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E-selectin can mediate the arrest type of adhesion of colon cancer cells under physiological shear flow

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Abstract

The aim of this study was to determine whether colon cancer cells flowing in blood exhibit the same adhesion pattern to the vascular bed as leucocytes using a flow adhesion system. In shear flow conditions, five colon cancer cell lines showed less tethering to E-selectin substrates than polymorphonuclear cells (PMN). However, some of the Colo201 cells formed complete arrest on E-selectin in continuous shear flow which was never observed in PMN cells. Colo201 cells expressed both sialyl Le-x and sialyl Le-a at similar levels in flow cytometry. However, the staining pattern showed marked contrast under the fluorescein microscope. The cell membrane of Colo201 cells was uniformly stained with anti-sialyl Le-a MAb, whereas anti-sialyl Le-x MAb only stained in the patchy areas. Pretreatment of Colo201 cells with anti-sLe-a decreased tethering, while anti-sLe-x significantly inhibited the arrest formation. Our data suggest that E-selectin alone can mediate colon cancer cell lodgement and subsequent metastasis without the contribution of integrin molecules and that the different distribution of E-selectin ligands may affect the adhesion behaviour of colon cancer cells in flow conditions.

Keywords: Metastasis; E-selectin; Colon cancer; Arrest; Rolling; Sialyl Le-x shear flow; Adhesion

1. Introduction

Haematogenous metastasis is an important factor affecting the prognosis of patients with colorectal cancer. Haematogenous metastasis is a complex process, and the attachment of circulating cancer cells to the vascular bed in target organs is one of the essential steps [1,2]. Previous studies have demonstrated that the attachment is mediated by various adhesion proteins. E-selectin and its counter receptors, sialyl Lewis-x (sLe-x) and sialyl Lewis-a (sLe-a), have been proposed to play a major role, since cancer cell adhesion to activated endothelial cells is largely inhibited by the blockade of these molecules with specific monoclonal antibodies (MAb) [3–5]. Consistently, histological studies have shown that metastatic lesions show a higher level of expression of these E-selectin ligands than primary lesions [6–8].

Leucocyte infiltration in inflammatory tissue or homing to lymphoid organs are similar biological phenomena in terms of the interaction with local endothelium. Leucocyte adhesion to endothelial cells is also a multistep process [9]. The first step of leucocyte emigration is tethering and rolling on local endothelial cells, followed by the development of firm adhesion, i.e. arrest, and then by diapedesis. The molecular mechanisms have been extensively studied, and it has been demonstrated that the initial tethering and rolling are supported by selectin–carbohydrate interactions, while integrins cannot bind to their ligands under shear flow conditions but function only at the later steps. In contrast to leucocytes, the effect of shear stress has not been carefully evaluated in cancer cell attachment to endothelial cells, although cancer cells and leucocytes have been shown to share some adhesion mechanisms [3,10].

In this study, we have, therefore, compared the adhesion patterns of colon cancer cells and polymorphonuclear cells (PMN) using a flow adhesion system [11,12].

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2. Materials and methods

2.1. Reagents, antibodies and preparation of adhesion substrates

Plasmids encoding E-selectin–IgG and P-selectin–IgG chimeras, were transfected into COS cells as previously described [13]. Filtered supernatant cultures were stored at 4°C with 0.1% azide. The selectin substrates were generated by spotting the polystyrene plates with 15 μl protein A (20 μg/ml in phosphate buffered serum (PBS), pH 9.1), incubation for 2 h at 37°C, followed by washing and quenching with 50 μl of 20 μg/ml human serum albumin (HSA) for a further 24 h. Then, the substrates were overlaid overnight at 4°C, with 50 μl of the supernatant culture of selectin–IgG chimera transfected COS cells. A peripheral part of the spotted area was selected and used for the observation of cell adhesion. MAbs to sialyl-Lewis-x (SNH3, mIgM) and to sialyl-Lewis-a (2D3, mIgM) were provided by R. Kannagi (Nagoya, Japan). MAb to PSGL-1 (G1, mIgG1) was from R.P. McEver (Oklahoma City, Oklahoma, USA).

