日本財団 助成事業
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２００３年度日中医学協会共同研究等助成事業報告書

－在留中国人研究者研究助成－

2004年 3月 10日

財団法人 日中医学協会
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1．研究テーマ 胚性幹細胞（ES 細胞）の細胞生物学解析
—マウス ES 細胞における細胞接着分子の３次元局在及び分化による初期的変化

2．本年度の研究業績

(1) 学会・研究会等における発表 (有) ・ 無 （学会名・演題）

2004年3月24日第3回日本再生医療学会総会にて第一演者として発表する。

演題： Spatial Distribution and Initial Changes of SSEA-1 and Other Cell Adhesion-Related Molecules on Mouse Embryonic Stem Cells Before and During Differentiation
（マウス ES 細胞における細胞接着分子の３次元局在及び分化による初期的変化）

(2) 学会誌等に発表した論文 (有) ・ 無 （雑誌名・論文名）

欧文雑誌〈Journal of Histochemistry and Cytochemistry〉に投稿して、今 revise 中。

Title：Spatial Distribution and Initial Changes of SSEA-1 and Other Cell Adhesion-Related Molecules on Mouse Embryonic Stem Cells Before and During Differentiation
（マウス ES 細胞における細胞接着分子の３次元局在及び分化による初期的変化）

3．今後の研究計画

これまで体得した手法をもちいながらES細胞の動態、特に細胞間相互作用を細胞生物学的に研究しております。ES細胞は、外、中、内胚葉すべての細胞に分化する能力（多分化能）を持っています。ES細胞はさらに、特定条件下でほぼ無限に増殖する能力（自己複製能）を有することから、各種細胞の発生メカニズムの解明に有用であるのみならず、再生医学への応用が期待されています。今後は、ES細胞を心筋細胞に分化誘導する方法を研究し、心筋梗塞等の心臓疾患に対する細胞療法への応用を図りたいと考えております。この分野の研究は、将来中国でも盛んに行われるようになると考えられますので、しっかり研究してまいりたいと思っております。

4．指導責任者の意見

Cuiさんは本講座のES細胞研究の立ち上げより関わり、その基盤を我々とともに築いてきた研究者の一人です。卓抜したES細胞培養のテクニックを持ち、マウス、サルES細胞培養法を確立し、教室への技術継承を極めて速やかに行ってくれました。本講座のES細胞研究のレベルはまさに彼女の情熱的な努力により高い状態に維持されていると思います。

一方、本人自らは、ES細胞のもっとも基本的な、そしてあまり注目されなかった細胞表面の抗原表出を詳細に検討しました。その結果細胞サイクルとある表面抗原表出がパラレルに連動することをつき止め、一般に信じられているES細胞のマーカーが必ずしも適切でないことを明らかにしました。本講座の研究者の中でも、研究への志向、テクニック、まとめあげていく能力には図抜けてすばらしいものがあります。さらに後輩に対する指導も的確で思いやりがあり、安心してまかせられます。今回得た助成金により、さらに彼女の能力は引き出されました。深く感謝いたします。Cuiさんはもうしばらく助手として本講座にとどまり、研究、大学院生の指導に専念する予定です。

指導責任者氏名 佐々木克典 

5．研究報告書

別紙「研究報告書の作成について」に倣い、指定の用紙で作成して下さい。

研究発表または研究状況を記録した写真を添付して下さい。

※研究成果を発表する場合は、発表原稿・抄録集等も添付して下さい。

※発表に当っては、*日中医学協会助成金による*旨を明記して下さい。

－日中医学協会助成事業－

胚性幹細胞（ES 細胞）の細胞生物学解析
――マウス ES 細胞における細胞接着分子の３次元局在及び分化による初期的変化

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Abstract　(要旨)

