells mainly express GM2 and GD2 (Fig. 1a). The antisera obtained by injection with ACHN rafts and Vero rafts were found to uniquely bound to sialylGb5, suggesting the development of mono-specific antibodies against sialylGb5 (Fig. 1b). As we previously showed, ACHN cells contain comparable amounts of Gb3, Gb4, Gb5, and sialyGb5, suggesting that the sialylGb5 of ACHN cells is strongly immunogenic [2]. The antisera obtained by injection with Karpas rafts were also found to specifically bind to a single glycolipid that has not yet been identified. Since the glycolipid was stained with resorcinol and not bound by choleratoxin even after digestion with *Clostridium perfringens* sialidase (data not shown), it is suggested that this antigen is a sialylated non-ganglioseries glycolipid. The observation that the anti-Karpas rafts antisera did not bind to any glycolipid extracted from mouse brains in which various kinds of gangliosides are abundantly contained (data not shown) should support this idea. The antisera obtained by injection with EL4 rafts were found to uniquely react with GD2. However, when antisera obtained by injection of rafts prepared from the other cell lines, *i.e.*, B16F1, P3U1, RL-2, Molt 4, Jurkat, or NALM-6, were tested, no such monoglycolipid-specific reactivity was observed (data not shown). These findings indicate that immunization with rafts prepared from some specific cell lines can induce the development of monoglycolipid-specific antibodies. Since we obtained identical results in a similar experiment in C57BL/6 mice (Fig. 1c), the development of monoglycolipid-specific antibodies is a common feature of immunization of these cell lines with rafts and not a phenomenon specific to a certain strain of mice.

Immunization of mice with a suspension of whole cells is one of the ways that is often used to obtain monoclonal antibodies against cell surface molecules [6, 7]. We therefore investigated whether whole-cell immunization is capable of inducing the development of monoglycolipid-specific antibodies in mice, the same as raft immunization does. The antisera obtained by injection with Vero, Karpas, and EL4 cell suspensions did not react with certain specific glycolipids, and only the antisera obtained by immunization with ACHN cell suspensions yielded a mono-specific reaction with sialylGb5 (Fig. 2). This suggests that immunization with suspensions of whole cells does not usually induce the development of monoglycolipid-specific antibodies and that the rafts on the cell surface of ACHN cells assemble in a manner that is favorable for inducing immune reactions against sialylGb5.

Next, we examined the quantitative and qualitative kinetics of the production of the specific antibodies in sera by immunization with rafts derived from ACHN cells. The antisera obtained after each immunization were examined by dot-blot immunostaining and TLC immunostaining (Fig. 3a). The relative amounts of antibodies that bound to rafts dot-blotted on a PVDF membrane or lipids separated on a TLC plate were shown as PSL (Fig. 3b). Production of IgM class anti-raft antibodies was detected after the second immunization, and it peaked after the third immunization, and then decreased. Production of IgG class anti-raft antibodies was also detected after the second immunization, but at a low level, and it continued to increase even after the fourth immunization. The specificity of the secondary antibodies used for typing the immunoglobulin class of anti-raft antibodies was confirmed in advance (data not shown). No anti-sialylGb5 antibodies were detected not after the first immunization (data not shown). They were faintly detected after the second immunization, and then increased in an immunization time-dependent manner. These results indicate that the production of anti-raft antibodies in mice is a typical immune response accompanying the class switch from IgM antibodies to IgG antibodies. Interestingly, the #3 antisera of the third immunization gave strong reactivity with the lower band glycolipid, while that of the fourth

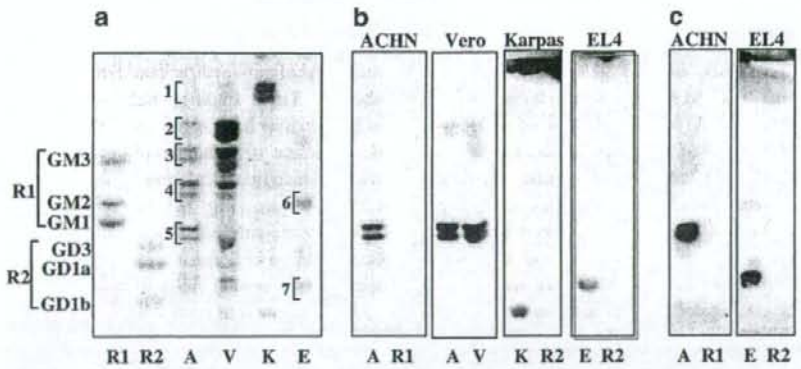

Fig. 1 TLC immunostaining with antisera against rafts components. The lipids extracted from ACHN (*A*), Vero (*V*), Karpas (*K*), EL4 (*E*) cells and the reference glycolipids (*R1* GM3, GM2, GM1; *R2* GD3, GD1a, GD1b) were separated by TLC and chemically stained with Orcinol reagent (**a**) or immunostained with antisera of Balb/c mice (**b**) and C57BL/6 mice (**c**) that had been immunized with rafts prepared from ACHN cells, Vero cells, Karpas cells and EL4 cells. Lipids extracted from 5×10^6 cells and 1×10^6 cells of each cell line are subjected to TLC for Orcinol staining and immunostaining, respectively. *1* LacCer; *2* Gb3; *3* Gb4; *4* Gb5; *5* sialylGb5; *6* GM2; *7* GD2

