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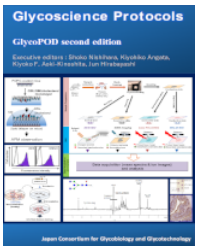

Selectin-binding analysis of tumor cells

Keiichiro Sakuma, M.D., Ph.D.[1] and Reiji Kannagi, M.D., Ph.D.[2]

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Introduction

Hematogenous metastasis of cancer is a highly complex process comprising multiple steps. It starts with the intravasation of cancer cells into the bloodstream from the primary tumor lesion. The cancer cells then travel in the blood stream, where they interact with various blood cells, such as leukocytes. Finally, they adhere to endothelial cells somewhere in the peripheral vessel walls. After extravasation, they enter the connective tissue and form a new metastatic lesion.

In the extravasation step at the peripheral vessel walls, cancer cells having a higher adhesive affinity for endothelium have more opportunity to develop metastasis, escaping the killing activity of the monocytes and NK cells. The cancer-associated glycans, sialyl Lewis a and sialyl Lewis x, serve as ligands for cell adhesion molecules of the selectin family, such as E-selectin, which is expressed on vascular endothelial cells. These glycans are involved in the adhesion of cancer cells to vascular beds and contribute to hematogenous metastasis of cancer (1–5). The degree of expression of these glycans at the surface of cancer cells is well correlated with the frequency of hematogenous metastasis and prognostic outcome of patients with cancers.

Protocol

In this chapter, we introduce two assays for quantifying the selectin-binding activity of cancer cells: A) nonstatic monolayer cell adhesion assay (6) and B) flow cytometric assay.

Materials

1. Nonstatic monolayer cell adhesion assay (**Note 1**)
    a. Endothelial cells (for instance, human umbilical vein endothelial cells: HUVECs)
    b. 24-well cell-culture plates
    c. Recombinant interleukin-1β (IL-1β)
    d. Appropriate culture media for the cells assayed
    e. BCECF-AM (2'7'-bis-(carboxyethyl)-5-(and-6)- carboxyfluorescein acetoxymethyl ester)
    f. Heat-inactivated fetal bovine serum (FBS)
    g. NP-40 (nonoxynol-40)

Author Affiliations: 1 Kindai University; Email: ksakuma@nara.kindai.ac.jp. 2 IBMS, Academia Sinica; Email: rkannagi@ibms.sinica.edu.tw.

Corresponding author.

2. Flow cytometric assay
    a. Appropriate culture media for the cells assayed
    b. Heat-inactivated FBS
    c. Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS(−)).
    d. 0.05% trypsin–0.5 mM of EDTA in Hanks' balanced salt solution
    e. Defined trypsin inhibitor (Thermo Fisher Scientific, Waltham, MA)
    f. PBS containing Ca^{2+} and Mg^{2+} (PBS(+))
    g. PBS(+) containing 1% bovine serum albumin (FACS buffer)
    h. Recombinant human E-selectin-Ig(Fc) chimera protein (R&D Systems, Minneapolis, MN)
    i. (Optional) Neutralizing antibodies for sialyl Lewis a (clone 2D3, murine IgM, Tokyo Chemical Industry, Tokyo, Japan) and sialyl Lewis x (clone SNH-3, murine IgM, Tokyo Chemical Industry)
    j. (Optional) Recombinant human P-selectin-Ig(Fc) chimera protein (R&D Systems)
    k. Fluorescein isothiocyanate (FITC)-conjugated anti-human Ig(Fc) antibody
    l. Cell strainer

Instruments

1. Nonstatic monolayer cell adhesion assay
    a. A rotary plate shaker
    b. Microplate reader
2. Flow cytometric assay
    a Flow cytometer

Methods

1. Nonstatic monolayer cell adhesion assay
    a. Appropriately cultured endothelial cells (for instance, human umbilical vein endothelial cells, HUVECs) are grown in the monolayer at the bottom of 24-well plates in the presence of 1.0 ng of IL-1β for 4 h (**Notes 2** and **3**).
    b. Cancer cells are harvested and resuspended in RPMI without FBS at 5×10^6 cells/mL and labeled with BCECF at a final concentration of 5 nM for 30 min at 37°C in the dark.
    c. After labeling, the cells are washed twice with RPMI with 10% FBS and are resuspended in the same medium.
    d. Remove the medium from the 24-well plate and add the labeled cancer cells at $0.5–1.0 \times 10^6$ cells/0.5 mL/well (**Note 4**).
    e. Place the 24-well plate on a rotating platform for incubation under shear (90–180 rpm) for 20 min at room temperature in the dark.
    f. Remove the medium containing nonadherent cells and wash the wells gently twice with PBS containing Ca^{2+} and Mg^{2+} (**Note 5**).
    g. Add 0.4 mL of 1% NP-40 in RPMI with 10% FBS to each well to lyse the adherent cells.
    h. Measure the fluorescence intensity of each well using a microplate reader and a filter set for BCECF-AM (the same filter set as used for FITC).
    i. Calculate the number of adherent cells in each well using a reference curve made from the fluorescence intensity of a known number of labeled cancer cells.
2. Flow cytometric assay
    a. Culture cells in a humidified CO_2 incubator at 37°C (**Note 6**).
    b. Aspirate the culture media and wash the cells using PBS(−) to remove FBS, which may inhibit the action of trypsin.
    c. Detach the cells with 0.05% trypsin–0.5 mM of EDTA and transfer the cells into conical tubes containing the trypsin inhibitor.