Surface molecules play an important role in a wide range of cellular functions. To develop an understanding of the role for cell adhesion-related molecules (CAMs) in undifferentiated ES cells, we examined the distribution of cell adhesion-related molecules (CAMs) among mouse ES cells. We also observed the spatial distribution on cell surfaces before and during differentiation. Flow cytometry showed that 51% of the undifferentiated cells were positive for SSEA-1, while PECAM-1 and ICAM-1 were present on 75% and 81% of the undifferentiated ES cells respectively. In contrast, 98% of the cells were positive for CD9. The cell-cell heterogeneity of SSEA-1, PECAM-1 and ICAM-1 among the undifferentiated cells in the ES colonies was evident by immunohistochemistry and immuno-SEM, supporting the flow cytometry findings. In contrast, microscopy showed that nearly all undifferentiated ES cells strongly expressed CD9. SSEA-1 was located preferentially on the edge of low protuberances and microvilli and formed clusters or linear arrays of 3-20 particles. PECAM-1 and ICAM-1 were randomly localized on the free cell surfaces, whereas CD9 was preferentially localized on the microvilli or protuberances, especially in the cell periphery. The SSEA-1$^+$ fraction of magnetic cell sorting (MACS) formed undifferentiated colonies 2 days after plating. Flow cytometry showed that SSEA-1 negative cells emerged again in these colonies. Differentiation induced by retinoic acid down-regulated the expression of all CAMs mRNAs as determined by RT-PCR, and their reduction or disappearance was also evident by flow cytometry. Immuno-SEM showed decreases of SSEA-1 in the differentiated ES cells, though some clustering still remained. Our findings help to elucidate the significance of these molecules in ES cell maintenance and differentiation, and suggest that cell surface antigens may be useful for defining the phenotype of undifferentiated and differentiated ES cells.

Key words: embryonic stem cells, SSEA-1, cell adhesion-related molecules, immuno-SEM and TEM, flow cytometry

Introduction　(緒言)

Embryonic stem (ES) cells are derived from the inner cell mass of blastocyst stage early embryos and have both pluripotency and capacity of self-renewal. Thus ES cells can serve as experimental models for studying early embryo development and differentiation, and may serve as sources for cell therapy of various tissues and organs. Mouse ES cells can be maintained in an undifferentiated state for long periods under given conditions and be induced to differentiate along various pathways, depending on culture condition. A common feature of mouse ES cells following induction of differentiation is a change of cell colony morphology from dome-shape to monolayer. This change in the cell-cell and cell-substratum interactions suggests that the expression of intercellular or cell/extracellular matrix adhesion molecules on these cells changes upon differentiation.

Embryonic cell surface molecules have been viewed generally as lineage markers and regulators of cell-cell interactions. Cell surface carbohydrates are implicated in a number of membrane-modulated phenomena such as cell aggregation and adhesion. They play a role in the cellular interactions of the immune system and in normal cell interactions during the embryogenesis of pre-implantation mouse embryos. Expression patterns of cell adhesion related molecules such as SSEA-1, ICAM-1, PECAM-1 and CD9 occur in undifferentiated and differentiated ES cells.

In the present study, we examined the surface ultrastructure of mouse ES cells, and the spatial distribution of SSEA-1, ICAM-1, PECAM-1, and CD9 on the cells. In addition, we further investigated the changes in the morphology and the expression of these CAMs upon the initiation of ES cell differentiation. We report for the first time the spatial distribution and expression levels of the above molecules on mouse ES cells.

Materials and Methods （材料と方法）

Murine ES cell lines and cell culture

Five karyotypically normal ES cell lines were used in the study. They are ES cell lines developed from 129/sv and DBA1 strain mice, ES cell lines AB1 and AB2.2 and ES cell line ES-D3. They were cultured as described previously (Johkura et al., 2003) with LIF, and subcultured every 3 days at a density of $2X10^4$ cells/cm^2 to maintain an undifferentiated phenotype.

Alkaline phosphatase staining and immunofluorescence labeling

For alkaline phosphatase staining ES cells were incubated with NBT/BCIP solution. For immunofluorescence double-staining, the following primary antibodies were used: rat anti-mouse ICAM-1, PECAM-1 and CD9, and mouse monoclonal antibody SSEA-1 (IgM). Specimens were incubated with DAPI for nucleic acid stain.