immunization reacted strongly with the upper band glycolipid (Fig. 3a). The result may indicate that ceramide structure is also involved in antigen presentation of glycolipid in rafts.

Next, we examined the correlation between the amounts of rafts injected and anti-raft antibody production. To do so, we immunized C57BL/6 mice with rafts prepared from various numbers of EL4 cells and evaluated the subsequent production of anti-EL4 raft antibodies by flow cytometry and TLC immunostaining. As shown in Fig. 4a, the amounts of anti-EL4 raft antibody increased with the amounts of EL4 rafts injected. Injection with the rafts prepared from 0.12×10^7 EL4 cells induced a slight elevation of reactivity, and the rafts prepared from 3×10^7 EL4 cells were sufficient to induce maximum reactivity. Rafts prepared from more than 0.6×10^7 EL4 cells appeared to be needed to obtain a significant level of anti-GD2 antibodies, (Fig. 4b).

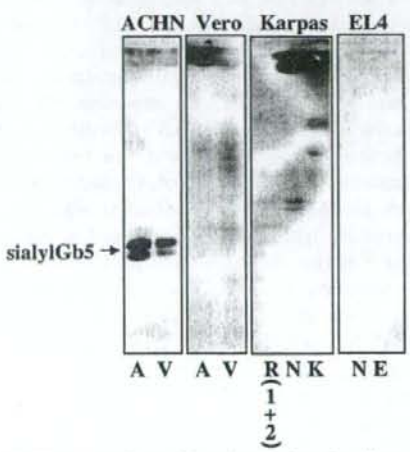

Fig. 2 TLC immunostaining with antisera against the cell suspension. The lipids were extracted from the cells as shown in the legends to Fig. 1 and from NALM-6 cells (*N*), and separated by TLC. The plates were immunostained with antisera from Balb/c mice immunized with the suspension of irradiated ACHN cells, Vero cells, Karpas cells, and EL4 cells

Since EL4 cells are derived from C57BL/6 mice, no immune responses to EL4 cells or EL4 cell components should be usually induced in syngeneic C57BL/6 mice. However, the injection of C57BL/6 mice with the EL4 rafts resulted in production of anti-raft antibodies in syngeneic mice as shown above. We therefore tried using flow cytometry to corroborate that injection of raft suspensions can induce anti-raft antibody production in syngeneic mice. The results showed that the antisera of C57BL/6 mice injected with rafts of syngeneic melanoma cell line B16F1 bound to B16F1 cells (Fig. 5a). Both mouse myeloma cell line P3U1 and lymphoma cell line RL-2 are derived from Balb/c mice, and antisera from Balb/c mice injected with rafts of these syngeneic P3U1 (Fig. 5b) and RL-2 rafts (Fig. 5c) were also confirmed to bind to P3U1 cells and RL-2 cells, respectively. Injection of mice with a PBS suspension of irradiated syngeneic cells did not result in the production of antisera that bound to syngeneic cells (data not shown).

Since repeated immunization of self- or syngeneic antigens is thought to induce autoimmune diseases, we repeated injection of C57BL/6 mice with EL4 rafts or Balb/c mice with the P3U1 rafts and investigated whether the mice produced anti-DNA antibodies by ELISA. The average A_{450} of anti-ssDNA IgM in the sera of the mice injected with PBS and the syngeneic rafts was 0.247±0.027

