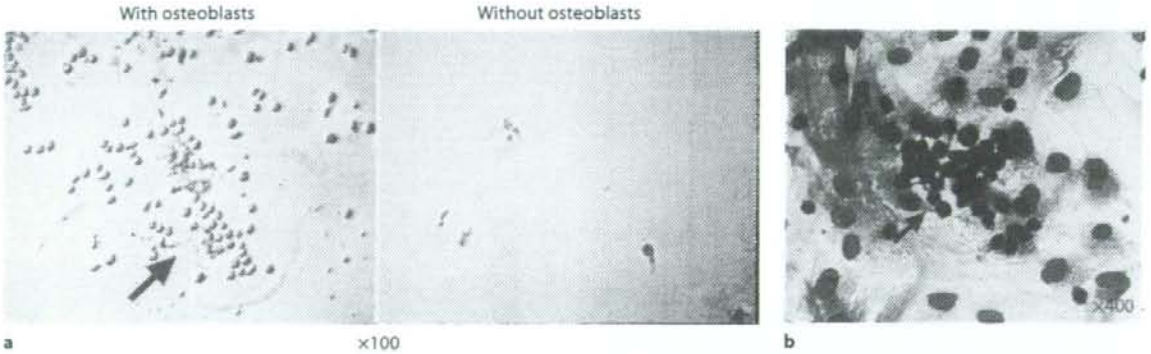


Fig. 2. Human CD34+ bone marrow cells grown on osteoblast primary cultures. **a** Human CD34+ bone marrow cells were cultured for 2 weeks on osteoblast primary cultures grown on coverslips and examined by phase-contrast microscopy (magnification ×100). **b** After May-Grünwald-Giemsa staining, cells were examined by light microscopy. The small nuclear cells gathered at the center are human CD34+ bone marrow cells (arrow), and the large nuclear cells present at the circumference are human osteoblasts (magnification ×400).

fect on the growth and differentiation of hematopoietic cells cultured on osteoblasts was observed (data not shown).

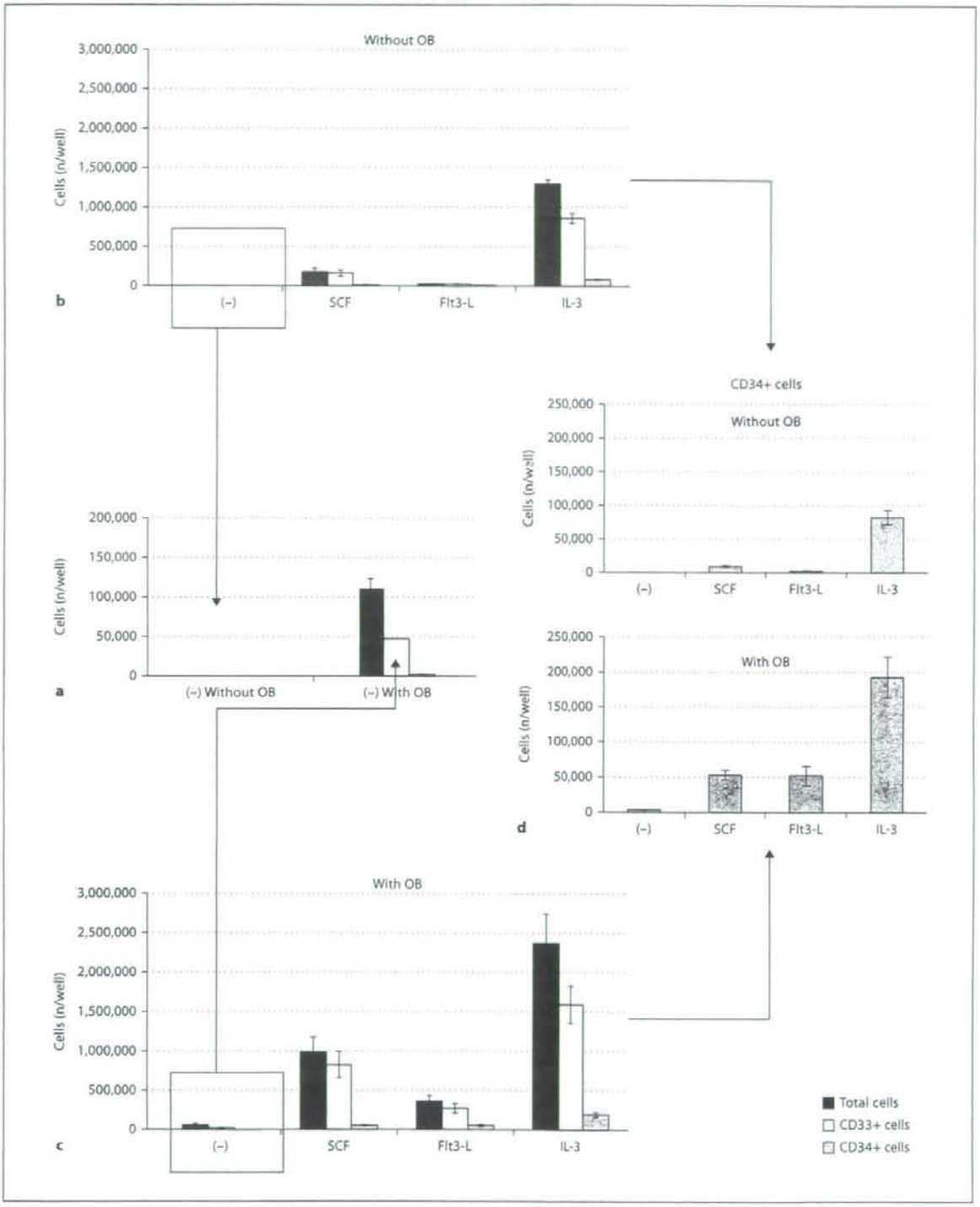
It was suggested that hematopoietic cells adhere to bone marrow stromal cells via binding between β_1- and α_4-integrin, a complex of CD29 and CD49d, expressed on hematopoietic cells and CD106 on stromal cells [8]. Consequently, the intracellular signaling required for cell survival and maintenance was initiated in hematopoietic cells [9]. As shown in figure 4, immunohistostaining experiments revealed that hematopoietic cells adhered to osteoblasts as they got twisted to the CD106 that filamentously expressed on osteoblasts. Immunohistostaining also showed that the CD166 staining pattern was similar to that of fibronectin and that some of the hematopoietic cells expressed CD166, and those cells looked like those adhering to osteoblasts that also express CD166. Both the hematopoietic cells and osteoblasts also expressed other signaling molecules, including paxillin, β-catenin, and vinculin (fig. 4). Paxillin expression in the hematopoietic cells was much greater than in osteoblasts (fig. 4).

