Vascular Cell Adhesion Molecule-1 Is a Key Adhesion Molecule in Melanoma Cell Adhesion to the Leptomeninges

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SUMMARY: Leptomeningeal metastases occur in up to 8% of patients with systemic malignancies and have a poor prognosis. A better understanding of the pathophysiologic processes underlying leptomeningeal metastases is needed for more effective treatment strategies. We hypothesized that tumor cells will have to adhere to the well-vascularized leptomeninges, because the cerebrospinal fluid lacks nutrients and growth factors for efficient tumor cell proliferation. Specific receptor-ligand interactions, which are unknown until now, will mediate this adhesion process. We determined the growth characteristics of B16F-10 melanoma cells in cerebrospinal fluid. The expression levels of specific adhesion molecules on both mouse leptomeningeal cells (MLMC) and murine B16F-10 melanoma cells were measured by immunofluorescence flow cytometry. We used mAbs to determine the function of these specific adhesion molecules on B16F-10 melanoma cell adhesion to a leptomeningeal cell layer under static and (cerebrospinal fluid-like) flow conditions. B16F-10 melanoma cells did not proliferate in cerebrospinal fluid because of a lack of nutrients and growth factors. MLMC expressed low levels of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), β_1 - and β_3 -integrin subunits, and CD44. VCAM-1 expression on MLMC was shown to be up-regulated by TNF- α . Blocking VCAM-1 on the MLMC with a mAb resulted in a 60% inhibition of melanoma cell adhesion to a leptomeningeal cell layer under flow but not under static conditions. No additive inhibitory effect on melanoma cell adhesion was found by concomitant blocking of the β_1 - and β_3 -integrin subunits and CD44 with mAbs. Our experiments indicate that cerebrospinal fluid does not support B16F-10 melanoma cell proliferation, suggesting the need for melanoma cell adhesion to the well-vascularized leptomeninges. VCAM-1, expressed on MLMC, is an important mediator of in vitro melanoma cell adhesion under (cerebrospinal fluid-like) flow conditions. (*Lab Invest* 2002, 82:1493-1502).

Leptomeningeal metastases (LM) occur in 0.8 to 8% of cancer patients, most frequently originating from myeloproliferative tumors, melanoma, and breast and lung cancer. New developments in cancer treatment have led to a prolonged survival of patients with several types of cancer, including myeloproliferative tumors and breast carcinoma. Consequently, the incidence of metastatic complications, such as LM, is increasing. Survival of patients with LM is usually less than 6 months despite intrathecal chemotherapy and radiotherapy of symptomatic sites (DeAngelis, 1998; Posner, 1995). More knowledge about the pathophys-

iologic processes may lead to treatment strategies that can improve the dismal outcome of LM.

LM can arise when tumor cells enter the subarachnoid space through direct growth of a tumor adjacent to the leptomeninges, via extravasation of tumor cells from the circulation, or when malignant cells spread via perineural spaces (Kokkoris, 1983; Mareel et al, 1998). The fate of tumor cells is unknown once they have reached the cerebrospinal fluid in the subarachnoid space. We hypothesized that cerebrospinal fluid lacks the nutrients and growth factors needed for tumor cell proliferation. Therefore, adhesion of tumor cells to the well-vascularized leptomeninges will be essential for further tumor growth. Histologic studies in the in vivo B16F-10 melanoma LM model cells indeed showed that tumor cells adhere to the leptomeninges in the early stages of LM (Reijneveld et al, 1999). Adhesion of tumor cells to surrounding cells or matrix proteins is mediated by cell surface adhesion molecules (Albelda, 1993; Zetter, 1993). These adhesion molecules are classified into several families, such as selectins, integrins, cad-

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herins, members of the immunoglobulin superfamily, and cell surface glycoproteins like CD44.

Adhesion molecules implicated in distant melanoma metastases include $\alpha_v\beta_3$ integrin, binding vitronectin, and a wide range of other matrix proteins (Marshall and Hart, 1996; Nip and Brodt, 1995). Furthermore, a high expression of CD44 on melanoma cells has been associated with a high metastatic potential in an experimental animal model (Birch et al, 1991). Finally, the expression of $\alpha_4\beta_1$ integrin (very late antigen 4; VLA-4) on melanoma cells has shown to be crucial in distant melanoma metastases via its binding to vascular cell adhesion molecule-1 (VCAM-1), expressed on cytokine-activated (IL-1 and TNF- α) endothelium (Dejana et al, 1988; Garofalo et al, 1995; Okahara et al, 1994; Rice et al, 1988; Rice and Bevilacqua, 1989). A comparison between melanoma cell adhesion to endothelium and leptomeninges may be envisioned, because in both situations melanoma cells have to adhere to mesenchyme-like cells under flow conditions (blood velocity in capillaries: 0.05–0.1 cm/seconds [Whitmore, 1968]; cerebrospinal fluid velocity: 0–7 cm/seconds [Henry-Feugeas et al, 1993]). Until now, adhesion of tumor cells to the leptomeninges has only been studied by Giese et al (1998), who demonstrated that glioma cell adhesion to a human leptomeningeal cell layer under static conditions could be blocked by antibodies against the β_1 subunit. Immunohistochemical studies have additionally shown that CD44 and the β_1 -integrin subunit are expressed on human leptomeninges (Besch et al, 1999; Figarella-Branger et al, 1997) and that intercellular adhesion molecule-1 (ICAM-1; ligand: $\alpha_L\beta_2$ integrin) is expressed on normal and traumatized rat spinal cord leptomeninges (Isaksson et al, 1999).

