

Mannose Receptor (MR) and Common Lymphatic Endothelial and Vascular Endothelial Receptor (CLEVER)-1 Direct the Binding of Cancer Cells to the Lymph Vessel Endothelium¹

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ABSTRACT

Although ~50% of cancers give rise to metastases via the lymphatic system, the mechanisms mediating this process have remained unknown. In this study, we have investigated the role of two lymphatic endothelial molecules, the mannose receptor (MR) and common lymphatic endothelial and vascular endothelial receptor (CLEVER)-1 in adhesion of malignant cells to the lymphatic endothelium, and analyzed their expression in two clinical series consisting of squamous cell cancers of the head and neck ($n = 17$) and breast cancers ($n = 72$). Affinity of the tested head and neck cancer cell lines to the lymphatic endothelium varied greatly, but adhesion of all cell lines was dependent on both the MR and CLEVER-1. Almost all cancer specimens contained peritumoral vessels that expressed CLEVER-1 and MR, and also the intratumoral lymph vessels often expressed them in both tumor types. However, only intratumoral expression of these molecules seems to be essential for metastatic spread to the regional lymph nodes. Only 8 (22%) of the 36 axillary node-negative breast carcinomas expressed the MR on the intratumoral lymph vessels as compared with 16 (50%) of the 32 node-positive carcinomas ($P = 0.017$), and all eight head and neck carcinoma patients with regional lymph node metastases at diagnosis had tumors that expressed CLEVER-1 on the intratumoral lymph vessels. These data suggest a role for both the MR and CLEVER-1 in directing the traffic of cancer cells within the lymphatic system.

INTRODUCTION

Growth of cancer beyond a tumor size of ~2 mm requires tumor angiogenesis (1), but much less is known about the need of tumor-associated lymph vessel growth. In general, vascular endothelial growth factor-C is an important inducer of lymphatic vessel growth (2, 3). It has long been known that an association exists between the tumor microvessel density and tumor metastatic potential at least in some histological types of human cancer (4), but the role of tumor lymphatic vessel density and the lymphatic vessel architectural pattern in dissemination of cancer has remained unexplored until recently.

Identification of lymphatic endothelial molecules, such as the β -chemokine receptor D6 (5), the hyaluronan-binding receptor LYVE-1 (6), VEGFR-3³ (7), podoplanin (8), and Prox-1 (9), has given novel tools for examining tumor lymphatic structures and their clinical significance. HNSCC and breast carcinoma are examples of human cancers that frequently metastasize via the lymphatic vessels and also give rise to lymph node metastases. Recent data suggest that the density of intratumoral LYVE-1-positive lymph vessels correlates with the presence of cervical lymph node metastases at the time of

diagnosis in oropharyngeal HNSCC (10, 11). Similarly, in some breast cancer models, a high density of vascular endothelial growth factor-C-induced LYVE-1-positive lymph vessels is associated with the presence of lymph node metastases (12, 13). On the other hand, there are also some data suggesting that intratumoral lymphatic vessels may not always be functional and that the presence of functional peritumoral lymphatic vessels alone may be sufficient for the development of metastases (14).

The mechanisms of cell traffic within the lymphatics are not known. According to our recent findings, both the MR and CLEVER-1 can be detected on the normal lymph endothelium, and they direct the traffic of lymphocytes within the lymphatic vessels (15, 16). Macrophage MR is known as an important molecule involved in the phagocytosis of microbes. MR expression on the vasculature, on the other hand, is strictly restricted to the lymphatic endothelium, where it supports lymphocyte adherence by binding to L-selectin on the lymphocyte surface (15). The function of CLEVER-1, in turn, has remained unknown until very recently. Its expression is not restricted to the lymphatic endothelium, because it is also expressed on the HEVs in the lymphatic tissues and on HEV-like vessels at the sites of inflammation (16).

In the present study, we investigated MR and CLEVER-1 expression in the lymphatic vessels of the head and neck and breast carcinomas and studied whether these adhesion molecules might be involved in the adhesive interaction between tumor cells and the endothelial cells. The results suggest that both the MR and CLEVER-1 may be important mediators not only in cancer cell adhesion to the lymphatic endothelium but that CLEVER-1 mediates tumor cell binding to HEVs as well. Therefore, these molecules might be potential targets for novel anticancer therapies.

MATERIALS AND METHODS

Tissue Samples and Cell Lines. Tumor tissue biopsies taken from 17 patients with primary HNSCC untreated previously were removed for diagnostic and/or therapeutic purposes. The tumor tissue specimens were divided into two pieces within a few minutes after their removal; a part of the specimen was formalin fixed for diagnostic purposes, and the remaining part was snap frozen in liquid nitrogen for additional analyses. In addition, lymph nodes were obtained from neck dissection specimens when they were not needed for diagnostic purposes. In addition, fresh unfixed tissue was taken from 72 women in conjunction of breast surgery performed for suspected breast cancer and stored in liquid nitrogen for further use. All cell and tissue sampling protocols used in this study were approved by an Institutional Review Board, and an informed consent was obtained from all study subjects.