d. Count the cell number while centrifuging the conical tube at 1,000 ×*g* for 5 min at 4°C.
e. Aspirate the supernatant.
f. Resuspend the pellet in FACS buffer and aliquot it into 1.5-mL centrifuge tubes of sample numbers at ~1.0×10^6 cells per tube.
g. Centrifuge the tubes at 3,000 ×*g* for 1 min at 4°C and aspirate the supernatant.
h. (Optional) Resuspend the cells in 100 µL of FACS buffer containing 100 µg/mL of antibody for sialyl Lewis a or sialyl Lewis x (**Note 7**).
i. (Optional) Incubate the samples on ice for 30 min.
j. (Optional) Centrifuge the cells at 3,000 ×*g* for 1 min at 4°C and aspirate the supernatant.
k. Resuspend the cells in 100 µL of FACS buffer alone or FACS buffer containing 5 µg/mL of recombinant selectin (**Note 8**).
l. Incubate the samples on ice for 30 min.
m. Centrifuge the cells at 3,000 ×*g* for 1 min at 4°C and aspirate the supernatant.
n. Resuspend all the samples in 100 µL of FACS buffer containing 1 µg/mL of secondary antibody.
o. Keep the samples on ice for 30 min.
p. Spin down the cells at 3,000 ×*g* for 1 min at 4°C and aspirate the supernatant.
q. Resuspend the cells in 100 µL of FACS buffer.
r. Pass the cells through the cell strainer.
s. Acquire data by flow cytometry.

Notes

1. This protocol is based on the property that carbohydrate-mediated cell adhesion is relatively resistant to mechanic shear stress, while cell adhesion mediated by proteins, such as integrins, generally cannot withstand mechanical stress.
2. HUVECs at 2–6 passages after isolation will show the best response to IL-1β. Endothelial cells cultured without IL-1β serve as negative control cells.
3. Cultured cells transfected with E-selectin cDNA (for instance, E-sel/CHO cells) can be used instead of endothelial cells. In such cases, mock transfectant cells can serve as control cells.
4. Inhibitory antibodies, such as anti-E-selectin (for treatment of endothelial cells), or antibodies directed to carbohydrate ligands (for treatment of cancer cells) can be added to the wells to ensure which molecules are involved in the adhesion, depending on the purpose of the experiments (Figure 1) (4).
5. The plate can be checked under a microscope to confirm that the washings were done gently enough, ensuring that adherent cells do not detach from endothelial cells at the bottom of the wells. After the washing procedure, the plate can be checked again under a microscope to ensure that any nonadherent cells do not remain floating in the medium.
6. In this assay, we used HT29 cells undergoing epithelial–mesenchymal transition (EMT) in which expression levels of sialyl Lewis a and sialyl Lewis x are markedly increased (7). EMT was induced in serum-starved DMEM supplemented with epidermal growth factor (20 ng/mL), basic fibroblast growth factor (10 ng/mL), recombinant insulin (25 µg/mL), holo-transferrin (100 µg/mL), putrescine dihydrochloride (10 µg/mL), and sodium selenite (5 ng/mL).
7. These antibodies are used to confirm whether the recombinant E-selectin-Ig(Fc) chimera protein bound to the cells through the specific interaction with sialyl Lewis a/x or through nonspecific fashion (Figure 2).
8. P-selectin can be used as a negative control because it selectively binds to sialyl Lewis x-harboring PSGL-1, a leukocyte-specific glycoprotein (Figure 2).