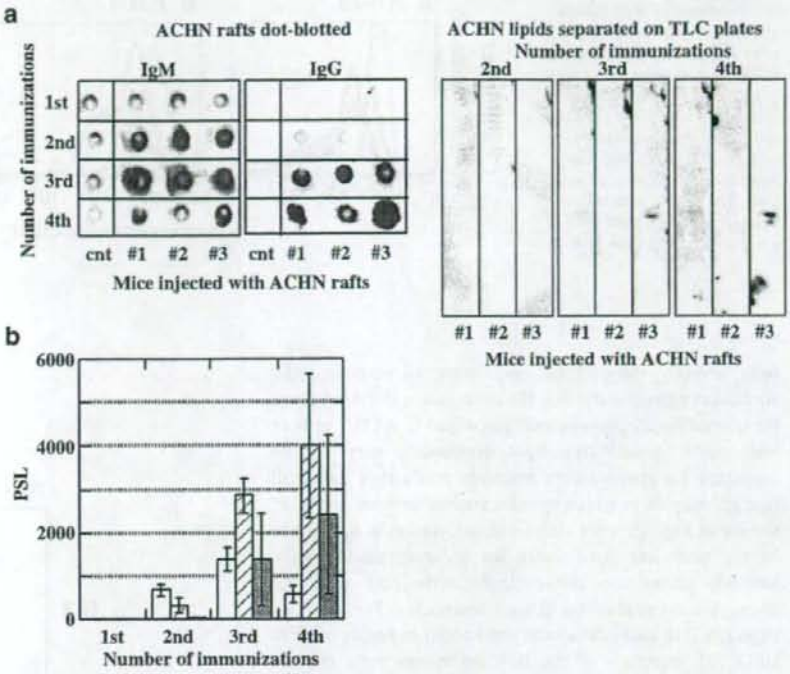

Fig. 3 Kinetics of production of antibody against ACHN rafts. Balb/c mice were injected with an ACHN raft suspension in triplicate (*#1, #2, #3*) or PBS (*cnt*) four times at 7 day intervals, and the sera were obtained 5 days after each immunization. The rafts dot-blotted on PVDF membranes were probed with each antiserum, and then probed with the HRP-conjugated anti-mouse IgM μ chain-specific antibodies or IgG γ chain-specific antibodies as secondary antibody. The lipids separated on the TLC plate were probed with each antiserum, and then with the HRP-conjugated anti-mouse IgG+M antibodies. **a** The images of dot-blot immunostaining of ACHN rafts (*left*) and TLC immunostaining of ACHN lipids (*right*) with the antisera. **b** Measurement of anti-raft IgM antibodies (*open column*), the anti-raft IgG antibodies (*striped column*), and anti-sialylGb5 antibodies (*shaded column*)

(column 1 in Fig. 6) and 0.240±0.043 (column 2 in Fig. 6), respectively, and the difference between the two groups was not significant. The A_{450} for anti-ssDNA IgM in the serum of NZB/WF1, which are well known to spontaneously develop autoimmune disease, was 0.325. No elevation of IgG class anti-DNA antibodies or anti-dsDNA antibodies was observed in the sera of either the immunized mice or NZB/WF1 mice (data not shown). No anti-DNA antibody production or other diagnostic signs of autoimmune disease were observed in these mice. These results show that the development of antibodies against syngeneic rafts components by the mice was not due to the development of an autoimmune disease.

The results of this study show that subcutaneous injection of mice with rafts prepared from specific cell lines induces production of antibodies that recognize single glycolipids, namely monoglycolipid-specific antibodies. For example, rafts prepared from ACHN cells and Vero

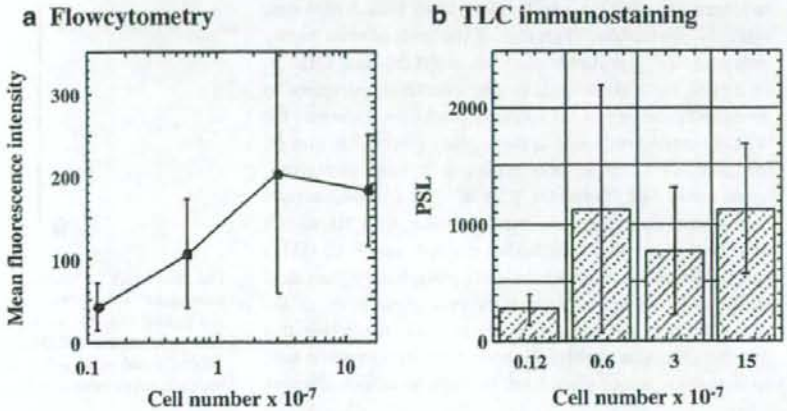

Fig. 4 Reactivity of mouse sera after immunization with the rafts prepared from various numbers of EL4 cells. The sera were obtained from C57BL/6 mice immunized with rafts prepared from 0.12, 0.6, 3 and 15×10^7 EL4 cells. The experiments were performed in triplicate. **a** Evaluation of antibody reactivity to EL4 cells by flowcytometry. **b** Evaluation of antibody reactivity to GD2 by TLC immunostaining