The cell lines used for the *in vitro* adhesion assays were KCA and IBW4 (B lymphoblastoid cell lines; gifts from Dr. E. Engelman and Dr. H. Kaplan, respectively, Stanford University, Stanford, CA), CRL-1648 (a Burkitt's lymphoma cell line, American Type Culture Collection, Manassas, VA), and 3 HNSCC cell lines: (a) UT-SCC-12A ($T_2N_0M_0$, grown from a grade 1 primary head and neck skin cancer, F81y); (b) UT-SCC-38 ($T_2N_0M_0$, grade 2 primary larynx cancer, M66y); and (c) UT-SCC-50 ($T_2N_0M_0$, grade 3 recurrent larynx cancer, M70y; Ref. 17).

Immunohistochemistry. Six- μ m-thick frozen sections were cut from the tumors for immunohistochemistry, air dried, and acetone fixed. The sections

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³ The abbreviations used are: VEGFR, vascular endothelial growth factor receptor; MR, mannose receptor; CLEVER-1, common lymphatic endothelial and vascular endothelial receptor-1; SCC, squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; HEV, high endothelial venule.

were overlaid with the first stage monoclonal antibody (50 $\mu\text{g/ml}$) and incubated for 30 min at room temperature in a humidified chamber. The primary antibodies used were 3-155 (anti-MR, IgG; Ref. 15), 3-372 (anti-CLEVER-1, IgG1; Ref. 16), and 3G6 (a negative control antibody against chicken T cells, IgG1; Ref. 18). After two washings in PBS, peroxidase-conjugated rabbit antimouse immunoglobulin in PBS containing 5% AB-serum was added. 3,3-diaminobenzidine (Polysciences, Inc., Warrington, PA) in PBS containing 0.03% hydrogen peroxide was used as the chromogen. Finally, the sections were counterstained with hematoxylin (Sigma Chemical Co., St. Louis, MO), dehydrated, cleared in xylene, and permanently mounted in DePex (BDH Limited, Pool, Dorset, England).

MR and CLEVER-1 expression of intratumoral and peritumoral lymphatic vessels was classified semiquantitatively. In this system, both the number and intensity of positive vessels were analyzed in the following manner: (a) score 0 was assigned to samples with no positive vessels; (b) +/- to very weak staining and the presence of a single positive lymph vessel within the tissue section; (c) + corresponds to weak staining of two to nine positive vessels in the section; and (d) +++ to abundant lymphoid vasculature (>20 brightly stained vessels in the tissue section). ++ was given to samples falling in between categories + and +++. Receptor expression was evaluated separately in the peritumoral and intratumoral areas. Vessels surrounded by malignant cells completely or partially were considered intratumoral, and vessels outside the foci of malignant cells were scored peritumoral. The minimum area examined was 50 mm^2 using an Olympus BX40 microscope (Olympus, Hamburg, Germany; $\times 200$).

Fluorescence Microscopy. FITC-conjugated antibody 3-155 (50 $\mu\text{g/ml}$), unconjugated 3-372 (50 $\mu\text{g/ml}$), anti-PAL-E (1:50; Abcam, Cambridge, United Kingdom), and the goat polyclonal anti-VEGFR-3 antibody (10 $\mu\text{g/ml}$, VEGFR-3; R&D Systems, Minneapolis, MN) were used as the primary antibodies in fluorescence immunostainings. Tetramethyl rhodamine isothiocyanate-conjugated rabbit anti-goat IgG (Zymed, San Francisco, CA) with 5% AB-serum was used as the secondary antibody for anti-VEGFR-3 stainings, and R-phycoerythrin conjugated antimouse IgG1 (Southern Biotechnology Associates, Inc.) with 5% AB-serum was used as the secondary antibody for anti-CLEVER-1 and R-phycoerythrin-conjugated antimouse IgG2a for anti-PAL-E. When both CLEVER-1 and VEGFR-3 or PAL-E was assessed from the same tissue section, CLEVER-1 was visualized with FITC-conjugated antimouse IgG1 (Southern Biotechnology Associates, Inc.). After staining, the samples were mounted with ProLong Antifade Kit (Molecular Probes, Inc., Eugene, OR). Negative controls in this staining were 3G6, FITC-conjugated 3G6, and normal goat serum (Vector Laboratories Inc., Burlingame, CA).