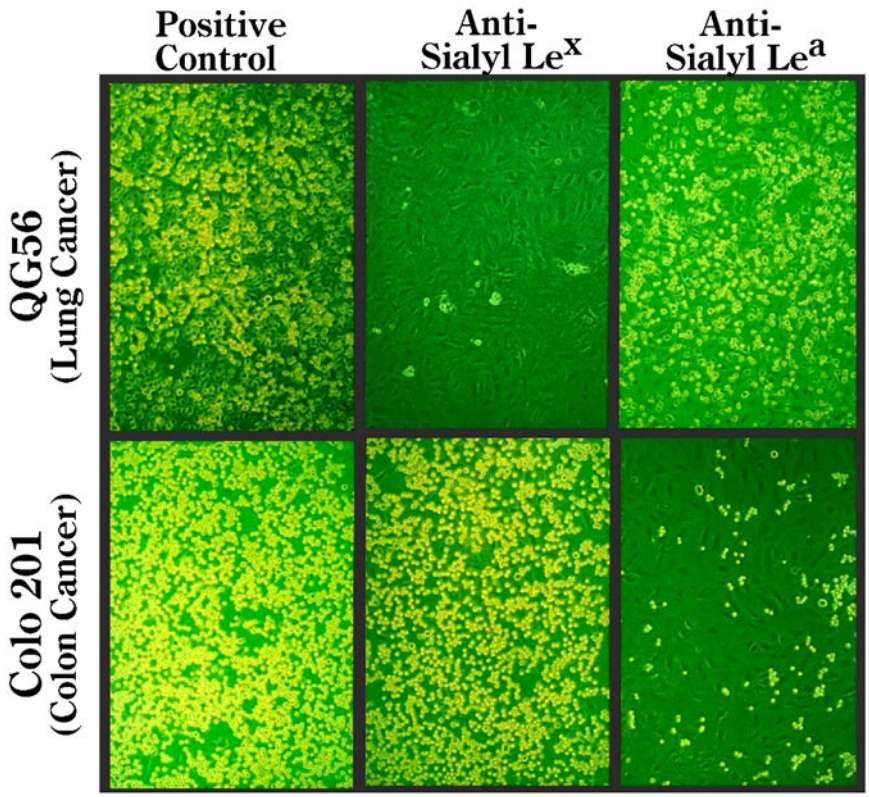

Figure 1: Nonstatic monolayer cell adhesion assay using human cancer cells and recombinant IL-1β-activated human umbilical vein endothelial cells. QG-56 cells (lung cancer) and Colo201 cells (colon cancer) were treated with anti-sialyl Lewis x (20 μg/mL) or anti-sialyl Lewis a (20 μg/mL) for 30 min before the adhesion experiment. Note that adhesion of QG-56 cells is inhibited by anti-sialyl Lewis x antibody, while that of Colo201 cells is inhibited by anti-sialyl Lewis a antibody.

This figure was originally published in the following article: Takada A, Kannagi R, *et al.* (1993) Contribution of carbohydrate antigens sialyl Lewis A and sialyl Lewis X to adhesion of human cancer cells to vascular endothelium. *Cancer Res.* 53(2), 354–61.

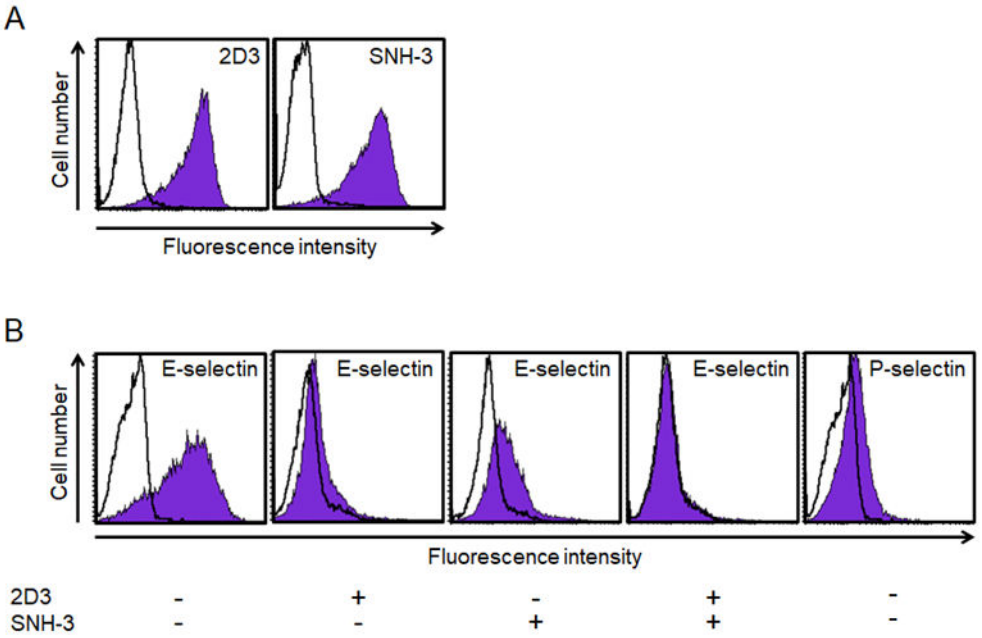

Figure 2: Flow cytometric analysis of HT29 cells for sialyl Lewis a/x expression levels and selectin-binding activity. A) Expression levels of sialyl Lewis a and sialyl Lewis x on HT29 cells were determined by flow cytometry using 2D3 or SNH-3 monoclonal antibody, respectively. B) E-selectin-binding activity of HT29 cells was assayed by flow cytometry using recombinant human E-selectin-Ig(Fc) chimera protein in the presence or absence of the anti-sialyl Lewis a/x antibodies. Recombinant human P-selectin-Ig(Fc) chimera protein was used as a negative control.

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The authors declare no competing or financial interests.

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