In this study we demonstrate that B16F-10 melanoma cells do not proliferate in cerebrospinal fluid because of a lack of nutrients and growth factors, suggesting the need for tumor cell adhesion to the leptomeninges. We show that primary cultures of mouse leptomeningeal cells (MLMC) express low levels of VCAM-1, which can be up-regulated by TNF- α . Using a newly developed in vitro adhesion model for LM, we provide evidence that VCAM-1 plays a key role in melanoma cell adhesion to a leptomeningeal cell layer under (cerebrospinal fluid-like) flow conditions.

Results

Growth Characteristics of B16F-10 Melanoma Cells in Cerebrospinal Fluid

B16F-10 melanoma cells had a doubling time of approximately 16 hours when cultured in DMEM containing 5% fetal bovine serum (FBS). Doubling time diminished to 13 hours when cells were cultured in DMEM with 40% FBS (data not shown). The proliferation of B16F-10 melanoma cells decreased in a linear way when cells were cultured in medium with an increasing percentage of cerebrospinal fluid (0 → 100%) and a decreasing percentage of DMEM supplemented with 40% FBS (100 → 0%) during 72 hours

(Fig. 1A). Interestingly, melanoma cells cultured in 100% cerebrospinal fluid hardly showed any proliferation, whereas no significant cell death occurred after 72 hours (trypan blue staining). The decreased cell proliferation in medium with increasing percentages of cerebrospinal fluid could be either a result of shortage of nutrients/growth factors in cerebrospinal fluid or of the presence of proliferation-inhibiting factors in the cerebrospinal fluid. To discriminate between these two factors, we cultured B16F-10 melanoma cells in medium containing 15% DMEM, 5% FBS, increasing amounts of cerebrospinal fluid (0% to 40% to 80% of total culture medium) and decreasing amounts of saline (80% to 40% to 0% of culture medium). Addition of cerebrospinal fluid did not result in decreased proliferation compared with addition of saline to normal culture medium (Fig. 1B). This finding suggests that cerebrospinal fluid does not contain specific factors that inhibit proliferation, but rather a shortage of nutrients and growth factors in cerebrospinal fluid and saline causes the diminished cell tumor cell proliferation.

Characteristics of Primary Culture of MLMC

Primary cell cultures grew readily from leptomeningeal tissue that was carefully dissected from the brains of 2-day-old mice. The cells were pleomorphic: approximately 60% of the cells displayed a broadly polygonal shape with cytoplasmic arcs and the remaining cells were spindle shaped (Fig. 2A). They maintained their morphologic characteristics at confluence (Fig. 2B). The cell morphology was comparable with earlier described primary cultures of human leptomeningeal cells (DeGiorgio et al, 1994; Rutka et al, 1986). Immunohistochemical stainings demonstrated that more than 95% of the cultured cells were positive for vimentin, which is an intermediate filament expressed on normal human leptomeningeal tissue (Kartenbeck et al, 1984). Moreover, the immunofluorescent cell staining for von Willebrand factor was negative, and no platelet endothelial cellular adhesion molecule-1 (PECAM-1) expression was found on the MLMC by immunofluorescence flow cytometry (Fig. 3A), indicating the absence of contaminating endothelial cells in the primary culture.

Identification of Cell Adhesion Molecules Present on MLMC and Murine B16F-10 Melanoma Cells

To determine the expression of surface adhesion molecules on MLMC and B16F-10 melanoma cells, immunofluorescence flow cytometry was performed. MLMC expressed low levels of VCAM-1, ICAM-1, β_1 - and β_3 -integrin subunits, and CD44. No expression of β_2 -integrin subunit, PECAM-1, L-selectin, and E-selectin was seen on MLMC (Fig. 3A, Table 1). No difference in expression levels of adhesion molecules was found between cells treated with trypsin during 2 minutes or with 10 mM EDTA (pH = 7.5) during 5 minutes, except for the CD44 expression (mean fluorescence intensity [MFI] for trypsin treated cells = 16

± 4 versus EDTA = 168 ± 21). B16F-10 melanoma cells expressed the α_4 -integrin subunit, β_1 - and β_3 -integrin subunits, and CD44. No expression of β_2 - and β_7 -integrin subunits, VCAM-1, ICAM-1, L-selectin, and E-selectin was seen (Fig. 3B, Table 1). Again the CD44 expression level was higher on the cell surface of EDTA-treated B16F-10 melanoma cells (MFI = 72 ± 2) as compared with trypsin-treated cells (MFI = 32 ± 9), without differences in expression levels of other adhesion molecules.