We also investigated the phosphorylation of signaling molecules in hematopoietic cells using activation-specific antibodies. As shown in figure 5, confocal microscopic analysis showed phosphorylation of FAK and AKT, and there was clear colocalization of phosphorylated FAK and AKT with CD49d and CD29, respectively (fig. 5, bottom panels).

Thus, we tested the effect of cell adhesion mediated by CD29 and CD49d on the maintenance of hematopoietic cells on osteoblasts. When the anti-CD29 antibody that can interfere in the binding between CD29 and CD106 was added to the coculture of CD34+ bone marrow cells and osteoblasts, subsequent hematopoietic cell proliferation was clearly reduced (fig. 6). Identical results were observed for the anti-CD49d antibody (fig. 6).

Since CD184 (CXCR4) has been suggested to contribute to the homing of hematopoietic cells, we investigated the effect of osteoblasts on CD184 expression by hematopoietic cells. As shown in figure 7a, human CD34+ cells purified from bone marrow did not express CD184. When cultured on osteoblasts, however, expression of CD184 was significantly increased and the hematopoietic cells were divided into several fractions based on the diverse patterns of CD184 and CD33 co-expression (fig. 7).

In the absence of osteoblasts, on the other hand, each cytokine also induced CD184 expression by hematopoietic cells in a distinct manner (fig. 7). When CD34+ bone marrow cells were cultured in the presence of osteoblasts, addition of cytokines further enhanced CD184 expression (fig. 7). We also examined the effect of SDF-1, a ligand for CD184, and anti-SDF-1 antibody on the proliferation and differentiation of CD34+ bone marrow cells cultured on osteoblasts in the presence of each cytokine, but no significant effect was observed (data not shown).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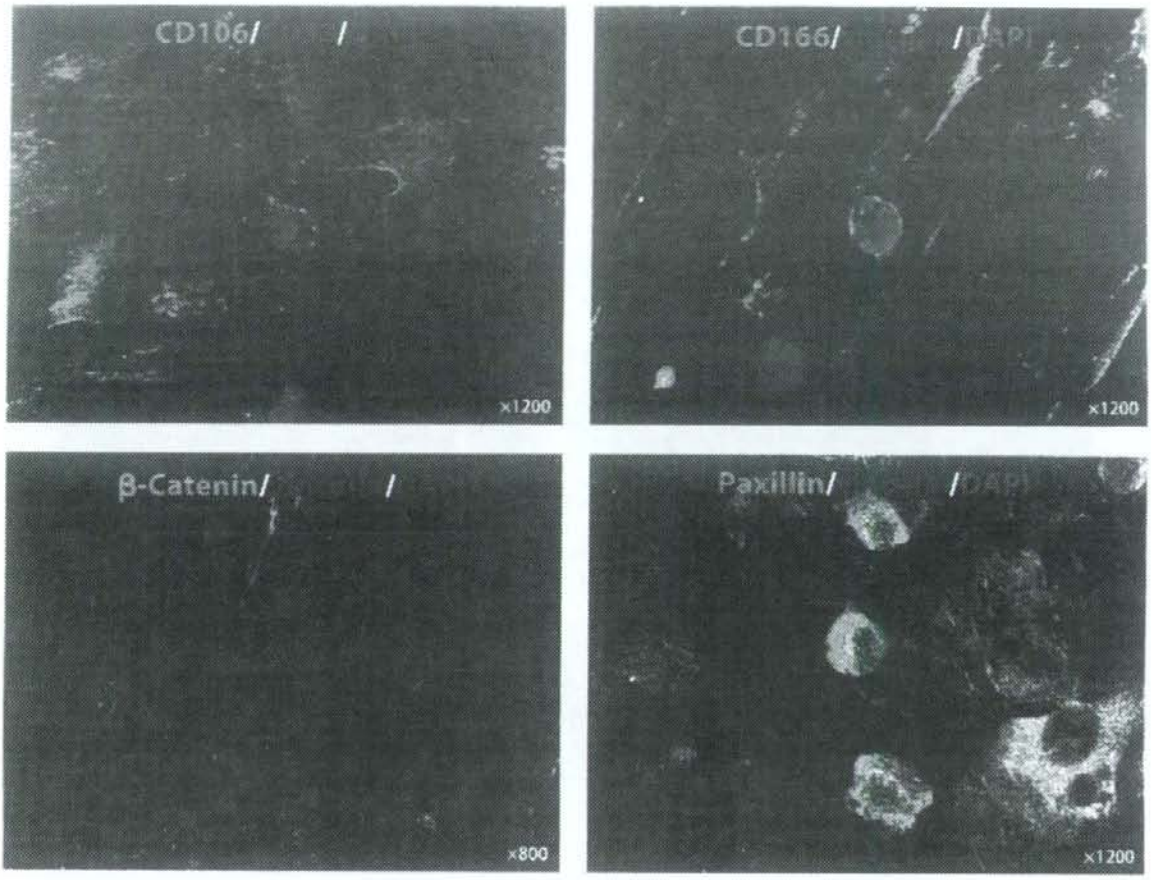
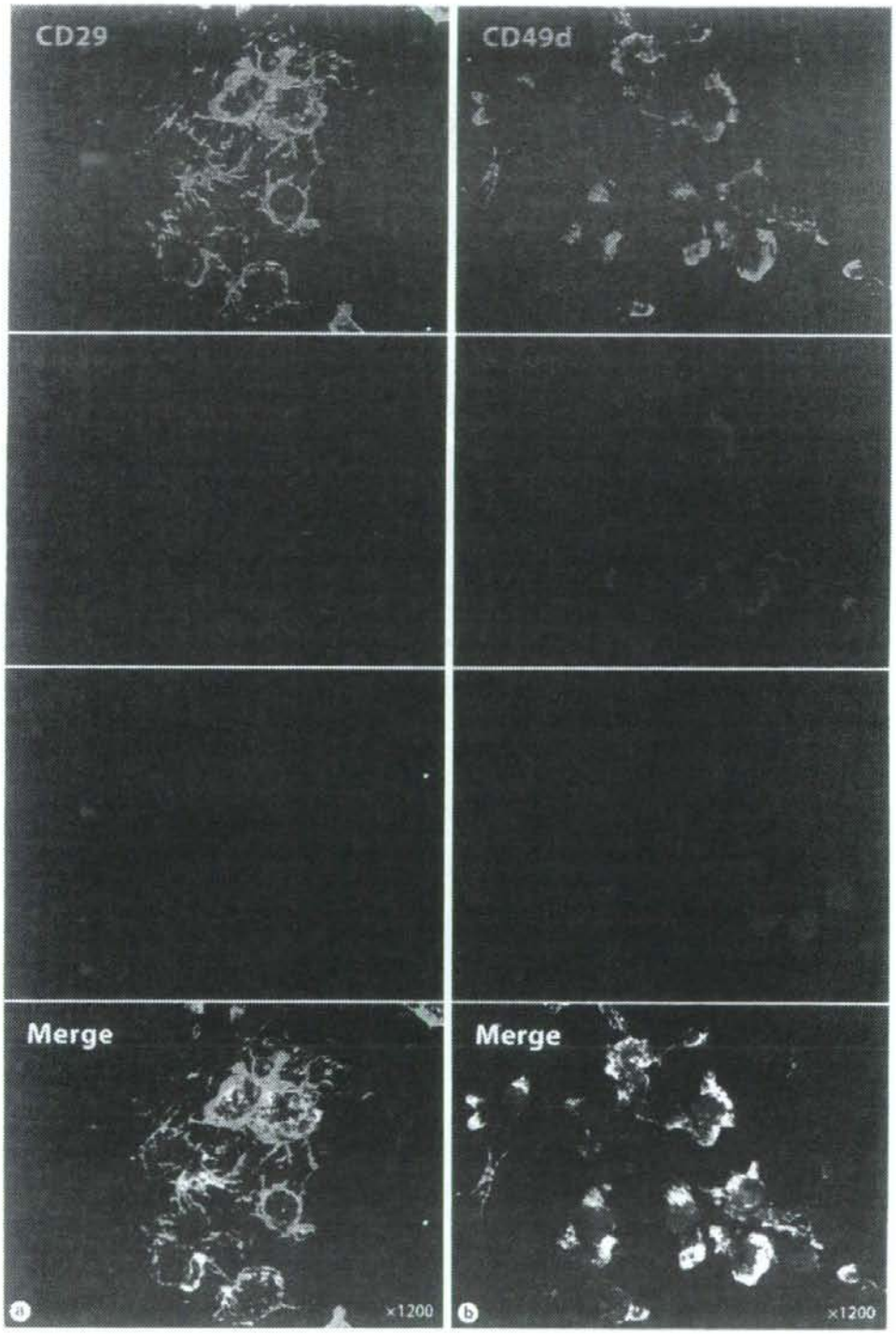