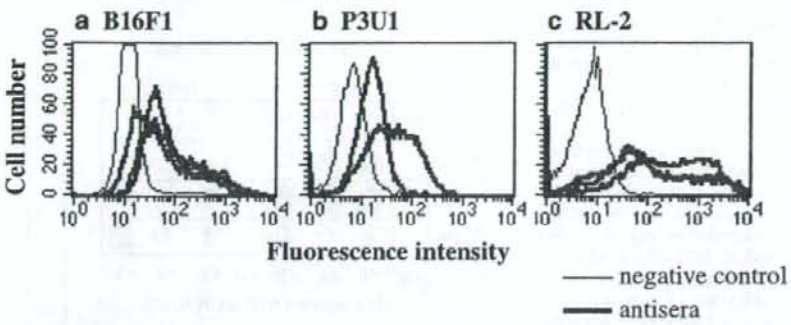

Fig. 5 Flow cytometric analysis of the antisera of mice immunized with syngeneic rafts. Cells were stained with the antisera of C57BL/6 mice immunized with B16F1 rafts (**a**), Balb/c mice immunized with P3U1 rafts (**b**) and Balb/c mice immunized with RL-2 rafts (**c**), and analyzed by flow cytometry (*bold line*). The sera of each mouse injected with PBS were used as a negative control (*thin line*)

cells strongly induced the production of mono-specific antibodies against sialyGb5. However, since sialyGb5 is not the quantitatively predominant glycolipid in ACHN cells or Vero cells, quantitative lipid dominance may not be necessary for monospecific antibody production. Since all four glycolipids to which specific antibodies were produced shown in Fig. 1b were sialylated, sialylation is thought to be the most important factor for inducing monospecific antibody production. However, the rafts from other cell lines gave no production of such antibodies. For example, although B16 melanoma cells are known to highly express GM3 [8], injection of the B16 melanoma rafts did not induce monoglycolipid-specfic antibody. Since Kawashima *et al.* [9] reported that when they intravenously injected ten strains of inbred mice with 100 μg of gangliosides adsorbed to *Salmonella minnesota*, gangliosides such as GD3, GD2, GD1b, GT1a, and GQ1b that have a trisaccharide sequence of NeuAcα2,8NeuAcα2,3Gal induced high-titer antibody responses, whereas gangliosides such as GM4, GM3, GM2, GM1, GD1a, and GT1b that have a disaccharide sequence of NeuAcα2,3Gal induced low-titer antibody responses, the diversity of immunogenicity among the glycolipids should be present. Since SSEA-4, an epitope carried by sialyGb5 has been well known highly immunogenic, a saccharide sequence of sailylGb5 can be thought to induce high-titer antibody production. Therefore, if the cells contain highly immunogenic glycolipids such as sialyGb5 and GD2 in lipid rafts, these glycolipids may be effectively presented as immunological targets for antibody production, whereas the rafts containing only low immunogenic glycolipids may be insufficient for antigen presentation to produce anti-glycolipids antibodies. Yamazaki Y. *et al.* [10] obtained several monoclonal antibodies by injecting mice with HL60 cell lipid rafts. One of the antibodies reacted with both GM1a and GD1b, and another reacted with phosphatidylglucoside. HL60 cells, however, mainly express glycolipids of the neolactoseries, not the ganglioseries [11], suggesting that raft immunization enables antibody production against such an extremely minor glycolipid. In order to induce effective

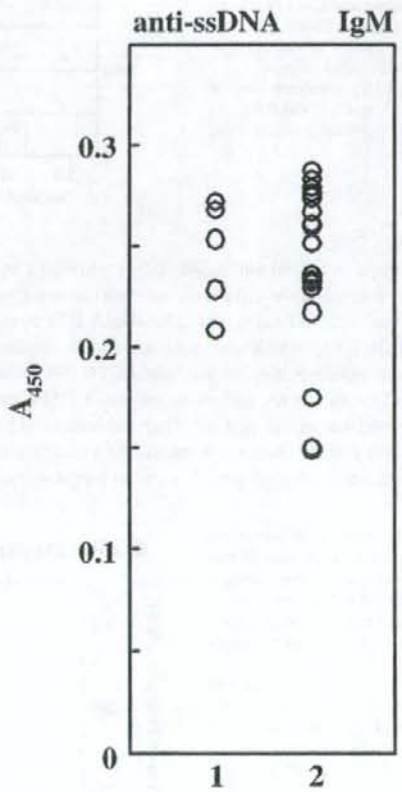

Fig. 6 ELISA of anti-ssDNA antibodies in the sera of mice immunized with syngeneic rafts. Calf thymus ss-DNA was coated and probed with the serum of C57BL/6 or Balb/c mice injected with PBS (*column 1*) and C57BL/6 mice injected with EL4 rafts or Balb/c mice injected with P3U1 rafts (*column 2*). The mean values of triplicate experiments are shown

immune responses against glycolipids in mice, a large amount of purified antigen usually must be immobilized by adsorbing it to the cell walls of bacteria, such as *Salmonella minnesota*, or by incorporating it into liposomes [12], whereas rafts themselves are insoluble and do not need to be immobilized. Furthermore, without mixing with Freund's adjuvant, rafts may retain adjuvant effects and be capable of inducing an immune response even in syngeneic mice.