2.2. Cell preparation

Polymorphonuclear cells (PMN) cells were isolated from citrate-anticoagulated whole blood by dextran sedimentation, Ficoll-Hypaque centrifugation and hypotonic lysis of red blood cells [14]. All the colon cancer cell lines, Colo201, Colo320, HT29, DLD-1 and WiDr, were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA) and maintained in Dulbecco's minimal essential medium (DMEM) with 10% fetal calf serum (FCS). For HT29, DLD-1 and WiDr, the monolayer cultures were incubated with 0.02% EDTA alone for 30 min at 4°C. Then, trypsin (1 ml/75 cm^2 flask) was added to the cells. After washing the surface of the monolayer with trypsin, shaking the flask for 30 s detached most of the cells from the flask. PMN cells treated by this treatment showed the same adhesion pattern as untreated PMN cells. Colo201 and Colo320 cells were detached from plastic flasks by 0.02% EDTA alone. These cells were washed twice and incubated for at least 1 h at 4°C before the measurement of adhesion. All the cells were warmed up to 37°C just before the experiments.

2.3. Laminar flow assay

A parallel-plate flow chamber [12] was mounted on the coated substrates, and placed on the stage of a phase contrast microscope and monitored with a 10× objective. Wall shear stress was calculated as previously described [11]. The flow chamber and the optical area of the microscope were covered with a plastic box and the temperature of the inside was strictly maintained at 37°C. 1×10^6 cells were suspended in 0.1 ml Hank's balanced salt solution (HBSS) with 10 mM Hepes (pH 7.4), kept at 4°C, and diluted with 1.0 ml of 37°C assay medium (HBSS with 10 mM Hepes including 2 mM Ca^{2+}, 1 mM Mg^{2+} and 10% FCS, pH 7.4) just before the experiments. Then, the cell suspensions were perfused on the substrates for 2 min through the flow chamber using an automated syringe pump (Harvard Apparatus, Natick, Massachusetts, USA) attached to the outlet side. For blocking experiments, each MAb was added to 1×10^6 cell suspensions in 0.1 ml HBSS + Hepes, at a final concentration of 50 μg/ml. After 1 h incubation on ice, the cells were diluted with 1.0 ml assay medium and used for the flow experiments.

2.4. Evaluation of tethering, rolling velocity and shear resistance

Cells interacting with the substrate during flow were analysed from the images videotaped with a TEC-470 CCD video camera (Hamamatsu Photonics, Hamamatsu, Japan) and a Victor CVD-1000 recorder. Tethered cells were defined as cells that maintained an adhesive interaction with the substrate for at least 1 s. The number of attached cells on coated selectin was counted over the 2 min after the flow became stable, and attached cells in three different fields were analysed. In some experiments, after 2 min perfusion of the cell suspension, the cells were subjected to higher shear stresses. On the video recording, the trace of each tethered cell was measured in the 30 s and the rolling velocity was calculated under each shear condition. Cells that were not displaced more than 1 cell diameter were regarded as arrested cells. The cells remaining attached to substrates were counted at the end of each shear stress, and shear resistance was evaluated as the percentage against the total number of tethered cells at the time point of 2 min perfusion. P values were calculated by paired Student's t-test and differences with a P value <0.05 were considered significant.

2.5. Flow cytometry and immunocytochemistry

1×10^6 PMN or Colo201 cells were suspended in 0.1 ml PBS containing 1% bovine serum albumin (BSA) and 0.02% EDTA (staining medium), and incubated with anti-sialyl-Lewis-x (SNH3, mIgM) or anti-sialyl-Lewis-a (2D3, mIgM). After 30 min incubation, the cells were washed twice and reincubated with FITC-conjugated goat anti-mouse immunoglobulin (Caltag, San Francisco, California, USA) for a further 30 min. After washing with the staining medium, the cells were analysed with FACScan (Becton-Dickinson). For immunocytometric studies, the cytospins were prepared with centrifugation at 300 rpm for 3 min. Then, the cells were fixed with 1% paraformaldehyde, mounted, and

observed with a confocal laser microscope (Fluoroview, Olympus, Tokyo, Japan).