VCAM-1 Expression on MLMC After TNF- α Activation

Because VCAM-1 expression on endothelium is known to be up-regulated by cytokines like TNF- α (Rice and Bevilacqua, 1989), we determined whether VCAM-1 expression on MLMC can also be modified by TNF- α . MLMC were activated with TNF- α (40 ng/ml) during 4, 6, and 16 hours, after which VCAM-1 expression was measured by FACS analysis. No significant up-regulation of VCAM-1 on MLMC was seen after 4 and 6 hours of TNF- α stimulation (data not shown). TNF- α activation of MLMC during 16 hours induced a 2.9 ± 0.5 -fold increase of VCAM-1 expression on MLMC (mean $\pm$ SEM; Fig. 4).

Figure 1.

A, Culture of B16F-10 melanoma cells in decreasing percentages of original culture medium (DMEM with 40% FBS) with increasing percentages of cerebrospinal fluid resulted in a decreased proliferation after 72 hours (bars represent mean $\pm$ SEM). A significant decrease in proliferation was reached when 40% cerebrospinal fluid was added to the medium ($n = 3$; independent sample t test: $p < 0.05$). B, Addition of cerebrospinal fluid instead of saline to the culture medium (DMEM with 5% FBS) did not lead to a significant decrease of cell proliferation after 72 hours of cell culture. Bars show mean $\pm$ SEM ($n = 3$; independent sample t test: $p > 0.1$).

B16F-10 Melanoma Cell Adhesion Under Flow Conditions—Effect of B16F-10 Cell Concentration, Perfusion Time, and Shear Stress

Because tumor cells have to adhere to the leptomeninges under cerebrospinal fluid flow conditions (0–7 cm/seconds) (Henry-Feugeas et al, 1993), we used a modified form of a perfusion setup (Sakariassen et al, 1983) to determine B16F-10 melanoma cell adhesion to a leptomeningeal cell layer under flow conditions. The perfusion setup was validated by measuring the effect of B16F-10 cell concentration, time of perfusion, and shear stress on B16F-10 melanoma cell adhesion. Melanoma cell adhesion increased in a linear way using cell concentrations varying from 5×10^5 to 4×10^6 cells/ml (shear stress = 50 mPa; perfusion time = 5 minutes). A cell concentration of 8×10^6 cells/ml did not further increase melanoma cell adhesion (Fig. 5A). Adhesion of melanoma cells increased in a linear way with perfusion times ranging from 5 to 20 minutes (50 mPa; 4×10^6 melanoma cells/ml) (Fig. 5B). Cells started to spread on the leptomeningeal cell layer after 7.5 minutes. An inverse relation was seen between shear stress (50–250 mPa) and B16F-10 melanoma cell adhesion (4×10^6 melanoma cells/ml; 5 minutes) (Fig. 5C). Shear forces did not induce rolling of the melanoma cells but rather a direct tethering followed by firm adhesion of the cells on the leptomeningeal cell layer.

B16F-10 Melanoma Cell Adhesion Under Flow Conditions—Effect of mAbs Against VCAM-1, β_1 - and β_3 -Integrin Subunits, and CD44

Based on the expression levels of adhesion molecules on MLMC and melanoma cells, measured by immunofluorescence flow cytometry and the known ligand-receptor interactions (Table 2), we evaluated the effect of mAbs against CD44, β_1 - and β_3 -integrin subunits, and VCAM-1 on B16F-10 melanoma cell adhesion to a leptomeningeal cell layer under flow conditions (1.0×10^6 cells/ml; 50 mPa; 5 minutes). MLMC also expressed ICAM-1, but no expression of its ligands (β_2 integrins) was found on B16F-10 melanoma cells, so we did not test mAbs against ICAM-1. Melanoma cell adhesion under flow conditions was reduced by 60% after pretreatment of the leptomeningeal cell layer by an anti-VCAM-1 mAb as compared with an isotype-

Figure 2.

A, Light microscopic photograph of the mouse leptomeningeal cell (MLMC) culture at Day 2 after isolation of leptomeningeal tissue showing polygonal cells with cytoplasmic arc structures (arrows). B, Light microscopic photograph of the MLMC culture at day 5 demonstrating the cobblestone-like appearance at confluence.

matched control antibody (Fig. 6). No additive inhibitory effect on B16F-10 melanoma cell adhesion was seen when B16F-10 melanoma cells were also pretreated with a mixture of mAbs against β_1 - and β_3 -integrin subunits and CD44 (Fig. 6). Furthermore, pretreatment of B16F-10 melanoma cells with single mAbs against either β_1 - or β_3 -integrin subunits or CD44, without blockade of VCAM-1, did not result in a significant reduction of adhesion (β_1 -integrin subunit: 15% reduction [$n = 3$; $p = 0.2$]; β_3 -integrin subunit: 0% [$n = 3$]; CD44: 0% [$n = 3$] (data not shown).

B16F-10 Melanoma Cell Adhesion Under Static Conditions—Effect of mAbs Against VCAM-1, β_1 - and β_3 -Integrin Subunits, and CD44

We also evaluated the effect of mAbs against CD44, β_1 - and β_3 -integrin subunits, and VCAM-1 on B16F-10 melanoma cell adhesion to a leptomeningeal cell layer under static conditions, because cerebrospinal fluid flow may be absent at some localizations in the subarachnoid space (cerebrospinal fluid flow 0–7 cm/seconds) (Henry-Feugeas et al, 1993). Static B16F-10 melanoma cell adhesion during 30 minutes was not inhibited by pretreatment of leptomeningeal cells with anti-VCAM-1 nor by additional pretreatment of the B16F-10 melanoma cells with antibodies against β_1 - and β_3 -integrin subunits and CD44 (Fig. 7).