Double-labeling immunoelectron microscopy

The second antibody conjugated with 10 nm and 20 nm gold particles respectively, were used to detected the distribution of CAMs and SSEA-1.

Preparation for scanning electron microscopy

The specimens were post-fixed with 1% OsO_4 for 4 h, dehydrated, and dried with the CO_2-critical point drying method, coated to 3 nm thickness with an osmium plasma coater, and observed with SEM with a backscatter electron (BSE) detector.

Preparation for transmission electron microscopy

The cells on coverslips were post-fixed with 1% OsO_4 for 1 h, dehydrated, and embedded into epoxy resin with a standard method. Sections 1.5 m thick were stained with 0.1% toluidine blue solution. Ultra-thin sections were stained with uranyl acetate and lead citrate solution, and observed with a TEM.

RT-PCR analysis

Total RNA was extracted from undifferentiated ES cells and from differentiated ES cells at various stages of differentiation using TRIzol® reagent. DNase-treated total RNA was used to prepare the first-strand cDNA with SuperScript II (Invitrogen). cDNA samples were subjected to PCR amplification with specific primers.

Magnetic cell sorting (MACS) separation

SSEA-1 positive cells were enriched using the magnetic cell sorting (MACS) system. The separated SSEA-1^+ fraction was cultured at a density of $2X10^4$ cells/cm^2 on feeder layers in ES culture medium with LIF.

Flow cytometry

Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM (Chemicon, Temecula, CA) or PE-Cy5-conjugated goat anti-rat IgG (Cederlane, Hornby, Ontario, Canada) were used to marked SSEA-1 and CAMs, respectively. The stained cells were analyzed on a FACS Calibur (Becton Dickinson).

Results （結果）

Characterization of undifferentiated mouse ES cells

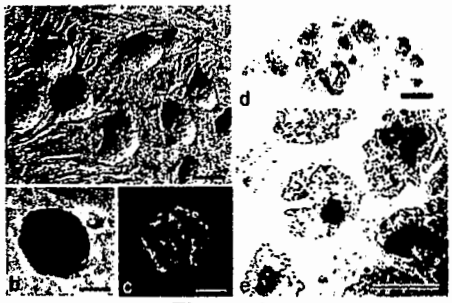

Figure 1

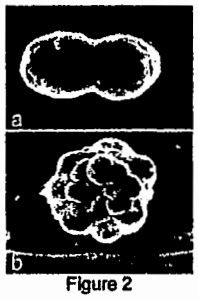

Figure 2

Hoffman modulation contrast microscopy showed high, dome-shaped ES colonies (Fig. 1a). Within the colonies, the undifferentiated ES cells have indistinct margins and were positive for ALP and SSEA-1 (Fig.1 b, c). The undifferentiated ES cells typically had high nucleus/cytoplasm ratios, prominent nucleoli, and were firmly and closely packed together in the colony (Fig. 1d, e). Dividing cells were present 12 h after plating (Fig. 2a), and many also displayed filopodia or lamellipodia. On Day 2 of culture, typical three-dimensional, tightly packed undifferentiated colonies were present (Fig. 2b).