3. Results

3.1. Colon cancer cells showed less tethering activity to E- and P-selectins than PMN cells

Five colon cancer cell lines, Colo201, Colo320, HT29, DLD-1 and WiDr, and PMN cells were perfused on immobilised E- and P-selectin chimeras. As previously reported [11], many PMN cells tethered and rolled on E- and P-selectin–IgG at a shear below 3.0 dyn/cm^2 (Fig. 1a, b). Although some of the colon cancer cells attached to E-selectin–IgG under a shear of 1.5 dyn/cm^2, the number of tethered cells was much less than that of PMN cells for all five cell lines (Fig. 1a). This difference was more prominent under high shear conditions. Although Colo201 cells showed a similar level of tethering to E-selectin as PMN cells at 0.5 dyn/cm^2, only a few Colo201 cells attached at a shear above 2.0 dyn/cm^2. In contrast, many PMN cells still tethered even at 3.0 dyn/cm^2 (Fig. 1b). On P-selectin–IgG, many PMN cells tethered, whereas only a small number of Colo201 cells tethered at 0.5 dyn/cm^2 and few cells attached at a shear above 1.0 dyn/cm^2 (Fig. 1b). The other four cancer cells also showed little attachment to P-selectin–IgG at 1.5 dyn/cm^2 (Fig. 1a).

3.2. Colo201 cells showed jerky rolling or arrests on E-selection

All the PMN cells showed subsequent rolling after tethering on E-selectin–IgG. Although more than half of tethered Colo201 cells rolled on the substrate the mode of rolling was clearly different from that of PMN cells. As shown in Figs. 2 and 3, PMN cells rolled on E-selectin with a relatively stable speed of 2.0 ± 1.28 μm/s at a shear of 1.5 dyn/cm^2, whereas most of the tethered Colo201 cells rolled and stopped on the substrate for several seconds (Fig. 2). Some of the rolling cells were completely arrested and the rolling velocity showed much variation among the tethered Colo201 cells (Fig. 3). Some Colo201 cells showed only a transient interaction with E-selectin and jumped over the substrates. Those cells were not counted as tethering cells. When the shear increased to 10 dyn/cm^2, more than 80% of the tethered PMN cells remained rolling with an average speed of 9.0 ± 2.5 μm/s, whereas all the rolling Colo201 cells were detached into the flow stream, and all the Colo201 cells that remained attached were completely arrested at 10 dyn/cm^2 (Fig. 3).

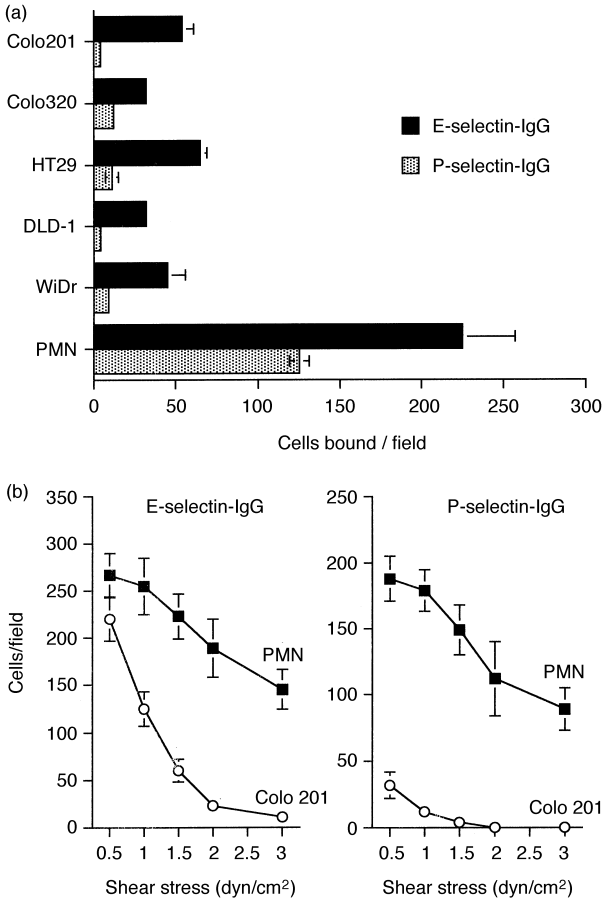

Fig. 1. Tethering activities of polymorphonuclear (PMN) and colon cancer cell lines on E- or P-selectin substrates. (a) Cells were perfused at a shear of 1.5 dyn/cm^2 as described in Materials and Methods. The total number of tethering cells was counted after 2 min perfusion on substrates E- or P-selectin. (b) The numbers of tethering PMN and Colo201 cells were compared at various shear conditions. Data show mean ± S.D. in three different experiments.