Discussion

For the first time, we demonstrate that tumor cells in cerebrospinal fluid do not proliferate because of a lack of nutrients and growth factors. This finding supports our hypothesis that tumor cell adhesion to the well-vascularized leptomeninges is needed for tumor cell proliferation. Earlier histologic studies of experimental LM showed that B16F-10 melanoma cell adhesion to the leptomeninges occurs in the early stage of LM, whereas in later stages larger well-vascularized tumor nodules were found (Reijneveld et al, 1999). Moreover, we have established that leukemia cell adhesion to the leptomeninges is crucial for the progression of leptomeningeal leukemia (JC Reijneveld et al, unpublished data). The reason why tumor cell adhesion to the leptomeninges would be so crucial in LM may be the efficient provision of nutrients and growth factors to the adhered tumor cells via the well-vascularized leptomeninges. Furthermore, the leptomeningeal cells themselves may provide survival and proliferation signals to the adhered tumor cells, a process called anchorage dependency (Ruoslahti, 1997; Schwartz, 1997).

Adhesion of tumor cells to the leptomeninges has to take place under cerebrospinal fluid flow conditions, with flow velocities varying from 0–7 cm/seconds, dependent on the localization in the subarachnoid space (Henry-Feugeas et al, 1993). In the underlying study we show that VCAM-1, expressed on primary cultures of murine leptomeningeal cells, plays a key role in B16F-10 melanoma cell adhesion to a leptomeningeal cell layer under (cerebrospinal fluid-like)

flow conditions. Since B16F-10 melanoma cells express α_4 - and β_1 -integrin subunits and no β_7 -integrin subunits, VCAM-1 on leptomeningeal cells interacts with $\alpha_4\beta_1$ integrin (VLA-4) on melanoma cells and not with the other identified VCAM-1 ligand, the $\alpha_4\beta_7$ integrin. We found that pretreatment of the leptomeningeal cells with an anti-VCAM-1 mAb led to a 60% inhibition of B16F-10 melanoma cell adhesion under flow conditions but not under static conditions. This strong reduction of melanoma cell adhesion under flow conditions by blocking VCAM-1 may be explained by the important role of the VCAM-1/VLA-4 interaction in the initial attachment of the B16F-10 melanoma cells with the leptomeningeal cell surface. VCAM-1 has been shown to support leukocyte tethering (=short transient interactions), rolling and rapid sticking, and arrest (Alon et al, 1995; Berlin et al, 1995), followed by firm adhesion, a process mediated by integrins (Butcher, 1991; Springer, 1994). An important role for VCAM-1/VLA-4 in the initial attachment of melanoma cells also explains the lack of effect of anti-VCAM-1 mAbs on B16F-10 melanoma cell adhesion under static conditions, since cell tethering can be overruled by firm adhesion during static cell-cell interactions. Surprisingly, no additional inhibitory effect on melanoma cell adhesion under static and flow conditions was found by concomitant pretreatment of B16F-10 melanoma cells with mAbs against β_1 - and β_3 -integrin subunits and CD44, molecules that are all important in firm adhesion. This lack of inhibitory effect under both conditions does not necessarily indicate that β_1 - and β_3 -integrin subunits and CD44 are not important in firm adhesion of melanoma cells to the leptomeninges. Rather, these results may be explained by a redundancy of other, unknown adhesion molecules important in the adhesion process.

The crucial role for VCAM-1 in *in vitro* melanoma cell adhesion to the leptomeninges, found in this study, is comparable with its role in *in vitro* melanoma cell adhesion to the endothelium (Bertomeu et al, 1993; Dejana et al, 1988; Rice et al, 1988). Interestingly, VCAM-1/VLA-4 interaction mediating melanoma adhesion to endothelium was shown to be critically involved in *in vivo* melanoma lung metastasis (Garofalo et al, 1995; Okahara et al, 1994). Okahara et al (1994) found that TNF- α intravenously administered before B16-BL6 inoculation significantly enhanced pulmonary metastasis in C57BL6 mice. Pretreatment of the B16-BL6s cells with an anti-VLA-4 mAb or administration of an anti-VCAM-1 mAb abolished this enhancement. Similarly, IL-1-mediated augmentation of pulmonary metastasis of A375M melanoma cells in nude mice was completely abrogated by an anti-VLA-4 mAb (Garofalo et al, 1995).

Our findings point to a model for LM in which VCAM-1 on leptomeningeal cells mediates tumor cell adhesion under cerebrospinal fluid flow conditions (Fig. 8). Tumor cells adhered to the well-vascularized leptomeninges start proliferating and ultimately form large-size tumor cell layers or nodules, which become dependent on angiogenesis (Reijneveld et al, 2002). In contrast, nonadhered cells in the cerebrospinal fluid

Figure 3.