It still remains unclear how monoglycolipid-specific antibodies are produced, which cells should be used for raft preparation, and to which glycolipids antibodies are predominantly produced. Although further experiments are certainly needed to answer these questions, raft immunization can be used as an effective method of producing monoclonal antibodies against glycolipids and can be applied as new approach in many fields.

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Menstrual Blood-derived Cells Confer Human Dystrophin Expression in the Murine Model of Duchenne Muscular Dystrophy via Cell Fusion and Myogenic Transdifferentiation[D]

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Duchenne muscular dystrophy (DMD), the most common lethal genetic disorder in children, is an X-linked recessive muscle disease characterized by the absence of dystrophin at the sarcolemma of muscle fibers. We examined a putative endometrial progenitor obtained from endometrial tissue samples to determine whether these cells repair muscular degeneration in a murine mdx model of DMD. Implanted cells conferred human dystrophin in degenerated muscle of immunodeficient mdx mice. We then examined menstrual blood–derived cells to determine whether primarily cultured nontransformed cells also repair dystrophied muscle. In vivo transfer of menstrual blood–derived cells into dystrophic muscles of immunodeficient mdx mice restored sarcolemmal expression of dystrophin. Labeling of implanted cells with enhanced green fluorescent protein and differential staining of human and murine nuclei suggest that human dystrophin expression is due to cell fusion between host myocytes and implanted cells. In vitro analysis revealed that endometrial progenitor cells and menstrual blood–derived cells can efficiently transdifferentiate into myoblasts/myocytes, fuse to C2C12 murine myoblasts by in vitro coculturing, and start to express dystrophin after fusion. These results demonstrate that the endometrial progenitor cells and menstrual blood–derived cells can transfer dystrophin into dystrophied myocytes through cell fusion and transdifferentiation in vitro and in vivo.

INTRODUCTION

Skeletal muscle consists predominantly of syncytial fibers with peripheral, postmitotic myonuclei, and its intrinsic repair potential in adulthood relies on the persistence of a resident reserve population of undifferentiated mononuclear cells, termed "satellite cells." In mature skeletal muscle, most satellite cells are quiescent and are activated in response to environmental cues, such as injury, to mediate postnatal muscle regeneration. After division, satellite cell progeny, termed myoblasts, undergo terminal differentiation and become incorporated into muscle fibers (Bischoff, 1994). Myogenesis is regulated by a family of myogenic transcription factors including MyoD, Myf5, myogenin, and MRF4 (Sabourin and Rudnicki, 2000). During embryonic development, MyoD and Myf5 are involved in the establishment of the skeletal muscle lineage (Rudnicki *et al.*, 1993), whereas myogenin is required for terminal differentiation (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). During muscle repair, satellite cells recapitulate the expression program of the myogenic genes manifested during embryonic development.

Dystrophin is associated with a large oligomeric complex of glycoproteins that provide linkage to the extracellular membrane (Ervasti and Campbell, 1991). In Duchenne muscular dystrophy (DMD), the absence of dystrophin results in destabilization of the extracellular membrane-sarcolemma-cytoskeleton architecture, making muscle fibers susceptible to contraction-associated mechanical stress and degeneration. In the first phase of the disease, new muscle fibers are formed by satellite cells. After depletion of the satellite cell pool in childhood, skeletal muscles degenerate progressively and irreversibly and are replaced by fibrotic tissue (Cossu and Mavilio, 2000). Like DMD patients, the mdx mouse lacks dystrophin in skeletal muscle fibers (Hoffman *et al.*, 1987; Sicinski *et al.*, 1989). However, the mdx mouse develops only a mild dystrophic phenotype, probably because muscle regeneration by satellite cells is efficient for most of the animal's life span (Cossu and Mavilio, 2000).

Myoblasts represent the natural first choice in cellular therapeutics for skeletal muscle because of their intrinsic myogenic commitment (Grounds *et al.*, 2002). However, myoblasts recovered from muscular biopsies are poorly expandable in vitro and rapidly undergo senescence (Cossu and Mavilio, 2000). An alternative source of muscle progenitor cells is therefore desirable. Cells with a myogenic potential are present in many tissues, and these cells readily

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form skeletal muscle in culture (Gerhart *et al.*, 2001). We report here that human dystrophin expression in the mdx model of DMD is attributed to cell fusion of mdx myocytes with human menstrual blood–derived stromal cells.