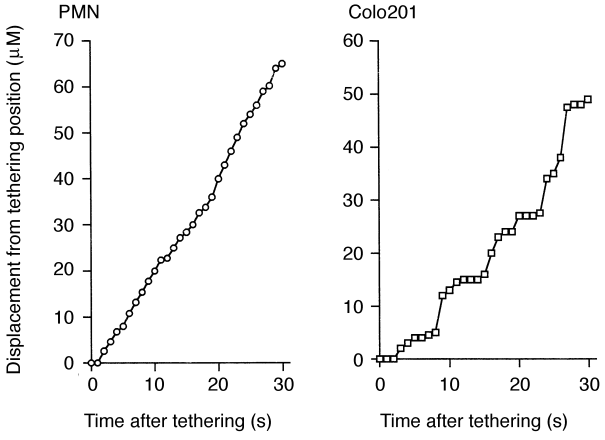

Fig. 2. The trace of polymorphonuclear (PMN) and Colo201 cells tethering on E-selectin–IgG at a shear of 1.5 dyn/cm^2. The locations of the centres of PMN and Colo201 cells interacting with coated E-selectin were monitored on the video movie, and the displacements from the original tethering position were measured at each time point. Data show the trace of two representative cells.

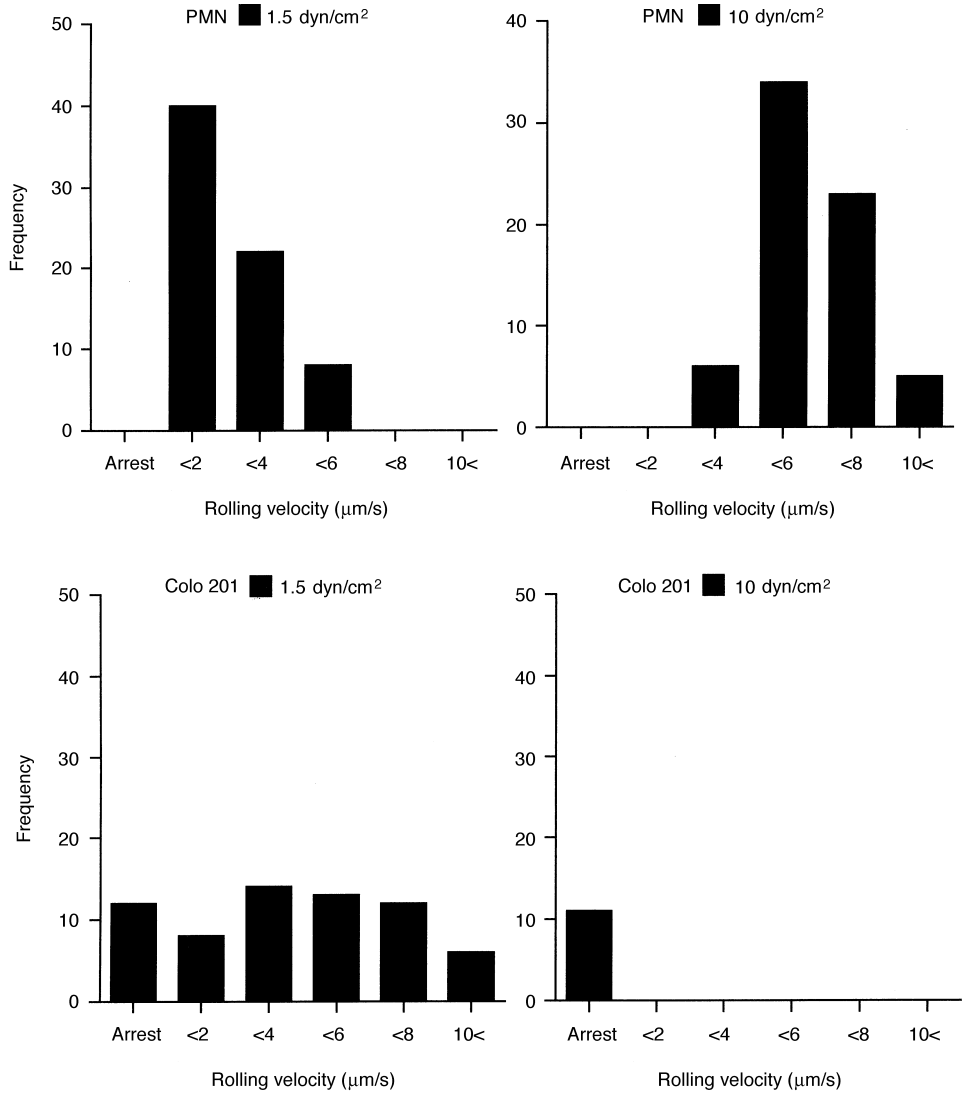

Fig. 3. Rolling velocities of polymophonuclear (PMN) and Colo201 cells on E-selectin–IgG at 1.5 and 10 dyn/cm^2. The displacements of 11–70 cells in 30 s were measured and the velocities were calculated in each shear condition. Data show a representative one in four different experiments.

3.3. E- and P-selectin ligands were differently expressed between PMN and Colo201 cells

Fig. 4a shows the flow cytometric profile of E- and P-selectin ligands. Both PMN and Colo201 cells expressed sLe-x, but the expression level was 10-fold lower in Colo201 than in PMN cells. However, sLe-a was only expressed on Colo201 cells and not on PMN cells. MAb to anti-sLe-a clearly stained the contour of the membrane of Colo201 cells (Fig. 4b). In contrast, anti-sLe-x MAb stained Colo201 cells with a more irregular staining pattern, with some patchy areas on the cell membrane being brightly, positivly stained, whilst other parts were totally negative in staining (Fig. 4c). The other four cancer cell lines showed a similar staining pattern to Colo201 cells of sLe-a and sLe-x (data not shown). This indicates that sLe-x and sLe-a antigens were differently distributed on the cell surface of Colo201 cells. PSGL-1, a major P-selectin ligand, was expressed strongly on PMN cells, but only weakly on Colo201 cells (Fig. 4a).

3.4. Arrest of Colo201 cells was partially mediated by sLe-x

The tethering and arrest of Colo201cells on E-selectin substrate pretreated with MAb to these E-selectin ligands were evaluated. As shown in Table 1, the total number of tethering Colo201 cells at 5 min perfusion was slightly decreased by anti-sLe-a, but not by anti-sLe-x. In contrast, the number of arrested cells was significantly reduced by anti-sLe-x ($P < 0.01$, $n = 3$), while anti-sLe-a had no effects on arrest formation.