Fig. 4. Immunohistochemical analysis of the interaction between osteoblasts and human CD34+ bone marrow cells. Human CD34+ bone marrow cells were grown on a primary culture of osteoblasts for 2 weeks and examined as in figure 1b.

Fig. 3. Effect of human osteoblasts and cytokines on the growth and differentiation of human CD34+ bone marrow cells. Human CD34+ bone marrow cells were cultured with or without human osteoblasts (OB) for 2 weeks in the presence or absence of the cytokines indicated. After cultivation, the ensuing hematopoietic cells were collected, counted, and positivity for CD33 and CD34 was determined by flow cytometry. The actual total cell numbers and the numbers of cells in each subpopulation are represented by bar graphs. **a** With or without OB (in the absence of cytokines); **b** without OB (in the presence of cytokines); **c** with OB (in the presence of cytokines); **d** CD34+ cells extracted from **b** and **c**.

Discussion

As reported in this paper, when cultured on human osteoblasts, human CD34+ bone marrow cells were able to survive without the addition of any cytokine, and differentiated into myeloid cells with slight proliferation, suggesting that human osteoblasts possess the ability to support the survival and differentiation of hematopoietic cells in vitro. Analysis by confocal microscopy suggested that cell adhesion molecules, including CD29/49d, CD106, and CD166, are involved in cell-to-cell interaction between hematopoietic cells and osteoblasts. Since we observed that FAK and AKT were colocalized with CD29/



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49d and phosphorylated in hematopoietic cells adhering to osteoblasts, we suspect that cell-to-cell interaction induces activation of integrin-bound kinases, leading to cell survival signals in hematopoietic cells in which AKT is involved. Although CD34+ bone marrow cells were cultured in the presence of 30% of the cultured supernatant of osteoblasts, most cells died over a 4-week culture period (data not shown), suggesting that the soluble factors derived from osteoblasts are not sufficient to support the survival of human CD34+ bone marrow cells, and adhesion to osteoblasts must be important for the survival of hematopoietic cells.

Human osteoblasts have been reported to produce several hematopoietic cytokines, including IL-1β, IL-6, IL-7, G-CSF, M-CSF, GM-CSF, tumor necrosis factor-α, LIF, OPG, receptor activator of NF-κB ligand, SDF-1, VEGF, and osteoclast differentiation factor [1, 2, 10–12], and not to produce IL-1α, IL-3, or SCF [10]. However, in our experiment, human osteoblasts did not produce IL-7, G-CSF, M-CSF, or GM-CSF. Although the precise reason for the discrepancy is not clear, it may be attributable to differences in cell culture conditions or donor age. Alternatively, different subsets or differentiation states related to differential cytokine production may be present among the osteoblasts.

Several cytokines have been shown to contribute to the maintenance, proliferation, and differentiation of HSCs. For example, Flt3-L and SCF play an important role in the early stage of hematopoiesis [13]. An in vivo study has demonstrated that SCF and IL-3 prevent unirradiated hematopoietic progenitors from undergoing apoptosis, and Flt3-L has been demonstrated to induce survival and proliferation of CD34+CD38– cells [14], suggesting the effects of these cytokines on hematopoiesis in vivo to some extent [15], but their effects in vitro, whether alone or in combination, are still a matter of controversy [2]. The results of this study demonstrate that SCF and IL-3, but not Flt3-L, induce proliferation of CD34+ bone marrow cells to some extent in our culture condition. When added to the coculture system of human osteoblasts and human CD34+ bone marrow cells, however, each of them significantly promoted the proliferation of hematopoietic cells. SCF and Flt3-L induced in particular significant growth of hematopoietic cells cultured on osteoblasts. Since our RT-PCR experiments revealed no expression of SCF and IL-3 mRNA in osteoblasts, the major role of osteoblasts in hematopoiesis could be to maintain HSCs as HSCs and therefore the lack of proliferation-inducing cytokines is appropriate for this role. In the context of the microenvironment, other cells should supply these factors to the niche. Alternatively, it is also possible that disaggregated osteoblasts do not produce these factors when they are grown in monocultures but do so in the niche when in the appropriate context.

