ABSTRACT

Numerous cell-based or cell-free systems for study of selectin adhesion use radiolabeled tracers. However, in addition to handling problems associated with the use of radioisotopes, these assays have difficulty relating a number of counts to a number of adherent cells. Here, we describe an assay that uses the natural fluorescence of the green fluorescent protein (GFP) to measure binding of cells to E-selectin. We elaborated an adhesion system composed of a cell monolayer expressing E-selectin ligand to which monodispersed fluorescent Chinese hamster ovary (CHO) cells expressing E-selectin are added. Due to GFP autofluorescence, adhered cells can be easily distinguished from cell monolayers by fluorescence microscopy, and adhesion can be measured by cytofluorometry. We applied this GFP-based adhesion assay to measure the adherence of a pancreatic tumor cell line and found that the binding parameters of these cells satisfy a number of E-selectin-specific criteria.

INTRODUCTION

E-selectin is a cytokine-inducible, calcium-dependent endothelial cell adhesion molecule, which is expressed on endothelial cells during the complex process of leukocyte recruitment (1). Like the other selectins, E-selectin binds to glycoconjugates carrying the tetrasaccharide sialyl Lewis x (sLe\(^x\), NeuAc\(\alpha\)2,3Gal\(\beta\)1,4[Fuc\(\alpha\)1,3]GlcNAc) and its isomer sialyl Lewis a (sLe\(^a\), NeuAc\(\alpha\)2,3Gal\(\beta\)1,3[Fuc\(\alpha\)1,4]GlcNAc) (4,6). The regulation of expression of selectins and their ligands provides an important level of control in determining patterns of leukocyte trafficking. Likewise, the process of tumor extravasation may involve a series of adhesive interactions through endothelial cell receptors, including selectins (reviewed in Reference 2). Therapy designed to block molecular mediators of metastasis may provide an effective treatment, and much interest exists in developing agents to inhibit the cell adhesion to endothelium as a way of modulating deleterious inflammatory reactions and tumor metastatic dissemination.

Numerous cell-based or cell-free systems have been used to study selectin adhesion. Most assays use radiolabeled tracers. In addition to handling problems associated with the use of radioisotopes, these assays have difficulty relating a number of counts to a number of cells. Other assays based on the use of immobilized glycolipid substrates (7) or immobilized selectin-IgG chimeras (5) have been used by several groups; however, the need for a large-scale purification of these molecules added to the artificial nature of the interaction raises the question of the functional relevance of the activities measured.

The in vitro evidence that E-selectin is involved in adhesion and transendothelial migration of several types of carcinoma cells, including pancreatic tumor cells (3), prompted us to develop a rapid cell-based assay for the screening of E-selectin-interacting tumor cells and to evaluate E-selectin inhibitors. The use of cultured cells offers the advantage of a physiologically relevant system that can mimic in vivo cell-cell interaction. So far, the in vivo adhesion assay model uses cytokine-activated human umbilical venule endothelial cells (HUVEC). However, the transient character of E-selectin expression by activated HUVEC cells often leads to variable results and adds to the complexity of the system. To overcome this problem, we made a Chinese hamster ovary (CHO) cell line stably expressing E-selectin and green fluorescent protein (GFP) from the jellyfish Aequorea victoria. The autofluorescence of GFP was used to monitor cells by fluorescence microscopy and flow cytometry. On the other hand, to serve as E-selectin-ligand carrying cells, we made another CHO cell line stably transfected with α(1,3)-fucosyltransferase VII (Fuc-TVII) that highly and homogenously expressed Sle\(^x\) carbohy-
drates and presumably the E-selectin ligand (8). This CHO model was then used to study the interaction of human pancreatic tumor cells with E-selectin.