FACS expression patterns of adhesion molecules on mouse leptomeningeal and murine B16F-10 melanoma cells. *Open curves* indicate FACS histogram plots of cells stained with a mAb against a specific adhesion molecule. *Solid curves* represent FACS histogram plots of cells stained with an isotype-matched antibody. MLMC express β_1 - and β_3 -integrin subunits, CD44, VCAM-1, and ICAM-1. No expression of the β_2 -integrin subunit, PECAM-1, L-selectin, and E-selectin was seen on the cell surface of leptomeningeal cells. B16F-10 melanoma cells express α_4 -, β_1 -, and β_3 -integrin subunits and CD44. No expression of β_2 - and β_7 -integrin subunits, VCAM-1, ICAM-1, L-selectin, and E-selectin was seen on the cell surface of B16F-10 melanoma cells. One representative FACS experiment of three experiments performed in duplicate is plotted.

do not multiply because of a lack of nutrients and growth factors. TNF- α , either produced by the tumor cells or macrophages entering the cerebrospinal fluid or systemically leaking in the cerebrospinal fluid, may facilitate this adhesion process via an up-regulation of VCAM-1 expression on the leptomeningeal cells. Immunocytochemical detection of TNF- α in tumor cells present in the cerebrospinal fluid in patients with LM (Nakamura et al, 1995) and high levels of TNF- α levels in the cerebrospinal fluid in children with central nervous system leukemia support this hypothesis (Ishii et al, 1991). Further research on the factors determining the process of tumor cell adhesion to the leptomeninges is in progress. This research may eventually direct the way to new prophylactic antiadhesive treatment strategies like anti-VCAM mAbs, intrathecally administered in patients with a high risk for LM, eg, patients who undergo surgery for a brain metastasis in the posterior fossa and consequently have an elevated risk of developing LM because of tumor cell shedding into the cerebrospinal fluid (van der Ree et al, 1999) and patients with acute lymphoblastic leukemia or high-grade non-Hodgkin's lymphoma currently

receiving potential toxic, prophylactic intrathecal chemotherapy.

Methods

Antibodies and Cytokines

The following functional blocking mAbs were purchased from PharMingen (San Diego, California): purified rat monoclonal IgGs against mouse integrin α_4 chain (CD49d; clone R1-2), mouse integrin β_1 chain (CD29; clone 9EG7), mouse integrin β_2 chain (CD18; clone GAME-46), mouse integrin β_7 chain (clone FIB27), mouse CD44 (clone KM114), mouse VCAM-1 (CD106; clone 429 [MVCAM.A]), mouse PECAM-1 (CD31; clone MEC 13.3), mouse E-selectin (CD62E; clone 10E9.6), and mouse L-selectin (CD62L; clone MEL-14), and purified hamster monoclonal IgGs against mouse ICAM-1 (CD54; clone 3E2) and mouse integrin β_3 chain (CD61; clone 2C9.G2). Furthermore, purified rat IgG₁ κ (clone R3-34), hamster IgG₁ (clone G235-2356), and rat IgG₂ α (clone R35-95) isotype control immunoglobulins and FITC-conjugated mouse anti-rat IgG₂ α (clone G28-5) and anti-hamster IgG_{1/2b}

Table 1. Expression of Specific Adhesion Molecule Expression on Mouse Leptomeningeal Cells and Murine B16F-10 Melanoma Cells^a

Adhesion molecule	Mean fluorescence intensity					
	Mouse leptomeningeal cells			Murine B16F-10 melanoma cells		
	Specific adhesion molecule	Isotype control	<i>p</i> value	Specific adhesion molecule	Isotype control	<i>p</i> value
α_4 integrin subunit	6 (2)	5 (0)	0.0826	17 (1)*	6 (0)	0.0003
β_1 -integrin subunit	26 (3)*	6 (1)	0.0011	19 (5)*	5 (0)	0.0290
β_2 -integrin subunit	6 (1)	7 (1)	0.6026	4 (0)	4 (0)	0.1526
β_3 -integrin subunit	10 (0)*	6 (0)	0.0001	11 (1)*	6 (1)	0.0005
β_7 -integrin subunit	5 (1)	5 (0)	0.9886	5 (0)	5 (0)	0.1676
VCAM-1	19 (4)*	6 (1)	0.0134	5 (0)	5 (0)	0.1447
ICAM-1	28 (1)*	6 (0)	0.0000	6 (1)	6 (1)	0.6906
PECAM-1	5 (0)	6 (1)	0.4894	Not tested	Not tested	
CD44	16 (2)*	9 (1)	0.0153	32 (5)*	4 (1)	0.0027
E-selectin	6 (1)	6 (1)	0.8214	5 (0)	5 (0)	0.0707
L-selectin	5 (0)	6 (1)	0.7201	5 (1)	5 (0)	0.3249

^a Measured by immunofluorescence flow cytometry after trypsin treatment. The mean fluorescent intensity ($\pm$ SEM) of the specific adhesion molecules and isotype controls of three experiments performed in duplicate is shown. Mean fluorescence intensities of specific adhesion molecules identified with asterisks are significantly higher than the isotype control values (independent sample *t* test: *p* < 0.05).