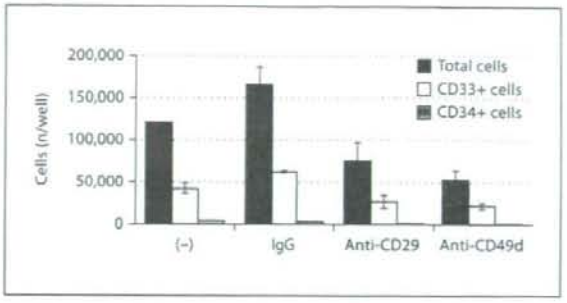
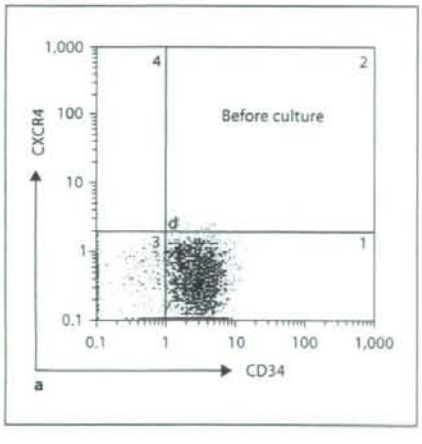


Fig. 6. Effect of anti-integrin antibodies on growth of CD34+ bone marrow cells on human osteoblasts. Human CD34+ bone marrow cells were cultured on osteoblasts for 2 weeks in the presence or absence of antibodies against CD29 or CD49d at a concentration of 5 μg/ml. Following cultivation, hematopoietic cells were collected, counted, and positivity for CD33 and CD34 was determined by flow cytometry (see fig. 3). Purified mouse IgG served as a negative control.

CD184, a receptor for CXC subfamily chemokines, was originally identified as an orphan receptor [16]. It was suggested that CD184 and its sole ligand SDF-1 play an important role in hematopoiesis and are required for homing of stem cells and progenitor cells from the liver to the bone marrow [2, 16–18], but their role at the molecular level remains unknown. Tokoyoda et al. [18] stated that contact between the earliest HSCs and SDF-1-expressing cells is necessary for B lymphopoiesis. In our study, the CD184 expression pattern was dramatically altered by cytokines and the presence of osteoblasts. Although the exact mechanism of action remains to be elucidated, the different expression pattern of CD184 may be related to the different function of hematopoietic cells,

Fig. 5. Phosphorylation of cell signaling molecules in hematopoietic cells cultured on osteoblasts detected by immunohistochemistry. Human CD34+ bone marrow cells were grown on a primary culture of osteoblasts for 2 weeks and stained with the combination of phospho-specific antibodies and anti-cell adhesion molecule antibodies and examined as in figure 4. **a** CD29 versus phosphorylated FAK; **b** CD49d versus phosphorylated AKT.



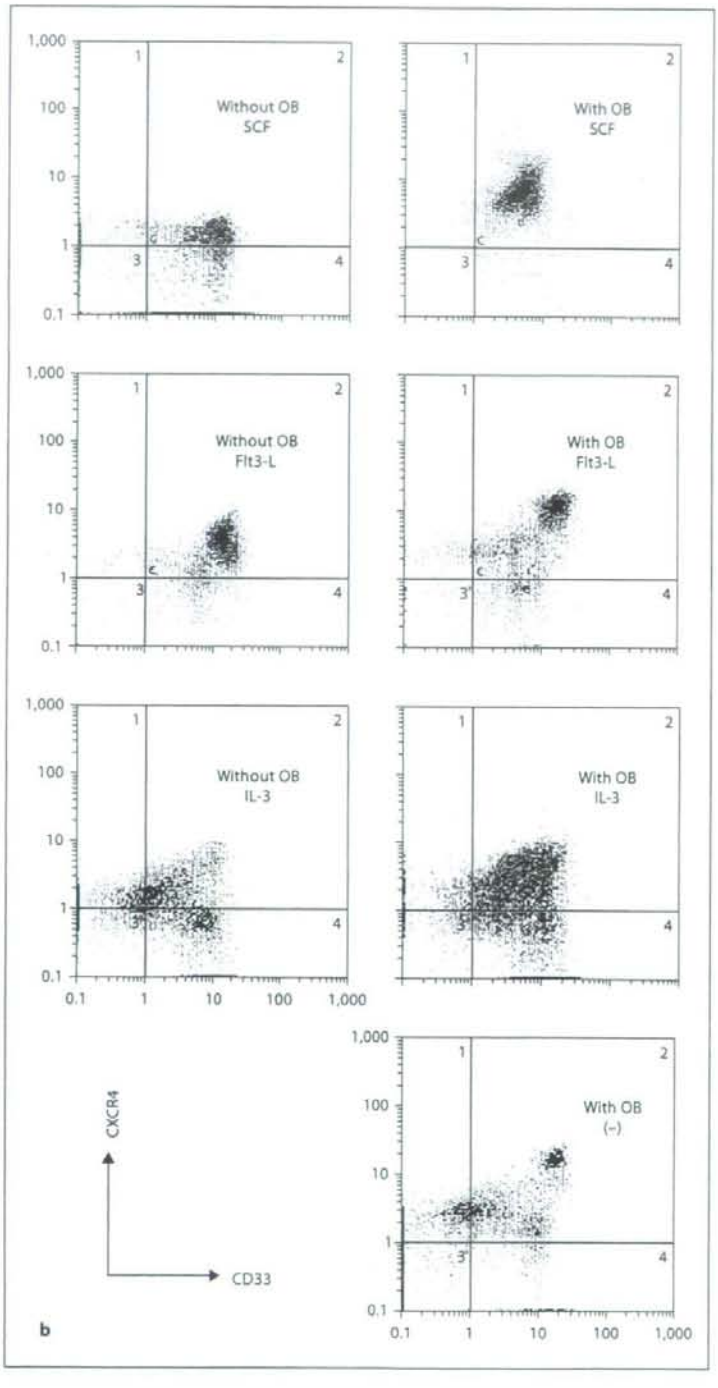


Fig. 7. Expression of CD184 in hematopoietic cells grown on human osteoblasts. Human CD34+ bone marrow cells were cultured for 2 weeks (see fig. 3). Hematopoietic cells were collected and examined by flow cytometry. Two-parameter histograms for CD184 versus CD34 (**a**) or CD33 (**b**) are shown.

e.g. homing. Further investigation to identify the role of CD184 expression in hematopoiesis is now underway.

In conclusion, human osteoblasts have the ability to support the survival and differentiation of human CD34+ bone marrow cells. Addition of cytokines to this culture system stimulates human CD34+ bone marrow cells to differentiate into various blood cells. Osteoblasts provide a useful in vitro model of the hematopoietic microenvironment, and further studies are required to elucidate the role of the microenvironment in early hematopoiesis.