Cell adhesion-related molecules on ES cells

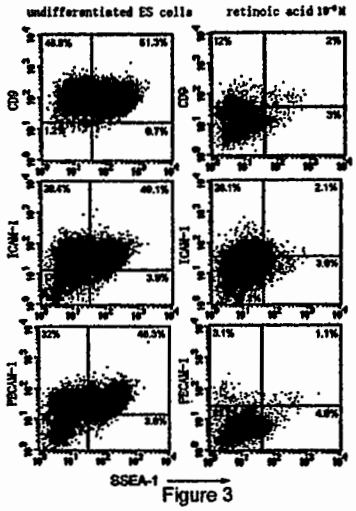

Figure 3

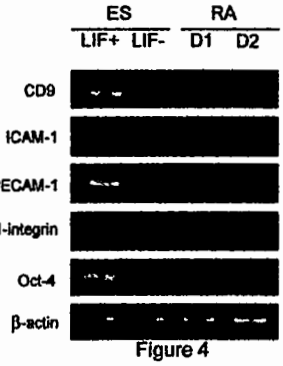

Figure 4

Cell adhesion-related molecules that may be involved in the maintenance of undifferentiated ES cells were assessed by flow cytometry (Fig. 3). Nearly all undifferentiated ES cells, 98.5 ± 0.6 % (mean ± SD), expressed high levels of CD9 antigen. The fluorescence intensity for SSEA-1, PECAM-1 and ICAM-1 varied from low to high. PECAM-1 and ICAM-1 patterns of distribution were similar to one another, i.e., 74.6 ± 1.4% and 80.7 ± 2.0% of the undifferentiated ES cells expressed the respective molecules. In contrast, only 51.5 ± 1.6% of the undifferentiated ES cells were positive for SSEA-1 (Fig. 3). Nearly all SSEA-1 positive cells expressed PECAM-1, ICAM-1 and CD9. Following differentiation for 2 days, flow cytometry showed that the expression level of SSEA-1 and cell adhesion-related molecules decreased or disappeared (Fig. 3). Fluorescence intensity was also generally reduced when viewed by CLSM (not shown).RT-PCR also confirmed the synthesis in undifferentiated ES cells of mRNA for PECAM-1, ICAM-1 and CD9 and the rapid decline in the expression levels of CD9, ICAM-1 and PECAM-1 mRNAs during initial cell differentiation in reference to β-actin expression (Fig. 4).

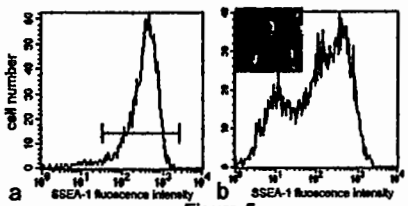

Figure 5

To further characterize SSEA-1 positive cells, we purified them from the undifferentiated ES colonies using MACS. By flow cytometry analysis, SSEA-1 positive cells were enriched to about 98% after MACS from 51% beforehand (Fig. 5a), whereas the percentage of CD9 positive cells before and after sorting was the same (not shown). Two days after plating, the morphology of colonies obtained from SSEA-1$^+$ fraction was the same as that of standard undifferentiated culture (Fig. 5b inset). SSEA-1 negative cell population emerged again in undifferentiated ES colonies derived from SSEA-1$^+$ fraction (Fig. 5b).

Spatial localization of cell adhesion-related molecules on the undifferentiated ES cells

When viewed by confocal laser scanning microscopy (CLSM), antibodies to SSEA-1, CD9, ICAM-1 and PECAM-1 were localized at the contact regions of undifferentiated ES cells as well as on the free surfaces (not shown), though the fluorescence intensity varied greatly from cell to cell. Cells stained for both CD9 and SSEA-1 showed that most were CD9-positive, but some of the same cells were SSEA-1 positive while others were SSEA-1 negative, consistent with the results from flow cytometry. SSEA-1 exhibited dot-like appearance on the surfaces of