Figure 4.

VCAM-1 expression on MLMC after 16 hours of TNF- α stimulation, as determined by FACS analysis. The solid curve represents a FACS histogram plot of cells stained with an isotype-matched antibody. The gray, open curve shows the basal VCAM-1 expression on MLMCs, and the black, open curve demonstrates the VCAM-1 expression after 16 hours of TNF- α stimulation. One representative FACS experiment of four single experiments is plotted. The mean fold up-regulation of VCAM-1 by 16 hours of TNF- α stimulation was 2.9 ± 0.5 (mean $\pm$ SEM; *n* = 4; independent sample *t* test: *p* < 0.05).

(clones G70-204 and G94-56) were purchased from PharMingen. FITC-conjugated donkey anti-rat IgG (H+L) was obtained from Jackson ImmunoResearch (West Grove, Pennsylvania). Polyclonal rabbit anti-human von Willebrand factor, cross-reacting with mouse tissue, was purchased from Dako (Glostrup, Denmark). Peroxidase-conjugated swine anti-rabbit antibody was obtained from Dako. Recombinant human TNF- α was purchased from SanverTech (Heerhugowaard, The Netherlands).

Reagents

Tissue culture supplies (culture media, FBS, antibiotics, and trypsin) were obtained from GIBCO Biocult

(Grand Island, New York). Soybean trypsin inhibitor and DNase I were purchased from Sigma Chemical Company (St. Louis, Missouri). EDTA was obtained from Riedel de Haen (Seelze, Germany).

MLMC

MLMC were obtained through a modified protocol for the isolation of rat leptomeningeal cells (Ness and David, 1997). Briefly, leptomeninges were carefully dissected from the cortical surface of 2-day-old neonatal balb/c mice and trypsinized with 0.25% trypsin for 30 minutes at 37° C. After neutralizing trypsin with DMEM containing 0.025% trypsin inhibitor and 0.004% DNase I, cells were centrifuged (1250 rpm, 5 minutes, room temperature), resuspended, and plated in poly-L-lysine-coated plates. MLMC were cultured in DMEM containing sodium pyruvate and nonessential amino acids, supplemented with 10% FBS, 1% penicillin/streptomycin/L-glutamine, and 0.1% amphotericin. Cells were incubated in 5% CO₂-95% air at 37° C and passaged two or three times before use. Von Willebrand factor staining was performed, and PECAM-1 expression levels were measured to exclude a possible contamination with endothelial cells. Leptomeningeal cell layers for adhesion assays were obtained by administering 3.5×10^5 MLMC (400 μ l, 8.75×10^6 cells/ml) in a meniscus on a poly-L-lysine-coated coverslip (18 $\times$ 18 mm) in a 6-well plate. After 20 minutes, 2 ml of culture medium was added to the well. Two days later, confluent cell layers were used in the adhesion assays.

Murine B16F-10 Melanoma Cells

The murine B16F-10 melanoma cell line was originally obtained from I. J. Fidler (M.D. Anderson Cancer Center, Houston, Texas) (Fidler, 1975). Melanoma

Figure 5.

B16F-10 melanoma cell adhesion to a leptomeningeal cell layer under flow conditions: effect of B16F-10 melanoma cell concentration (A), perfusion time (B), and shear stress (C). Number of adherent melanoma cells/mm² are plotted on the y axis. A, Melanoma cell adhesion increased in a linear way using cell concentrations from 5×10^5 to 4×10^6 cells/ml (50 mPa, 5 minutes). Cell concentration of 8×10^6 cells/ml did not further increase melanoma cell adhesion. B, Adhesion of melanoma cells increased in a linear way with perfusion times ranging from 5 to 20 minutes (50 mPa; 4×10^6 melanoma cells/ml). C, An inverse relation was seen between shear stress (50–250 mPa) and B16F-10 melanoma cell adhesion (4×10^6 melanoma cells/ml; 5 minutes). Data represent the mean of three independent experiments $\pm$ SEM.

cells were maintained as adherent monolayers in noncoated, plastic culture flasks in DMEM, supplemented with 5% FBS and 1% penicillin/streptomycin/L-glutamine and incubated in 5% CO₂-95% air at 37° C. For adhesion assays, adherent melanoma cells were washed twice with PBS and incubated with trypsin at 37° C for 2 minutes. Trypsin was neutralized with serum-supplemented DMEM, and the detached cells were centrifuged (1500 rpm, 5 minutes, room temperature) and resuspended in DMEM supplemented with 1% FCS and 1% penicillin/streptomycin/L-glutamine. B16F-10 melanoma cells cell counts

were performed using a cell counter (Particle Counter; Coulter, Becton Dickinson, San Jose, California).

Cerebrospinal Fluid

Cerebrospinal fluid samples were obtained from patients with hydrocephalus or non-neurologic disease at the department of Neurology of the University Medical Center Utrecht, The Netherlands. Cell count and protein and glucose levels were within normal limits in these samples. Informed consent was obtained from these patients. The specimens were centrifuged at room temperature (10 minutes, 1250 rpm), and supernatants were immediately stored in polyethylene tubes at -80° C.