MATERIALS AND METHODS

Isolation of Human Endometrial Cells from Menstrual Blood

Menstrual blood samples (n = 21) were collected in DMEM with antibiotics (final concentrations: 100 U/ml penicillin/streptomycin) and 2% fetal bovine serum (FBS), and processed within 24 h. Ethical approval for tissue collection was granted by the Institutional Review Board of the National Research Institute for Child Health and Development, Japan. The centrifuged pellets containing endometrium-derived cells were resuspended in high-glucose DMEM medium (10% FBS, penicillin/streptomycin), maintained at 37°C in a humidified atmosphere containing 5% CO_2, and allowed to attach for 48 h. Nonadherent cells were removed by changing the medium. When the culture reached subconfluence, the cells were harvested with 0.25% trypsin and 1 mM EDTA and plated to new dishes. After 2–3 passages, the attached endometrial stromal cells were devoid of blood cells. Human EM-E6/E7/hTERT-2 cells, endometrium-derived progenitors, were obtained from surgical endometrial tissue samples and were immortalized by E6, E7, and hTERT (Kyo *et al.*, 2003). C2C12 myoblast cells were supplied by RIKEN Cell Bank (The Institute of Physical and Chemical Research, Japan).

Flow Cytometric Analysis

Flow cytometric analysis was performed as previously described (Terai *et al.*, 2005). Cells were incubated with primary antibodies or isotype-matched control antibodies, followed by additional treatment with the immunofluorescent secondary antibodies. Cells were analyzed on an EPICS ALTRA analyzer (Beckman Coulter, Fullerton, CA). Antibodies against human CD13, CD14, CD29, CD31, CD34, CD44, CD45, CD50, CD54, CD55, CD59, CD73, CD90, CD105, CD117 (c-kit), CD133, HLA-ABC, and HLA-DR were purchased from Beckman Coulter, Immunotech (Marseille, France), Cytotech (Hellebaek, Denmark), and BD Biosciences PharMingen (San Diego, CA).

In Vitro Lentivirus-mediated Gene (EGFP) Transfer into EM-E6/E7/hTERT-2 Cells

Infection of EM-E6/E7/hTERT-2 cells with lentivirus having a CMV promoter and enhanced green fluorescent protein (EGFP) reporter resulted in high levels of EGFP expression in all cells. Cells were analyzed for EGFP expression by flow cytometry (Miyoshi *et al.*, 1997, 1998).

In Vitro Myogenesis

Menstrual blood–derived cells or EM-E6/E7/hTERT-2 cells were seeded onto collagen I–coated cell culture dishes (Biocoat, BD Biosciences, Bedford, MA) at a density of 1×10^4/ml in growth medium (DMEM, supplemented with 20% FBS). Forty-eight hours after seeding onto collagen I–coated dishes, cells were treated with 5-azacytidine for 24 h. Cell cultures were then washed twice with PBS and maintained in differentiation medium (DMEM, supplemented with either 2% horse serum (HS) or 1% insulin-transferrin-selenium supplement [ITS]). The differentiation medium was changed twice a week until the experiment was terminated.

RT-PCR Analysis of EM-E6/E7/hTERT-2 Cells and Menstrual Blood–derived Cells

Total RNA was prepared using Isogen (Nippon Gene, Tokyo, Japan). Human skeletal muscle RNA was purchased from TOYOBO (Osaka, Japan). RT-PCR of Myf5, MyoD, desmin, myogenin, myosin heavy chain-IIx/d (MyHC-IIx/d), and dystrophin was performed with 2 μg of total RNA. RNA for RT-PCR was converted to cDNA with a first-stand cDNA synthesis kit (Amersham Pharmacia Biotechnology, Piscataway, NJ) according to the manufacturer's recommendations. The sequences of PCR primers that amplify human but not mouse genes are listed in Supplementary Table 1. PCR was performed with TaKaRa recombinant Taq (Takara Shuzo, Kyoto, Japan) for 30 cycles, with each cycle consisting of 94°C for 30 s, 62°C or 65°C for 30 s, and 72°C for 20 s, with an additional 10-min incubation at 72°C after completion of the last cycle.

Immunohistochemical and Immunocytochemical Analysis

Immunohistochemical analysis was performed as previously described (Mori *et al.*, 2005). Briefly, the sections were incubated for 1 h at room temperature with mouse mAb against vimentin (Cone V9, DakoCytomation, Fort Collins, CO). After washing in PBS, sections were incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin, diluted, and washed in cold PBS. Staining was developed by using a solution containing diaminobenzidine and 0.01% H_2O_2 in 0.05 M Tris-HCl buffer, pH 6.7. Slides were counterstained with hematoxylin. In the cases of fluorescence, frozen sections fixed with 4% PFA were used. The antibodies against human dystrophin (NCL-DYS3; Novocastra, Newcastle upon Tyne, United Kingdom) or anti-human nuclei mouse mAb (clone 235-1, Chemicon, Temecula, CA) was used as a first antibody, and goat anti-mouse IgG conjugated with Alexa Fluor 488 or goat anti-mouse IgG antibody conjugated with Alexa Fluor 546 (Molecular Probes, Eugene, OR) was used as a second antibody.