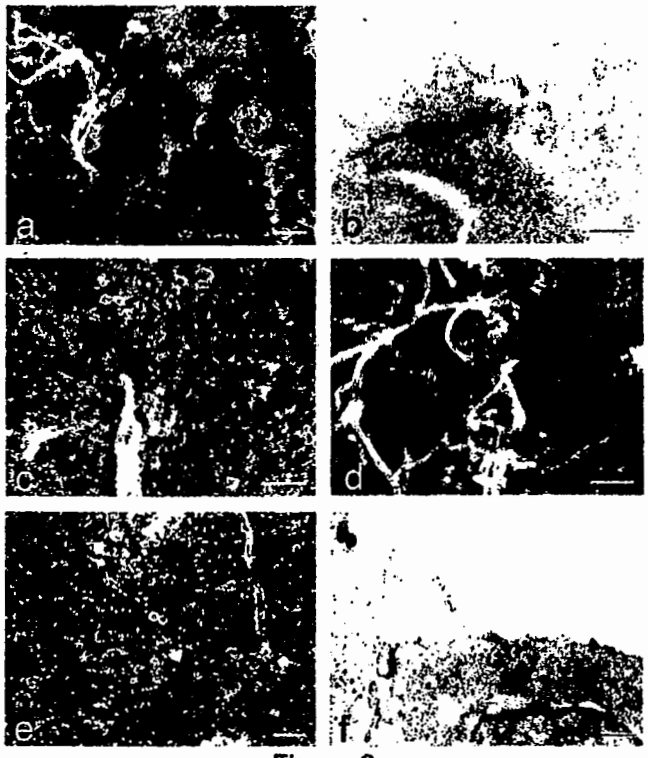

Figure 6
expressed during initial differentiated ES cells.

ES cells.

Immuno-SEM and TEM showed that CD9 was preferentially localized at cell boundaries, where the gold particles were mainly restricted to the microvilli or low protuberances and often formed clusters (Fig. 6a, b). In contrast, the gold particles for PECAM-1 and ICAM-1 were randomly distributed on the cell free surface (Fig. 6c, d, e, f). In the undifferentiated ES cells, the distribution of SSEA-1 varied greatly from cell to cell (Fig. 6d). On SSEA-1 positive cells, the 10-nm gold particles were located preferentially on the edge of low protuberances and microvilli, forming clusters ranging from 3-20 particles or in linear arrays (Fig. 6c, d, e, f). β1-integrin was also highly expressed in the undifferentiated ES cells and did not significantly change throughout the time course of initial differentiation. Oct-4, a marker of undifferentiated ES cells, was also

Discussion (考察)

In this study we have demonstrated the intercellular heterogeneity of SSEA-1, ICAM-1 and PECAM-1 distribution and ubiquitous expression of CD9 in the undifferentiated mouse ES cells. The range of SSEA-1, ICAM-1 and PECAM-1 expression varied from low to high levels. Based on these observations, we hypothesize that SSEA-1 is involved in the formation of multilayered and tightly compacted colonies of mouse ES cells through highly specific Le^x-Le^x interaction.

Despite the discernible expression of ICAM-1, ligands for this molecule, i.e., LFA-1 and Mac-1, were not present in the ES cells (Tian et al., 1997). Thus the function of this molecule in the mouse ES cells remains unclear. Distribution of PECAM-1 in cell boundaries suggests that PECAM-1 may play a role in ES cell aggregation via its homophilic adhesion. SEM showed random distribution of PECAM-1 on the free surface of ES cells, but its accumulation at the cell-cell borders could not be verified because they are inaccessible to observation by immuno-SEM. The random distribution of PECAM-1 observed by immuno-SEM may reflect the diffusion of molecules not involved in homophilic binding.

Because most undifferentiated ES cells are positive for CD9, and it quickly disappeared following initial differentiation, CD9 may be a more suitable for marker of undifferentiated ES cells than SSEA-1. CD9 is a cell adhesion-related molecule and may play a role in cell-extracellular matrix or cell-cell interactions as a cofactor of integrin. The preferential localization of CD9 on microvilli and protrusions of the cellular periphery suggest that it is associated with attachment of adjacent cells. It may also regulate cytoskeletal organization, thus affecting the cell-ECM or cell-cell interactions.

The association of cell surfaces containing microdomains of adhesion molecules plays an important role in the three-dimensional cell-cell interactions that affect differentiation of ES cells. The data presented here allow us to further understand the roles of these cell adhesion-related molecules in cell-cell interactions and in self-renewal of ES cells. In addition, the present study indicates that these antigens may be used as markers of cell status to test the phenotypic stability of long-term ES cell cultures. Simultaneous use of immunoreactivity for multiple surface antigens will assist in the identification of positive or negative selection of target cells derived from ES cells.

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注： 本研究は、2004年3月24日日本再生医療学会にて発表する予定、<Journal of Histochemistry and Cytochemistry>に論文revision中。

作成日： 2004年 3月 10日