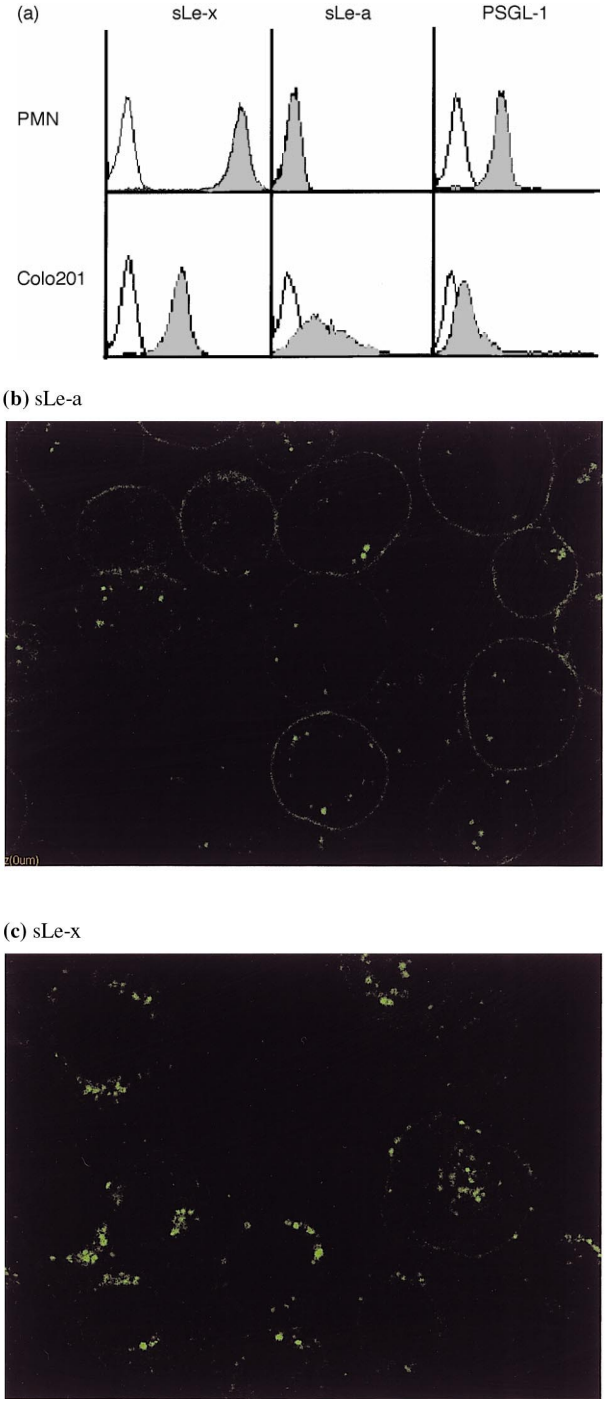

Fig. 4. (a) Flow cytometric profiles of sLe-x, sLe-a and PSGL-1 on polymorphonuclear (PMN) and Colo201 cells. Distribution of sLe-a (b) and sLe-x (c) on Colo201 cells under the fluorescein microscope (×400).

Table 1
The effects MAb to sLe-a and sLe-x on the tethering and arrest of Colo201 cells on E-selection substrate[a]

	Number of tethering Colo201 cells	Number of arrested Colo201 cells
No MAb	96 ± 14	16 ± 5.5
Anti-sLe-a	68 ± 218	15 ± 4.8
Anti-sLe-x	102 ± 18[b]	4.5 ± 1.3[c]

[a] After pre-incubation with each MAb cells were perfused on E-selectin–IgG at a shear of 1.5 dyn/cm^2. The total number of tethered and arrested cells were counted after 5 min perfusion. Data show mean ± S.D. in three different experiments.
[b] $P < 0.05$.
[c] $P < 0.01$.

4. Discussion

Before the development of haematogenous metastasis, circulating cancer cells need to lodge in the vascular bed of the target organ against the blood flow. This lodgement must occur in the continuous presence of shear stress, similar to leucocyte tethering and rolling on inflammatory endothelium. Leucocytes have been shown to utilise E- and P-selectins induced on endothelial cells in the initial step of extravasation [9]. Previous studies have demonstrated that the carbohydrate antigen, sLe-x on leucocytes is the major E-selectin ligand [15–18]. Others have shown that sLe-a, a cancer-associated antigen, is another E-selectin ligand that mediates vigorous adhesion to E-selectin on activated endothelial cells under static or stirring conditions [18–21].

In this flow study, we directly compared binding activities between colon cancer cells and PMN cells, and found that colon cancer cells exhibited much less tethering to E-selectin–IgG than PMN cells. This difference was prominent under physiological shear conditions above 1.0 dyn/cm^2. Moreover, colon cancer cells showed markedly different movements on E-selectin substrate as compared with PMN cells. Importantly, some Colo201 cells: completely arrested in continuous shear conditions, although others jumped on the substrates or detached in flow. Similar jerky movements were also observed in the other four cancer cell lines (data not shown). When high shear was subjected to the attached cells, Colo201 cells were detached more easily than PMN cells. However, the arrested Colo201 cells remained adherent even at higher shears. This adhesion pattern was previously described for lymphocytes [22], monocytes [23], and eosinophils [12] perfused on purified VCAM-1 or activated HUVEC monolayer. Those studies have suggested that the tethering is mediated by $\alpha 4\beta 1$ integrin on leucocytes. However, there is no report to show that E-selectin interaction can support the arrest of any types of leucocytes. This suggests that E-selectin alone can mediate colon cancer cell lodgement and subsequent metastasis under blood flow without any contribution from the integrins. This is in marked contrast to leucocyte recruitment that requires integrin molecules for arrest formation under flow conditions.