Immunocytochemical analysis was performed as previously described (Mori *et al.*, 2005), with antibodies to skeletal myosin (Sigma, St. Louis, MO; product no. M 4276), MF20 (which reacts with all sarcomere myosin in striated muscles, Developmental Studies Hybridoma Bank, University of Iowa, IA), α-sarcomeric actin (Sigma, product no. A 7811), and desmin (Bio-Science Products, Emmenbruecke, Switzerland; no. 010031, clone: D9) in PBS containing 1% bovine serum albumin. As a methodological control, the primary antibody was omitted. In the cases of fluorescence, slides were incubated with Alexa Fluor 546–conjugated goat anti-mouse IgG antibody.

Western Blotting

Western blot analysis was performed as previously described (Mori *et al.*, 2005). Blots were incubated with primary antibodies (desmin, myogenin [Clone F5D, Santa Cruz Biotechnology], and dystrophin [NCL-DYSA, Novocastra]) for 1–2 h at room temperature. After washing three times in the blocking buffer, blots were incubated for 30 min with a horseradish peroxidase–conjugated secondary antibody (0.04 μg/ml) directed against the primary antibody. The blots were developed with enhanced chemiluminescence substrate according to the manufacturer's instructions.

Fusion Assay

EM-E6/E7/hTERT-2 cells ($2500/cm^2$) or EGFP-labeled EM-E6/E7/hTERT-2 cells ($2500/cm^2$) were cocultured with C2C12 myoblasts ($2500/cm^2$) for 2 d in DMEM supplemented with 10% FBS and then cultured for 7 additional days in DMEM with 2% HS to promote myotube formation. The cultures were fixed in 4% paraformaldehyde and stained with a mouse anti-human nuclei IgG1 mAb and the mouse anti-human dystrophin IgG2a mAb (or anti-myosin heavy chain IgG2b mAb MF-20). The cells were visualized with appropriate Alexa-fluor–conjugated goat anti-mouse IgG1 and IgG2a (or IgG2b) secondary antibodies (Molecular Probes). Total cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole).

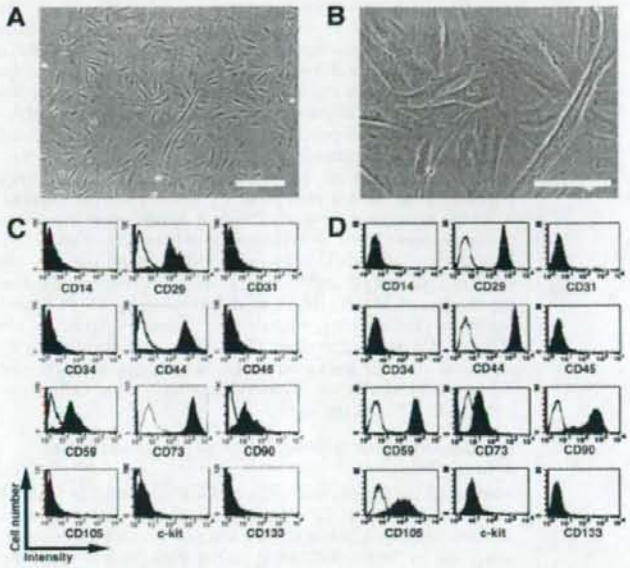
In Vivo Cell Implantation

Six- to 8-wk-old NOD/Shi-scid/IL-2 receptor −/− (NOG, CREA, Shizuoka, Japan) mice and 6- to 8-wk-old mdx-acid mice were implanted with EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells in seven independent experiments. The cells (2×10^5) were suspended in PBS in a total volume of 100 μl and were directly injected into the right thigh muscle of NOG mice or mdx-scid mice. The mice were examined 3 wk after cell implantation, and the right thigh muscle was analyzed for human vimentin and dystrophin by immunohistochemistry. The antibodies to vimentin and dystrophin (NCL-DYS3) react with human vimentin and dystrophin-equivalent protein, but not murine protein.

