**Preferential localization of SSEA-4 in interfaces between blastomeres of mouse
preimplantation 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.

Key words: sialylGb5; SSEA-4; embryonic stem cells; embryonal carcinoma cells; preimplantation embryo; immunostaining

1. Introduction

Embryonal carcinoma (EC) cells isolated from teratocarcinomas have been shown to possess pluri- or multi-potency 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 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 ¹²⁵I-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.

2. Materials and methods

2.1. 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) (Gibco, Grand Island, NY) supplemented with 10% FBS, non-essential amino acid solution (NEAA) (Gibco), and Insulin-Transferin-Sodium Selenite media (Gibco). The cynomolgus monkey ES cell line MCK-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 mitomycin C in DMEM/F12 (Gibco) supplemented with 20 % Knock-out serum replacement (Invitrogen), penicillin/streptomycin (GIBCO), Glutamax (GIBCO), NEAA (GIBCO), sodium pyruvate (GIBCO), 2-mercaptoethanol (GIBCO), SR (GIBCO), hSPS (GIBCO), and bFGF

(GIBCO). The cultures were performed at 37 °C in a 5% CO₂ incubator. The human venous blood from a healthy consenting volunteer was drawn in a heparin-coated syringe. The blood was spun at 3,000 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 IgG₃, 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 was purchased from Molecular probes.

BDF₁ mouse were purchased from Clea Japan (Tokyo, Japan).

2.2. 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 GM1b was kindly provided by Dr. Nakamura of RIKEN, Saitama, Japan [10].

2.3. 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).

2.4. 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.

2.5. Indirect immunostaining of cynomolgus monkey ES cells

Cells were grown on a glass bottomed dish (IWAKI) for 3days and then these cells were

fixed for 30min with 4% paraformaldehyde in PBS and permeabilized with 0.2 % TritonX-200 in PBS for 20min. Subsequently, the cells were washed three times with PBS for 5min and blocked with 5% normal goat serum in PBS for 30min. The fixed cells were incubated with anti-SSEA-4 antibodies or isotype-matched mouse IgG at a dilution of 1:300 for 2h, followed by incubation with Alexa Fluor® 488-conjugated goat anti-mouse IgG at a dilution of 1:300 for 30min. DAPI was used for counter staining of nuclei.

2.6. Immunostaining of mouse preimplantation embryos

Mouse preimplantation embryos were collected from superovulated mice. Seven-week-old BDF1 female mouse 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 μ l drops of M16 medium containing 0.45 μ g 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 μ l drops of M16 medium. All staining steps were carried out at 37 °C in a CO₂ 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 x objective lens.

3. Results and Discussion

3.1. 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 GM1b (Fig. 1C). These results indicate that 6E2 specifically binds to sialylGb5 and thus is an anti-SSEA-4 Mab. The 80 kDa protein might be associated with sialylGb5 in NCR-G3 cells and thus co-immunoprecipitated by 6E2.

3.2. 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 IgG₃, 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-sialylGb5 Mabs do not [15]. However, our data indicate that 6E2 is more reactive than MC813-70. Next we compared the reactivity of the two 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.

3.3. 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.

3.4. 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 sialylGb5 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.

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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6. Legends

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-sulfuric 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 gangliosides nomenclature of Svennerholm [12] was used.

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)

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 μ m.

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 μ m.

Fig. 1

Fig.2

Fig. 3

Fig. 4