Proliferation Assay

For proliferation assays, B16F-10 melanoma cells were seeded in low density (10^4 cells per well) in noncoated 24-well plates and cultured overnight. The next day, 8 to 12 randomly chosen wells were gently washed twice with PBS and incubated with trypsin (15 minutes, 37° C). The cell suspension was transferred to 10 ml of Isoton (Baker-Mallincrodt, Deventer, The Netherlands). The number of cells in each well was assessed using a cell counter (Particle Counter). The average of these counts was used as the number of cells per well at the start of the experiment ($t = 0$ hours). Subsequently, wells were incubated with the selected culture medium ($n = 3$ per condition). After 72 hours, cell numbers were determined in the same way as at $t = 0$ hours. Vitality of the cells was determined by trypan blue staining.

Immunofluorescence Flow Cytometry

Expression of surface adhesion molecules was determined using immunofluorescence flow cytometry. Expression levels were measured after both trypsin and EDTA treatment, because trypsin is known to decrease the expression of adhesion molecules such as CD44 (Gardner et al, 1995). MLMC or B16F-10 melanoma cells were treated with trypsin (2 minutes) or with 10 mM EDTA (pH = 7.5, 5 minutes) and neutralized with DMEM supplemented with FBS, centrifuged, and washed twice in cold PBS (4° C). Cells were resuspended in cold PBS supplemented with 1% BSA (4° C) and distributed in a concentration of 1 to 2×10^5 cells/sample in a 96-well plate. Cells were centrifuged (1250 rpm, 3 minutes, 4° C) and incubated in 50 μ l of 1:10 diluted antibody in PBS/1% BSA (60 minutes, 4° C). Subsequently, cells were washed three times in PBS supplemented with 1% BSA (4° C) and incubated in 50 μ l of 1:50 diluted, FITC-labeled, secondary antibody (30 minutes, 4° C). After washing twice with PBS/1% BSA (4° C), stained cells were analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, California). Of each sample, forward scatter, side scatter, and fluorescence of 5×10^3 cells were recorded. Samples that were incubated with first step, isotype-matched, nonreactive antibodies in PBS/1% BSA served as negative controls. The

Table 2. Known Receptor-Ligand Interactions of Cell Surface Adhesion Molecules, of Which Expression Was Determined on B16F-10 Melanoma and Leptomeningeal Cells by FACS Analysis

Cell adhesion molecule (receptor)	Cell adhesion molecule (ligand)
VCAM-1	$\alpha_4\beta_1$ integrin (VLA-4), $\alpha_4\beta_7$ integrin
$\alpha_1\beta_1$ - $\alpha_6\beta_1$ integrins	Laminin, collagen, fibronectin
$\alpha_4\beta_1$ integrin (VLA-4)	VCAM-1, fibronectin
$\alpha_v\beta_1$ integrin	Fibronectin
$\alpha_L\beta_2$ integrin (LFA-1)	ICAM-1
$\alpha_M\beta_2$ integrin (MAC-1)	ICAM-1
$\alpha_v\beta_3$ integrin	Vitronectin, fibrinogen, thrombospondin, von Willebrand's factor, fibronectin
CD44	Hyaluronic acid
L-selectin	Sialic acid, sialyl Lewis X and A, carbohydrate chains
E-selectin	Sialyl Lewis X and A, LECAM-1
PECAM-1	PECAM-1, Sialyl Lewis A

Figure 6.

Melanoma cell adhesion to a leptomeningeal cell layer under flow conditions: effect of blocking adhesion molecules with mAbs. The leptomeningeal cell layers were either incubated with isotype-matched control antibodies, anti-VCAM-1, or anti-VCAM-1 and concomitant incubation of B16F-10 melanoma cells with mAbs against β_1 - and β_3 -integrin subunits and CD44 (10 μ g/ml) for 15 minutes at 37° C. Melanoma cells (10⁶ cells/ml) were perfused over a leptomeningeal cell layer for 5 minutes at shear stress 50 mPa, and the number of adhered melanoma cells/mm² was measured. Blocking VCAM-1 on the leptomeningeal cell layer resulted into a 60% inhibition of melanoma cell adhesion under flow conditions as compared with the isotype-matched control (bar 2 versus bar 1; $n = 9$; independent sample t test: $p < 0.001$). No additive effect on melanoma cell adhesion was seen by also blocking β_1 - and β_3 -integrin subunits and CD44 on the B16F-10 melanoma cells as compared with blocking VCAM-1 on the leptomeningeal cells alone (bar 3; $n = 9$; independent sample t test: $p = 0.7$).

adult aorta-derived mouse endothelial tumor cell line served as a positive control for E-selectin, VCAM-1, and PECAM-1 expression. The leukemia L1210 adherent subcell line, cultured in our laboratory, was used as a positive control for α_4 -, β_1 -, and β_3 -integrin subunit, CD44, and ICAM-1 expression. Mouse leukocytes served as positive controls for the expression of L-selectin and β_2 - and β_7 -integrin subunits.

In Vitro Tumor Cell Adhesion Model Under Flow Conditions

Adhesion experiments under flow conditions were performed in a modified form of a transparent perfusion chamber (Sakariassen et al, 1983). The microchamber has a slit height of 0.2 mm and width of 2 mm

Figure 7.