**MATERIALS AND METHODS**

**Plasmids, Cell Lines and Transfection**

The Human E-selectin cDNA in pCDM8 plasmid (BBG 57) was purchased from R&D Systems (Abingdon, England, UK), and the p-TracerCMV vector carrying the selection marker Zeocin® fused to the GFP gene was obtained from Invitrogen (Carlsbad, CA, USA). The cDNAs for the human Fuc-TVII in pCDM8 were kindly provided by Dr. J. Lowe (Howard Hughes Medical Institute, Ann Arbor, MI, USA). All the following transfections were made using LIPOFECTAMINETM as described by the manufacturer (Life Technologies, Gaithersburg, MD, USA). E-selectin-expressing cells (CHO/E) were made by transfecting CHO-K1 cells (Catalog No. CCL 61; ATCC, Rockville, MD, USA) with 5 µg of selectin plasmids and 1 µg pSVneo. Transfectants were selected in complete Ham’s F-12 medium containing 1 mg/mL G-418 (Life Technologies). A homogenously CHO/E clone was obtained and again transfected with 1 µg of p-Tracer-CMV, and selection was performed in complete Ham’s F-12 medium containing 200 µg/mL G-418 and 500 µg/mL Zeocin to generate the clone CHOgfp/E expressing both GFP and E-selectin. Fuc-TVII-expressing CHO cells (CHO/FT7) were made by co-transfecting CHO-K1 cells with 5 µg of FucTVII/pCDM8 and 1 µg of pSVZeo plasmids (Invitrogen); transfectants were selected in complete Ham’s F-12 medium containing 500 µg/mL Zeocin. Control cells were made by transfecting CHO-K1 cells by pSVZeo vector alone (CHO/Zeo). The human pancreatic cancer cells BxPC-3 were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 µg/mL penicillin and streptomycin.

**Fluorescence Microscopy**

Clones expressing GFP were visualized by direct fluorescence microscopy and isolated using cloning cylinders. E-selectin expression by the GFP-positive cells was assessed by indirect immunofluorescence using the anti-E-selectin monoclonal antibody (MAb) BBA-1 (R&D systems) at a 1/200 dilution for

![Figure 1. Immunofluorescence staining of CHOgfp/E cells. CHOgfp/E cells expressing GFP (A) were stained with mouse anti-E-selectin MAb (BBA-1) followed by rhodamine-labeled, anti-mouse IgG (B).](image-url)
30 min followed by another 30-min incubation with rhodamine-labeled anti-mouse IgG (Sigma, St. Louis, MO, USA). All incubations were carried out at 4°C in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). After washing, slides were mounted with PBS/75% glycerol containing 1% p-phenylenediamine and observed with a Zeiss Axiosplan® fluorescence microscope (Carl Zeiss, Thornwood, NY, USA).

Flow Cytometric Analysis of Adhesion Assay

CHO/Zeo, CHO/FT7 and the human pancreatic tumor cells BxPC-3 were plated in 6-well plates one day before adhesion assay. The CHOgfp/E cells were harvested using a nonenzymatic dissociation solution (Sigma) at 37°C for 15 min, washed 3× with cold serum-free RPMI-1640 and resuspended at 0.5 × 10^6 cells/mL in RPMI containing 1 mg/mL BSA. The cell suspension (1 mL) was loaded onto prechilled CHO/Zeo, CHO/FT7 or BxPC-3 cell monolayers, and incubation was carried out for 15 min on ice. After 3 rinses with cold RPMI-1640, adherent CHOgfp/E cells were harvested by incubation with 2 mM EGTA in calcium and magnesium-free PBS (PBS/-) for 5 min at room temperature. GFP fluorescence was determined on a FACSScan™ Flow Cytometer (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA). Data were collected using the CellQuest™ program (Becton Dickinson) to analyze a total of 5000 light scatter events and plotted as histograms of log fluorescence intensity.

Specificity of Selectin-Mediated Cell-Cell Interaction

To evaluate the specificity of our adhesion assay, we used neuraminidase or EGTA treatments. For neuraminidase treatment, cell monolayers were incubated at 37°C for 1 h with 0.1 U/mL *Vibrio cholerae* neuraminidase (Boehringer Mannheim GmbH, Mannheim, Germany) in RPMI-1640. For EGTA treatment, CHOgfp/E cell suspension was adjusted to 0.25 mM EGTA before loading onto cell monolayers, and adhesion was performed as described above.

RESULTS

Co-expression of GFP and E-selectin

The aim of this work was to study interaction of cancer cells with E-selectin using a fluorescent adhesion assay. We initially investigated whether the GFP could be used as a reporter protein in living cells to allow a direct and noniso-

![Figure 2. Flow cytometric and fluorescence microscopic analysis of CHOgfp/E adhesion.](image)
topic evaluation of cell adhesion using either the FACScan Flow Cytometer or a fluorescence microscope. To this end, it was necessary to establish a CHO cell line that stably expressed both E-selectin and GFP. As shown in Figure 1, a clone of CHO-K1 cells was obtained that homogeneously co-expressed GFP and E-selectin, as assessed by the green fluorescence (Figure 1A) and BBA1 MAb staining (Figure 1B). This clone (named \textit{CHO}gfp/E) was regularly checked for GFP and E-selectin expression, and no change in either parameters was observed over more than one year after its production.