A previous study has shown that sLe-x is expressed on both PMN cells and colon cancer cells, while sLe-a is expressed only on colon cancer cells and not on PMN cells [3]. In that study, the authors showed that colon

cancer cell adhesion to activated HUVEC was effectively inhibited by MAb to sLe-a, but not by MAb to sLe-x, indicating the adhesion of Colo201 cells to E-selectin was mainly mediated by sLe-a. In our flow assay, we could also detect the partial inhibition of Colo201 cells tethering by this anti-sLe-a MAb but not by the anti-sLe-x MAb. However, the number of arrested cells was significantly reduced by anti-sLe-x, but not by anti-sLe-a. This clearly indicates that arrest is dependent on sLe-x molecules, although tethering and rolling are more dependent on sLe-a. As shown previously, rolling interaction alone does not lead to leucocyte accumulation and requires another adhesive interaction to mediate the arrest formation at the local vasculature. Our data, however, suggest that the interaction between E-selectin and sLe-x may be enough to mediate the colon cancer cell lodgement in the vasculature of the target organ.

Using flow cytometry we confirmed that Colo201 cells used in this study were positive for these E-selectin ligands, although the expression level of sLe-x was significantly less than that on PMN cells. However, our study with fluorescein microscopy showed that sLe-x is not distributed equally but concentrated at certain areas on the surface membrane of Colo201 cells and the other four cancer cell lines. In PMN cells, the surface membrane was stained by anti-sLe-x mAb more evenly (data not shown). In fact, sLe-x has been shown to be localised on the top of microvilli and evenly distributed on the cell membrane of PMN cells, which enables their stable rolling on E-selectin [24]. In contrast, sLe-a was uniformly localised on the surface membrane of Colo201 cells. The difference of membrane localisation of these E-selectin ligands may result in the preferential usage of sLe-a as a tethering molecule for E-selectin in Colo201 cells. In contrast, the concentrated localisation of sLe-x may be beneficial for the arrest on E-selectin under continuous flow. However, these differences might be caused by the long-term culturing of these colon cancer cells since they were used after multiple passages whilst PMN cells were freshly isolated. Nevertheless, the leukaemia cell line, HL60, showed a similar adhesion behaviour to PMN cells with an even distribution of sLe-x (data not shown), suggesting that the arrested adhesion on E-selectin is a specific event for the cancer cells.

Previous studies in static conditions have shown that P-selectin specifically binds to a variety of cancer cells including colon cancer cells [25–27]. In our flow assay, however, all the colon cancer cells showed very weak binding to P-selectin–IgG under physiological shear conditions, and scarcely expressed PSGL-1. Although the ligands for P-selectin in cancer cells have not yet been clarified, our data suggest that P-selectin interaction may not have a major role in colon cancer cell attachment to vascular endothelium under shear flow conditions. However, Kim and associates showed that intravenous injection of colon carcinoma cells generated fewer lung metastases in P-selectin-deficient mice [28]. In this study, they suggested the possibility that the attenuated metastasis was due to the diminished platelet function to bind to tumour or endothelial cells. In fact, Dardik and associates reported that activated platelets could bind to endothelium via P-selectin which resulted in the augmentation of tumour cell deposition under flow conditions [29]. Taken together, the role of P-selectin in tumour metastasis is considered to be mostly dependent on platelet function.

In summary, adhesive interactions to E- and P-selectins in laminar flow conditions are generally weaker in colon cancer cells than in PMN cells. However, some colon cancer cells formed complete arrest on E-selectin without any additional adhesion pathways in continuous flow conditions. This suggests the possibility that the colon cancer cell can make stable adhesion and subsequent metastasis in target organs only through E-selectin at the vasculature of target organs. The distribution of the selectin ligands on each cancer cell may be another important factor in evaluating the metastatic potential.

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