Acknowledgments

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MINIREVIEW

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Functional Significance of Stage-Specific Embryonic Antigens in the Development of Preimplantation Embryos

初期胚における Stage-Specific Embryonic Antigens（SSEA 抗原）

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Key Words: SSEA, sialylGb5Cer, raft, preimplantation mouse embryo

Abstract

In preimplantation embryos, stage-specific embryonic antigen (SSEA)-1, SSEA-3, and SSEA-4 are well-known carbohydrate marker antigens that show a stage-specific expression. SSEA-1 is usually carried by glycoproteins and/or glycolipids, while SSEA-3 and SSEA-4 are mostly carried by glycosphingolipids, which are the major constituents of raft microdomains. Biochemical analysis and confocal laser-scanning microscopic study of raft microdomains have been conducted in cultured cell lines. Here, we show the dynamic movement of SSEA-4 in the raft microdomains of preimplantation mouse embryos by immunostaining with 6E2, a novel anti-SSEA-4 monoclonal antibody, and subsequently discuss the role of raft microdomains in early embryogenesis.

要　約

SSEA-1, -3, -4は胚発生に伴い発現が変化することで知られている糖鎖抗原マーカーである。SSEA-1は糖タンパク糖脂質どちらにもみられるが，SSEA-3と-4は通常はスフィンゴ糖脂質のみにみられ，ラフトマイクロドメインの構成分子となる。ラフトマイクロドメインの生化学的解析や共焦点レーザー顕微鏡による観察は，これまで主に培養細胞でなされており，哺乳動物初期胚でのラフトマイクロドメイン像は検出されていない。筆者らは，新規抗SSEA-4単クローン抗体（Mab）6E2を使って生きたままのマウス初期胚のSSEA-4の動態を解析した。SSEA-4は割球表面を自由に且つ迅速に動く一方で，一部は細胞内部へも取り込まれるなど，その動きはダイナミックである。胚表面のSSEA-4を中心とするラフトマイクロドメインの時-空間的変化は，マウス胚初期発生においても，ラフトマイクロドメインが重要な働きをしている事を示唆するものである。

A. Introduction

Simons had postulated the hypothesis that raft microdomains act as scaffolds for signaling molecules more than 10 years ago (1). Raft microdomains isolated as detergent-insoluble and low-density fractions of membranes have been implicated in signal transduction, because they have been associated with a variety of signaling molecules such as Src-family kinase, heterotrimeric G proteins, and glycosylphosphatidylinositol (GPI)-anchored proteins. We have previously shown that the binding of Shiga toxin (Stx) to Gb3Cer in raft microdomains temporally activates the Src-family kinase Yes in the human renal cancer cell line ACHN (2). In this previous study, we produced monoclonal antibodies (Mabs) against the raft microdomains of ACHN cells in order to search the molecules involved in signal transduction via the raft

A. はじめに

Simons(1)がシグナル伝搬の足場としてのラフトマイクロドメインの概念を提唱して以来，10年以上が経過した。界面活性剤不溶性で，低密度画分として得られるラフトマイクロドメインは，Src型キナーゼ，三量体型Gタンパク，GPIアンカー型タンパクなど，シグナル関連分子群が局在していて，シグナル伝達に関与している事が示されてきた。筆者等は，ヒト腎癌由来細胞株ACHN細胞のラフトマイクロドメインに局在するGb3Cerにシガ毒素が結合すると一過性にSrc型キナーゼYesが活性化される事を報告した(2)。ラフトマイクロドメインを介するシグナル伝達に関与する分子の機能解明を目的としてACHN細胞のラフトマイクロドメインに対するMabの作製にとりかかった。その過程でラフトの脂質成分に反応するクローンはすべてsialylGb5Cerを認識抗原としてい

microdomains. While producing Mabs, we observed that all the clones reacting with raft lipids recognized the SSEA-4-carrying sialylGb5Cer and that sialylGb5Cer possessed marked immunogenicity (3,4). Raft.2 (IgM, κ), one of these clones, also bound to the laminin-binding protein (LBP) of the mouse embryonal carcinoma (EC) cell line F9 on a western-blotted nitrocellulose membrane (5). The possibility that LBP carries the SSEA-4 epitope is an attractive topic for researchers studying mammalian embryogenesis, because laminin is one of the most important components of the extracellular matrix during embryogenesis and LBP is thought to act as a laminin receptor as well as an integrin in embryos. In the late 1970s, SSEAs were first mentioned by Solter and Knowles (6) as developmentally regulated cell-surface antigens that exhibit a lineage-restricted pattern of expression. In this review, we present our recently obtained data on the distribution of SSEA-4 in preimplantation mouse embryos and discuss the role of raft microdomains and the functional significance of SSEAs during the early embryogenesis.

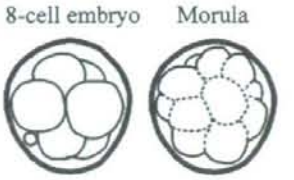
B. SSEA Expression in Preimplantation Embryos and Mouse Embryonic Stem (ES) Cells and EC Cells

Cell-surface molecules play an important role in cellular recognition and interaction, and they are thought to be involved in regulatory processes during embryogenesis. Changes in the expression of cell-surface molecules during the course of murine embryonic development have been analyzed by mainly using immunological methods. In order to induce the production of antibodies against certain subpopulations of early embryonic cells, EC cells, which resemble the pluripotent cells of early embryos, have been used as immunogens. SSEA-1, which was identified by Mab MC480 raised against F9 cells, appeared on cleavage-stage mouse embryos, and its expression was found to be restricted to the inner cell mass (ICM), embryonic ectoderm, and migrating primordial germ cells (6). SSEA-1 is intensely expressed in ES/EC cells, but it disappears from most of the cells that have differentiated from ES/EC cells in vitro. Thus, SSEA-1 has been conveniently used as a marker to identify EC cells in a differentiating cell population. The antigenic determinant of SSEA-1 is Galβ1,4(Fucα1,3)GlcNAc (7), also known as Lewis X antigen (Le^x). SSEA-1 is usually carried by neolacto-series glycolipids and high-molecular-weight glycoproteins in early embryos. SSEA-2, which was identified by an antiserum raised against the hybrids of SV40-transformed human cells and Balb/c mouse macrophages, has not been reported since the time Shevinsky et al. first documented it (8). SSEA-3 was identified by Mab MC631 raised against mouse 4- to 8-cell embryos (9), and SSEA-4

ること，また，sialylGb5Cerが突出して免疫原性が高いことがわかった(3,4)。sialylGb5CerはSSEA-4エピトープを有するグロボ系糖脂質である。これらのクローンの一つRaft.2（IgM, κ）はマウスEC細胞F9のlaminin binding protein（LBP）に結合する事がウエスタン解析でわかった(5)。ラミニンは初期発生における重要な細胞外基質の一つで，LBPはインテグリン同様，ラミニンの受容体として機能していると考えられており，LBPがSSEA-4エピトープを担っているという可能性は大変興味深い。1970年代後半Solter等(6)により提唱された分化抗原としてのSSEAは発生にともない発現様式が変化する。本総説では6E2で解析したマウス着床前胚におけるSSEA-4の分布とラフトマイクロドメイン形成に関する筆者等の最近のデータを紹介し，初期発生におけるラフトマイクロドメインとSSEAの機能と意義について述べる。