RESULTS

Surface Marker Expression of Endometrium-derived Cells

We investigated myogenic differentiation of primary cells without gene introduction from menstrual blood, because menstrual blood on the first day of the period is considered to include endometrial tissue. We successfully cultured a large number of primary cells from menstrual blood. Menstrual blood–derived cells showed at least two morphologically different cell groups: small spindle-like cells and large stick-like cells, regarded as being passage day (PD) 1 or 2 (Figure 1, A and B, respectively). Surface markers of the menstrual blood–derived cells were evaluated by flow cytometric analysis. Surface markers of EM-E6/E7/hTERT-2 cells (Figure 1C) and menstrual blood–derived cells (Figure 1D) were evaluated by flow cytometric analysis (Figure 1E). In these experiments, the cells were cultured in the absence of any inductive stimuli. EM-E6/E7/hTERT-2 cells were positive for CD13, CD29 (integrin β1), CD44 (Pgp-1/ly24), CD54, CD55, CD59, CD73, and CD90 (Thy-1), implying that EM-E6/E7/hTERT-2 cells expressed mesenchymal cell–related antigens in our experimental setting. Menstrual blood–derived cells were positive for CD13, CD29, CD44, CD54, CD55, CD59, CD73, CD90, and CD105, implying that prolif-



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Surface marker	EM-E6/E7/ hTERT-2 cells	Menstrual blood cells	Surface marker	EM-E6/E7/ hTERT-2 cells	Menstrual blood cells
CD13	+	++	CD55	++	++
CD14	-	-	CD59	++	+++
CD29	++	+++	CD73	+++	+
CD31	-	-	CD90	++	+++
CD34	-	-	CD105	-	++
CD44	+++	+++	c-kit	-	-
CD45	-	-	CD133	-	-
CD50	-	-	HLA-ABC	++	++
CD54	+	++	HLA-DR	-	-

Figure 1. Surface marker expression of endometrium-derived cells. (A and B) Morphology of menstrual blood–derived cells, regarded as being PD 1 or 2. Scale bars, 200 μm (A), 100 μm (B). (C and D) Flow cytometric analysis of cell surface markers of EM-E6/E7/hTERT-2 cells (C) and menstrual blood–derived cells (D). (E) Further phenotypic analysis in EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells are summarized. Peak intensity was estimated in comparison with isotype controls. +++, strongly positive (>100 times the isotype control); ++, moderately positive (<100 times but more than 10 times the isotype control); +, weakly positive (<10 times but more than twice the isotype control); −, negative (less than twice the isotype control).

erated and propagated cells express mesenchymal cell-related cell surface markers. Unlike EM-E6/E7/hTERT-2 cells, the menstrual blood–derived adherent cells were positive for CD105. EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells expressed neither hematopoietic lineage markers, such as CD34, nor monocyte-macrophage antigens such as CD14 (a marker for macrophage and dendritic cells), or CD45 (leukocyte common antigen). The lack of expression of CD14, CD34, or CD45 suggests that EM-E6/E7/hTERT-2 cells and the menstrual blood–derived cell culture in the present study is depleted of hematopoietic cells. The cells were also negative for expression of CD31 (PECAM-1), CD50, c-kit, and CD133. The cell population was positive for HLA-ABC, but not for HLA-DR. These results demonstrate that almost all cells derived from endometrium are of mesenchymal origin or stromal origin.

Implanted Endometrium-derived Cells Induce De Novo Myogenesis in Immunodeficient NOG Mice

EM-E6/E7/hTERT-2 cells originate from the endometrial gland and are considered as endometrial progenitor cells or bipotential cells capable of differentiating into both glandular epithelial cells and endometrial stromal cells (Kyo *et al.*, 2003). To determine whether EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells generate complete endometrial structure in vivo, like endometriosis, the cells without any treatment or induction were injected into the right thigh muscle of immunodeficient NOG mice. PBS without cells was injected into the left thigh muscles as a control. We failed to detect any endometrial structure in the cell-injected site. Immunohistochemical analysis using an antibody specific to human vimentin, an intermediate filament associated with a mesenchymal cell, revealed that the injected EM-E6/E7/hTERT-2 cells (Figure 2, A–F) or menstrual blood–derived cells (Figure 2, G–J) extensively migrated or infiltrated between muscular fibers (Figure 2, arrowheads). To investigate if the donor cells between muscular fibers occur as a result of cell migration, we performed a time-course analysis of implanted cells, as probed by human-specific antibody to vimentin (Supplementary Figure 1). Donor cells at 3 h after implantation are observed at the injection site, which is considered to be due to just injection of cells. Cells at 1–3 wk after implantation are detected between myocytes in the muscle bundle or muscular fascicle as well as in the interstitial tissue, implying that the donor cells between myotubes result from cell migration. Interestingly, some of the vimentin-positive implanted cells exhibited round-shaped structure (Figure 2, D, F, and J, arrows), suggesting that endometrium-derived cells are capable of differentiating into myoblasts/myotubes, and can contribute to skeletal muscle repair in patients suffering from genetic disorders such as DMD, similar to previous reports for marrow stromal cells (Dezawa *et al.*, 2005) and synovial membrane cells (De Bari *et al.*, 2003).