Melanoma cell adhesion to a leptomeningeal cell layer under static conditions: effect of blocking adhesion molecules with mAbs. Leptomeningeal cell layers were either incubated with isotype-matched control antibodies, anti-VCAM-1, or anti-VCAM-1 and concomitant incubation of B16F-10 melanoma cells with mAbs against β_1 - and β_3 -integrin subunits and CD44 (10 μ g/ml) for 15 minutes at 37° C. The mean number of melanoma cells on a leptomeningeal cell surface (6.5 mm²) after 30 minutes of static adhesion was measured. B16F-10 melanoma cell adhesion was not inhibited by pretreatment of leptomeningeal cells with anti-VCAM-1 nor by additional pretreatment of the B16F-10 melanoma cells with antibodies against β_1 - and β_3 -integrin subunits and CD44. The mean number of adhered melanoma cells/mm² $\pm$ SEM of seven independent experiments was shown.

Figure 8.

Schematic model of the pathophysiologic processes in the cerebrospinal fluid in leptomeningeal metastases (LM). I. Tumor cells (t) enter the cerebrospinal fluid and move through the subarachnoid space under varying cerebrospinal fluid flow (0–7 cm/seconds). Some of the tumor cells adhere to the leptomeningeal cells via receptor-ligand interactions between both cell types. II. Adhered tumor cells proliferate as a result of growth signaling induced by tumor cell adhesion to the leptomeninges and/or the provision of nutrients via the leptomeningeal vasculature. III. Large tumor nodules/layers are being formed, which become dependent on angiogenesis for further growth. CSF = cerebrospinal fluid.

and contains a plug on which a coverslip (18 mm × 18 mm) with confluent leptomeningeal cells can be mounted. B16F-10 melanoma cells in suspension were aspirated from a reservoir through the perfusion chamber with a Harvard syringe pump (Harvard Apparatus, South Natic, Massachusetts). In this way, the flow rate through the chamber could be controlled precisely. The wall shear stress (t) was calculated from the equation: $t = (6Q \cdot \eta) / (w \cdot h^2)$ in which Q is the flow rate, η is the suspending medium viscosity, w is the slit width, and h is the slit height. Shear stress was calculated in milli-Pascal. During the perfusion, the flow chamber was mounted on a microscope stage (DM RXE; Leica, Wetzlar, Germany), equipped with a B/W CCD-video camera (Sanyo, Osaka, Japan) coupled to a VHS video recorder. Perfusion experiments were recorded on videotape. Video images were analyzed for the number of adherent melanoma cells with a Quantimet 570C image-analysis system (Leica Cambridge, Cambridge, United Kingdom). The number of surface-adherent melanoma cells per square millimeter was measured after 5 minutes of perfusion at a minimum of 25 fields (total surface $\geq 1.0 \text{ mm}^2$) using custom-made software developed in Optimas 6.1 (Media Cybernetics Systems, Silver Spring, Maryland). To determine the effect of blocking specific adhesion molecules on melanoma cell adhesion under flow conditions, B16F-10 melanoma cells and the leptomeningeal cell layer were preincubated with specific or isotype-matched control mAbs (10 $\mu\text{g/ml}$, 15 minutes at 37° C).

In Vitro Tumor Cell Adhesion Model Under Static Conditions

For in vitro adhesion assays under static conditions, B16F-10 melanoma cells were washed with PBS, treated with trypsin (37° C, 2 minutes), and neutralized with DMEM supplemented with FBS. Cells were centrifuged, washed with PBS again, and resuspended in DMEM without phenol red and sodium pyruvate (GIBCO Biocult) supplemented with 10% FCS. B16F-10 melanoma cells were labeled with 0.5 μM calcein (Molecular Probes, Leiden, the Netherlands; 5 minutes, 37° C). After the labeling procedure, cells were centrifuged (1250 rpm, 5 minutes, room temperature) and resuspended in DMEM without phenol red and sodium pyruvate supplemented with 10% FCS. Leptomeningeal cells and B16F-10 melanoma cells were incubated with specific or isotype-matched control antibodies (10 $\mu\text{g/ml}$, 15 minutes, 37° C). Static adhesion assays were performed by adding 4.0×10^5 fluorescently labeled, pretreated B16F-10 cells (>90% viability) to a well containing a coverslip with a confluent pretreated leptomeningeal cell layer. After 30 minutes of adhesion, cells were washed three times with PBS and fixated with 2% paraformaldehyde. Five FITC images (1.3 mm^2/image) per coverslip were obtained of the central area of the leptomeningeal cell layer using a fluorescence microscope (Leica DM IRHC; Leica Microsystems BV, Rijswijk, The Netherlands). The confluence of the leptomeningeal cell layer

was checked by light microscopy. The number of adherent B16F-10 melanoma cells per FITC image was determined by quantitative analysis using Leica WIN software (Leica Microsystems BV).

Statistical Analysis

Results of the adhesion assays are expressed as mean number of adherent cells $\pm$ standard error of the mean (SEM). Statistical analysis of the data was performed using the independent sample Student's *t* test. *p* values < 0.05 were considered to be significant.

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