B. 着床前胚及びES/EC細胞におけるSSEAの発現

細胞表面分子は細胞認識や細胞間相互作用において重要な機能を果たしており，発生の制御機構にも深く関わっていると考えられている。マウス初期胚発生過程での細胞表面分子発現の変化は，主に免疫学的手法で解析されてきた。マウス初期胚の細胞群を認識する抗体を得るには，初期胚と類似した多分可能を有するEC細胞が免疫原として用いられた。F9細胞を免疫原として得られたMab MC480によって規定されるSSEA-1は卵割期に出現し，やがてその発現は内部細胞塊（ICM），胚性外胚葉，及び移動期の始原生殖細胞に限局される。マウスES/EC細胞はSSEA-1を高発現するが，分化誘導を受けると消失するので，SSEA-1はES/EC細胞の分化誘導の判定のマーカーとして頻繁に用いられてきた。SSEA-1の抗原決定基はGalβ1,4(Fucα1,3)GlcNAc(7)でルイス抗原X（Le^x）のエピトープとして知られている。SSEA-1は通常はネオラクト系糖脂質にみられるが，初期胚においては高分子糖タンパクにも存在する。SSEA-2(8)はSV40形質転換ヒト由来細胞とBalb/cマウスマクロファージのハイブリッドを免疫して得られた抗血清で規定される抗原であるが，以降言及されることはない。一方，マウス4～8細胞期胚を免疫原として得られたMab MC631で規定されるSSEA-3(9)と，ヒトEC細胞EC2102Epを免疫原として得られたMab MC813-70で規定されるSSEA-4(10)はマウス初期発生においてはSSEA-1に先行して出現し，ヒト胚盤胞のICM，ヒトES/ECに発現する。両抗原とも通常はグロボ系糖脂質にみられる。抗SSEA-3抗体はGb4, Gb5, sialylGb5の内部糖鎖構造R-GalNAcβ1,3



Human					
SSEA-1	-	+	-	+	-
SSEA-3	-	-	++	-	++
SSEA-4	-	-	++	-	++
Mouse					
SSEA-1	+	+	++	+	+
SSEA-3	+	+	-	-	-
SSEA-4	+	++	-	-	-

Fig. 1. Cell surface expression of SSEAs in the preimplantation embryos and ES/EC cells.

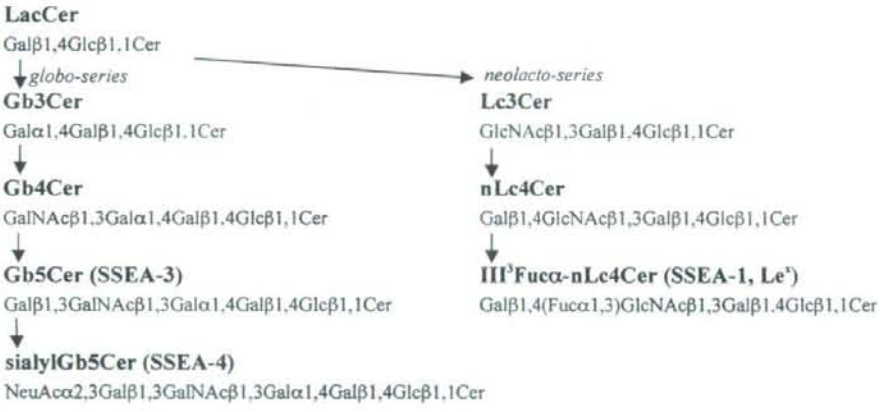


Fig. 2. The biosynthetic pathway of the carbohydrate chains of the globo- and neolacto-series glycosphingolipids carrying the SSEA epitopes. The glycosphingolipid nomenclature is according to the recommendations of IUPAC-IUB commission (21), and the ganglioside nomenclature is according to that reported by Svennerholm (22).

was identified by Mab MC813-70 raised against human EC 2102Ep cells (10). Both SSEA-3 and SSEA-4 are expressed earlier than SSEA-1 during mouse embryogenesis, namely, in early cleavage embryos, and are expressed on human EC/ES cells and the ICM of blastocysts. SSEA-3 and SSEA-4 are usually expressed on globo-series glycolipids. MC631 recognizes the internal oligosaccharide sequence of Gb4, Gb5, and sialylGb5, i.e., R-GalNAc β1,3Galα1,4-R′, whereas MC813-70 recognizes the terminal structure of sialylGb5, i.e., NeuAcα2,3Galβ1,3GalNAc (10). The SSEA expression in preimplantation mouse and human embryos and the biosynthetic pathway of the Galα1,4-R′を認識エピトープとしているのに対し，抗SSEA-4抗体はsialylGb5の還元末端3糖NeuAcα2,3Galβ1,3GalNAcを認識エピトープとしている(10)。ヒト及びマウス着床前胚とES/EC細胞におけるSSEAの発現を図1に，SSEAエピトープを担うグロボ系及びネオラクト系糖脂質の糖鎖合成経路を図2に示した。

carbohydrate chains of the globo- and neolacto-series glycosphingolipids carrying the SSEA epitopes are shown in Fig. 1 and Fig. 2, respectively.

C. Functional Significance of SSEAs

Since SSEA-1 appears at the onset of compaction and disappears at around the 32-cell stage, i.e., after the completion of the compaction process, SSEA-1 has been thought to be involved in compaction or cell-cell interaction during early embryogenesis. Indeed, a divalent SSEA-1 compound was reported to decompact mouse morulae and inhibit the formation of blastocysts (11). However, there was no conclusive evidence for the physiological significance of the role of SSEA-1 in compaction until mice lacking SSEA-1 synthase were produced. Kudo et al. cloned and characterized a cDNA that encoded mouse α1,3-fucosyltransferase IX (Fuc-TIX), i.e., SSEA-1 synthase, from a mouse brain cDNA library (12). Furthermore, they generated Fut9-deficient ($Fut9^{-/-}$) mice and showed that Fuc-TIX is an enzyme responsible for the synthesis of SSEA-1 in preimplantation embryos and that $Fut9^{-/-}$ mice develop normally (13).

Furukawa and his colleagues cloned a cDNA that encoded mouse α1,4-galactosyltransferase (α1,4GalT), i.e., Gb3Cer/CD77 synthase, which initiates the synthesis of globo-series glycosphingolipids (14). They showed that α1,4GalT null mutant mice are resistant to Stx because these mice lack Gb3Cer, the receptor for Stx (15). Although $\alpha1,4\text{-GalT}^{-/-}$ mutant mice could not synthesize SSEA-3 and SSEA-4, which are carried by Gb5Cer and sialylGb5Cer respectively, they could develop normally.