Analysis of \textit{CHO}gfp/E Adhesion to Cell Monolayers

Zöllner and Vestweber have reported previously that the E-selectin ligand-1 (ESL-1) is selectively activated in CHO cells upon transfection with
cDNAs for $\alpha(1,3)$-fucosyltransferase IV (Fuc-TIV) or Fuc-TVII (8). Therefore, it was not necessary to transfect the ESL-1 cDNA in our CHO/FT7 cells. In fact, as shown in Figure 2, the CHOgfp/E cells adhered well to CHO/FT7 as assessed by fluorescence microscopy (Figure 2E) and by the FACSscan Flow Cytometer (mean 23%; Figure 2B), while the adhesion was minimum to control cells CHO/Zeo (mean 1.6%; Figure 2, A and D). CHOgfp/E cells adhered equally well to the pancreatic tumor cells BxPC-3 (mean 31%; Figure 2, C and F).

**Specificity of Selectin-Mediated Cell-Cell Interaction**

We examined calcium and sialic acid dependence of E-selectin-mediated cell-cell interaction by performing the assay in the presence of the calcium-specific chelator EGTA or by pretreating cell monolayers with neuraminidase. As shown in Figure 3, in the presence of EGTA, the adhesion to CHO/FT7 and BxPC-3 was decreased by 80% (column e) and 78% (column h), respectively. Desialylation with neuraminidase also reduced adhesion on both cell lines by 64% (column f) for CHO/FT7 and 69% (column i) for BxPC-3 cells. In another series of experiments, CHOgfp/E cells were first preincubated with the E-selectin MAb P6E2 to inhibit E-selectin-dependent adhesion by internalization of the selectin and found that this treatment dramatically reduced adhesion to either CHO/FT7 or BxPC-3 (data not shown). Taken together, these data indicate that the binding parameters of our adhesion system satisfy E-selectin-specific criteria.

**DISCUSSION**

We describe here a GFP-based assay to evaluate E-selectin-mediated adhesion. To this end, a series of transfected CHO-K1 cells were developed and compared to the human pancreatic tumor cells BxPC-3, on the basis of their ability to interact with E-selectin. There are at least two major advantages of this approach compared to previously reported assays. First, and most importantly, it is a safe nonisotopic and noninvasive technique. Also, cells stably expressing GFP are continuously fluorescent, thus no additional manipulation is needed to differentiate adherent cells from cell monolayer ones by fluorescence microscope or FACSscan analysis. Second, to perform the adhesion assay, only one cell line, i.e., CHOgfp/E, is harvested and loaded onto cell monolayers. In many other cell lines, including BxPC-3, cells are difficult to dissociate into monodispersed cells, resulting in inaccurate estimation of the number of those attached to selectins. In contrast, CHO cells can be easily dissociated into monodispersed cells. Consequently, several cell monolayers can be tested for their ability to interact with E-selectin, and the assay can be adapted to 96-well plates for fluorescence quantitation.

A defining feature of selectin-mediated adhesion is a dependence on the presence of calcium. Likewise adhesion of CHOgfp/E cells to monolayers was found to be completely calcium-dependent (Figure 3). This adhesion involves sialylated moieties such as Sle$^\alpha$ and Sle$^\beta$, since neuraminidase completely abolished the adhesive interaction between cells and E-selectin. Although it has been previously reported that the ESL-1 is selectively and functionally activated in CHO cells upon transfection with Fuc-TVII cDNAs (8), the specific counter receptors carrying Sle$^\alpha$ and Sle$^\beta$ structures on pancreatic tumor cells remain to be determined.

In conclusion, our GFP-based assay is a useful tool to determine the avidity of adhesion of various cells to E-selectin and to discover new selectin inhibitors. The adaptation of this technique to a fluorescence-based enzyme-linked immunosorbent assay (ELISA) is under investigation in our laboratory and should be useful as a rapid high-throughput adhesion assay.

**ACKNOWLEDGMENTS**

We thank Dr. Minoru Fukuda (The Burnham Institute, La Jolla, CA, USA) for his critical review of the manuscript and M. Christian Crotte for his technical assistance. This work was supported by Grant No. 6122 awarded from the Association pour la Recherche sur le Cancer (ARC, Villejuif, France) to D.L.

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Received 23 June 1998; accepted 11 December 1998.

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