These results obtained with gene-targeted mice reveal that SSEAs are not necessarily essential for embryogenesis. However, frequently, the dysfunction induced by the gene targeting of certain molecules is overcome by other molecules. Glycosphingolipids such as Gb5Cer and sialylGb5Cer are expected to form raft microdomains and are considered to be involved in the dynamic changes or membrane trafficking on the cell surface during embryogenesis. Hereafter, we show the preferential localization of SSEA-4 between the blastomeres of mouse preimplantation embryos and discuss the role of raft microdomains in early embryogenesis.

D. Localization of SSEA-4 in Mouse Preimplantation Embryos

Recently, Sato et al. showed that 6E2 (mouse IgG_3, κ), a Mab raised against the human EC cell line NCR-G3 (16), recognizes sialylGb5Cer and that the binding of 6E2 to sialylGb5Cer in preimplantation mouse embryos causes clustering and consequent accumulation of sialylGb5Cer

C. SSEA の生理的意義

SSEA-1 はコンパクション時に発現が強くなり，コンパクション完了から 32 細胞期にかけて消失するので，コンパクションや細胞と細胞の相互作用に深く関わっていると推測されてきた。現に，2 価の SSEA-1 化合物をマウス桑実胚に加えるとコンパクションが解消され胚盤胞への分化が阻害されるが(11)，決定的な確証を得るまでにはいたらなかった。しかしながら，SSEA-1 合成酵素を欠損したマウスの出現はそれまでの仮説を無惨にも否定した。Kudo 等は SSEA-1 合成酵素である α1,3 フコース転移酵素 IX（Fuc-TIX）の cDNA をマウス脳 cDNA ライブラリーからクローニングし(12)，ついで，Fut9 欠損マウスを作製して，Fuc TIX が着床前胚における SSEA-1 合成酵素であること，また，Fut9 欠損マウスは正常に発生する事を報告した(13)。

Furukawa 等は，マウス α1,4 ガラクトース転移酵素（α1,4GalT），すなわち Gb3Cer/CD77 合成酵素の cDNA をクローニングした(14)。グロボ系糖脂質合成の起点となる酵素である。さらに α1,4GalT 欠損マウスを作製し，シガ毒素の受容体である Gb3Cer が全く発現していないこのノックアウトマウスは，シガ毒素に対して抵抗性である事を示した(15)。α1,4GalT 欠損マウスは Gb5Cer も sialylGb5Cer も合成できないので，SSEA-3, SSEA-4 のいずれも発現していない。それにもかかわらず，正常に発生した。

これら遺伝子ターゲッティングマウスから得られた結果は，SSEA は必ずしも発生に必須ではないという事を示している。しかしながら，ある分子を欠損してもその機能が他の分子で補われる事は，しばしばみられることである。Gb5Cer や sialylGb5Cer はラフトマイクロドメインを形成し，発生においても，細胞表面上での膜のダイナミックな変化や細胞内への移動に関与していると予測される。次章では，マウス着床前胚の割球間に SSEA-4 が優先して分布する事を示し，初期発生におけるラフトマイクロドメインの機能について論じる。

D. 着床前胚マウスにおける SSEA-4 の分布

Sato 等は，ヒト EC 細胞 NCR-G3 を免疫原として得た Mab 6E2（マウス IgG_3, κ）(16) が sialylGb5Cer を認識抗原としていること，6E2 のマウス着床前胚への結合は sialylGb5Cer のクラスタリングとそれに続く割球界面への集積を誘導する事を見いだした(17)。生きた状態での SSEA-4 の分布の変化

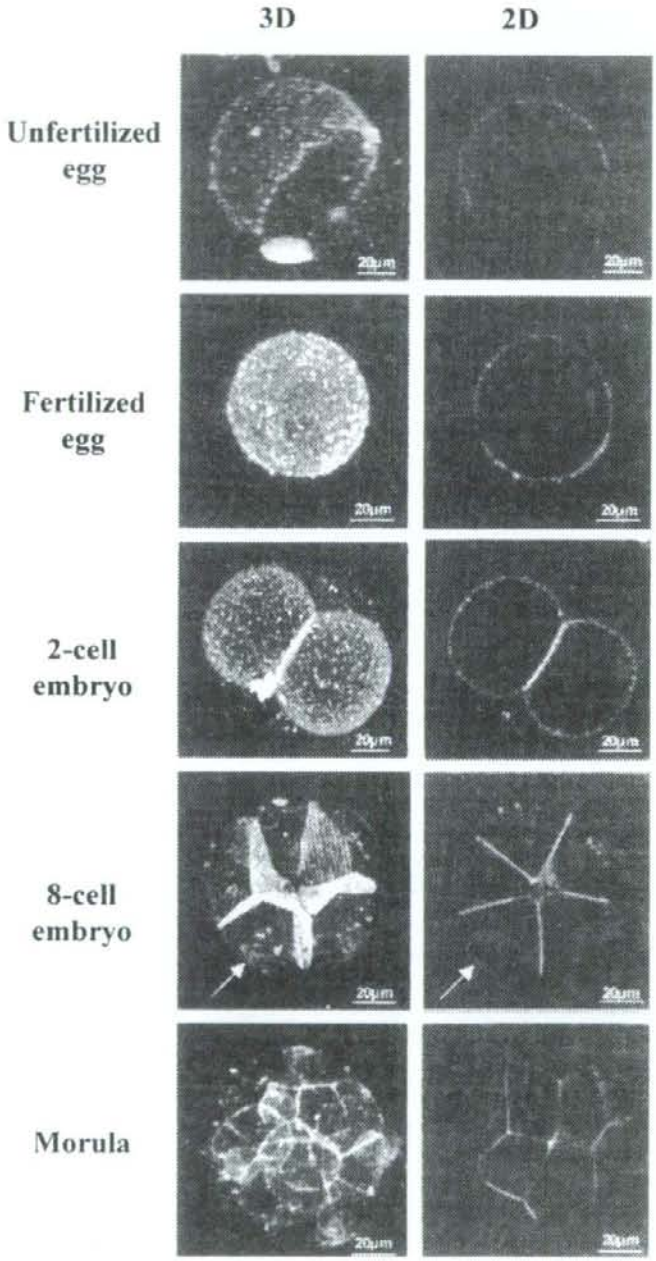


Fig. 3. Immunostaining of SSEA-4 in mouse unfertilized egg and preimplantation embryos by using 6E2. The column designated 3D is a three-dimensional image reconstructed by stacking optical images (2D) with an LSM software.

at the interface between the blastomeres (17). In order to establish the time-course of the distribution of SSEA-4 in a viable state, we immunostained viable preimplantation

を観察するため，未固定の着床前胚を 6E2 で免疫染色した。この染色方法により，マウス卵や分裂中の着床前胚の膜マイクロドメインいわゆる脂質ラフトを検出することができる。

embryos by using 6E2. With this approach, we succeeded in detecting the membrane lipid rafts in mouse oocytes and cleaving preimplantation embryos. The movement of SSEA-4 in mouse preimplantation embryos is depicted in the three-dimensional and two-dimensional images in Fig. 3. In an unfertilized egg, SSEA-4 is dispersed at the vegetal pole side (represented as dots). Immediately after the fertilization of the egg, SSEA-4 moves to the surface and accumulates at the interfaces of the blastomeres. In a compacted 8-cell embryo, the amount of SSEA-4 accumulated at the interfaces is further increased. The blastomeres appeared to be separated by planer membranes; some large SSEA-4 patches were internalized but other patches remained on the surface membranes. Raft microdomains were also enriched at the apical end of each blastomere (indicated by arrows). The amount of SSEA-4 concentrated at the interfaces in the morulae was not as significant as that in the 8-cell embryos; however, the internalization of some patches was evident in the former. These results suggest that SSEA-4 actively moves to the cell surface and readily accumulates between the blastomeres after binding to 6E2. This dynamic change in the staining patterns of SSEA-4 seems to reflect the movement of raft microdomains during cell cleavage. Comiskey et al. stained preimplantation mouse embryos with cholera toxin β (CTβ) to detect raft microdomains and showed that the membranes of the mouse embryonic cells are rich in lipid rafts with a heterogeneous and stage-dependent distribution (18). The staining pattern of CTβ is different from that of the anti-SSEA-4 Mab, indicating that GM1Cer and sialylGb5Cer are localized in different raft microdomains and are involved in distinct signaling pathways.

Recently, Steelant et al. reported that sialylGb5Cer clustering induced the activation of sialylGb5Cer-associated kinases in the raft microdomains of human mammary carcinoma cells and consequently initiated downstream signaling events. They authors also proposed the importance of the organization of crucial molecules in raft microdomains (19,20). In preimplantation embryos, the spatiotemporal position of raft microdomains and their associated molecules is considered important for the functioning of these elements. Currently, SSEA-4 is an important cell-surface marker for the undifferentiated human and simian ES cells; however, SSEA-4 may not only be a surface marker but also function as a signaling molecule via raft microdomains, thus participating in the regulation of early embryogenesis.

マウス着床前胚上のSSEA-4の動きを3次元及び2次元像で図3に示す。未受精卵では，SSEA-4はドット状に植物極側に分布している。受精するとSSEA-4は卵表面全体に広がり，卵割が始まると割球界面に集積してくる。コンパクションをおこした8細胞期胚では界面への集積はさらに進行し，平面で割球を隔てているように見える。パッチも観察され，一部は細胞内にとりこまれている。また，各割球の頂点域にも弱いながらも集積が見られる（図中矢印）。桑実胚の割球界面への集積は8細胞期胚程ではないが，依然として明確に観察され，パッチの細胞内への取り込みも見られる。未固定胚の6E2による免疫染色解析から，SSEA-4は細胞表面上を活発に移動し，割球界面に容易に集積することがわかった。SSEA-4染色パターンのダイナミックな変化は，卵割期のラフトマイクロドメインの動きを反映しているものと思われる。Comiskey等は，コレラ毒素Bを用いてマウス着床前胚のラフトマイクロドメインを検出し，マウス胚の膜には脂質ラフトが豊富にあり，不均一でステージ依存的に分布していると報告している(18)。抗SSEA-4抗体とコレラ毒素とは異なる染色パターンを呈するので，sialyGb5CerとGM1Cerは異なるラフトマイクロドメインに存在し，異なるシグナル経路に関与している可能性が考えられる。

Steelant等は，ヒト乳がん細胞株のラフトマイクロドメインに局在するsialylGb5Cerを抗体で架橋すると下流へのシグナル伝達が誘導される事を見いだし，ラフトマイクロドメインの鍵となる分子の統合の重要性を指摘している(19,20)。着床前胚において，ラフトマイクロドメインとその関連分子は時-空間的に配置をかえながら重要な機能を果たしていると考えられる。SSEA-4はヒトやサルES細胞の“未分化である”事の細胞表面マーカーの一つと考えられているが，単なる表面マーカーとしてだけではなく，ラフトマイクロドメインを介するシグナル関連分子として機能し，初期発生の制御に関与していると考えられ，今後の解析が期待される。

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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

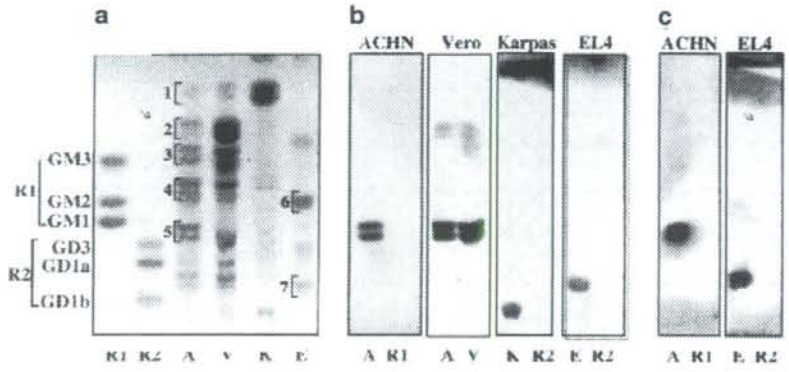

Fig. 1 TLC immunostaining with antisera against rafts components. The lipids extracted from ACHN (*A*), Vero (*V*), Karpas (*K*), EL4 (*E*) cells and the reference glycolipids (*R1* GM3, GM2, GM1; *R2* GD3, GD1a, GD1b) were separated by TLC and chemically stained with Orcinol reagent (**a**) or immunostained 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).

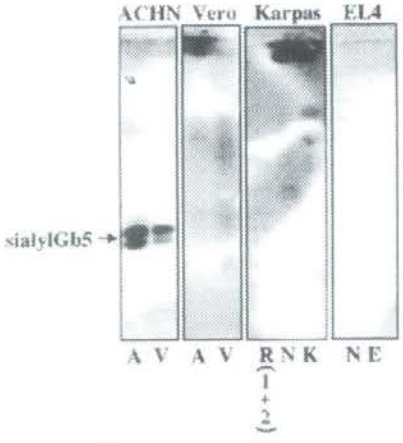

Fig. 2 TLC immunostaining with antisera against the cell suspension. The lipids were extracted from the cells as shown in the legends to Fig. 1 and from NALM-6 cells (*N*), and separated by TLC. The plates were immunostained with antisera from Balb/c mice immunized with the suspension of irradiated ACHN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