In Vitro Adhesion Assay. To evaluate the binding of malignant cells to HEVs, we used *in vitro* Stamper-Woodruff adhesion assay as described in detail elsewhere (19). Additional modifications were done to customize the conditions for cell binding to the lymphatic endothelium (15). In brief, 8- μm frozen sections were cut from the lymph nodes. Sections were incubated with saturating levels of 3-155 (only in the static assays), 3-372, or a control antibody directed against human HLA ABC (HB 95; American Type Culture Collection). Antibody incubations were performed under constant rotation for 30 min at 7°C. In the classical type of the adhesion assay that assesses binding of cells to HEVs, the malignant cell suspension was overlaid on the HEVs, and the sections were incubated under constant rotation at 60 rpm for another 30 min at 7°C. In the static assay, which mimics the conditions on the lymphatic endothelium, the malignant cells were overlaid, and the sections were incubated in static conditions for 15 min, followed by rotation at 60 rpm for 5 min, and again without rotation for another 15 min at 7°C. The adherent cells were fixed in 1% glutaraldehyde. The number of malignant cells bound to the vessels was counted under a dark-field illumination microscope ($\times 200$; Leitz Aristoplan, Oberkochen, Germany). In each experiment, the binding of each cell line after different antibody treatments was analyzed to two to four lymph node sections from one patient. Lymph nodes from three different patients were used in these assays. In each static assay, all lymphatic sinusoids (~30/sample) were counted, and in each nonstatic assay, ≥ 80 HEVs were counted for each cell type after each treatment. To be able to compare experiments performed on different days, the results of the inhibition assays are presented as a percentage of binding found in control slides, where the number of cells adherent to the vessels in the presence of the control monoclonal antibody was taken as 100% adherence.

Statistical Analyses. Student's *t* test was used to compare the numbers of adherent cells to the endothelium in the presence of a blocking antibody or control antibody. The frequency tables were analyzed using the χ^2 test or Fisher's exact test.

RESULTS

Adhesion of Malignant Cells to the Lymphatic Endothelium and HEVs Vary. The binding of malignant cells to the lymphatic endothelium and HEVs was investigated using *in vitro* adhesion assays. Adhesion of different types of malignant cells both to the HEVs and lymphatic endothelium varied considerably (Table 1). When comparing the binding to lymphatic endothelium and HEVs, it is important to notice that in general, lymphatic sinusoids are much larger structures than HEVs, which vary considerably by their size in the section. Many are so small that no more than two to three malignant cells have space to bind to them. Cells of SCC cell lines (UT-SCC-12A and UT-SCC-38) bound very efficiently both to the lymphatic endothelium and HEVs (Table 1; Fig. 1a). In contrast, UT-SCC-50 cells bound poorly (less than one cell per a sinusoid and practically none per one HEV; Fig. 1c), so that no meaningful antibody inhibition assays could be done using this cell line.

Both α -MR and α -CLEVER-1 Antibodies Effectively Reduced Adhesion to the Lymphatic Endothelium. Adhesion of all malignant cell lines tested to the lymphatic endothelium was reduced statistically significantly by both the anti-MR (3-155) antibody and anti-CLEVER-1 (3-372) antibody (Figs. 1b and 2, a and b). This indicates that both the MR and CLEVER-1 may play a key role in malignant cell binding to the lymphatic endothelium of lymph nodes in lymphomas and squamous cell head and neck carcinoma.

CLEVER-1 Mediates Adhesion of Malignant Cells Also to the HEVs. In contrast to the MR, CLEVER-1 is expressed also on the endothelium of the HEVs. We tested involvement of CLEVER-1 in adhesion of malignant cells to the HEVs under shear using the classical Stamper-Woodruff adhesion assay (Fig. 2c). The α -CLEVER-1 antibody 3-372 reduced significantly adhesion of both lymphoblastoid cell lines, KCA and IBW4, and that of UT-SCC-38 to HEVs, indicating that CLEVER-1 on blood vessel endothelium is also able to bind different types of malignant cells.

Peritumoral and Intratumoral Lymphatic Vessels Express Commonly MR and CLEVER-1 in Head and Neck and Breast Carcinomas. After studying the role of the MR and CLEVER-1 in tumor cell adhesion to the lymphatic endothelium and HEVs (only CLEVER-1), we investigated HNSCC and breast cancer tissue samples with immunohistochemistry for the MR and CLEVER-1 expression (Table 2). The peritumoral lymphatic vessels expressed both the MR and CLEVER-1 in all 17 HNSCCs (Fig. 3). Both adhesion molecules were also detected on some enlarged, thin walled, almost sinusoidal-appearing lymphatic lacunas (Fig. 3d). As expected, the HEV-like vessels expressed CLEVER-1 (Fig. 3b). However, the intensity of immunostaining and proportion of stained vessels varied considerably from one tumor to another, and the staining pattern was

Table 1 Binding of malignant cells to lymphatic endothelium and HEV

Cell line	Binding to	
	Lymphatic endothelium ^a	HEV ^b
UT-SCC-38	13.9 \pm 3.2	2.3 \pm 0.6
UT-SCC-12A	15.6 \pm 5.4	1.5 \pm 0.07
IBW4	6.5 \pm 3.4	0.8 \pm 0.2
KCA	5.7 \pm 3.3	0.6 \pm 0.15
CRL-1648	10.5 \pm 4.1	1.2 \pm 0.2

^a Mean number of bound cells/lymphatic sinusoid (number of experiments = 4).

^b Mean number of bound cells/HEV (number of experiments = 3).

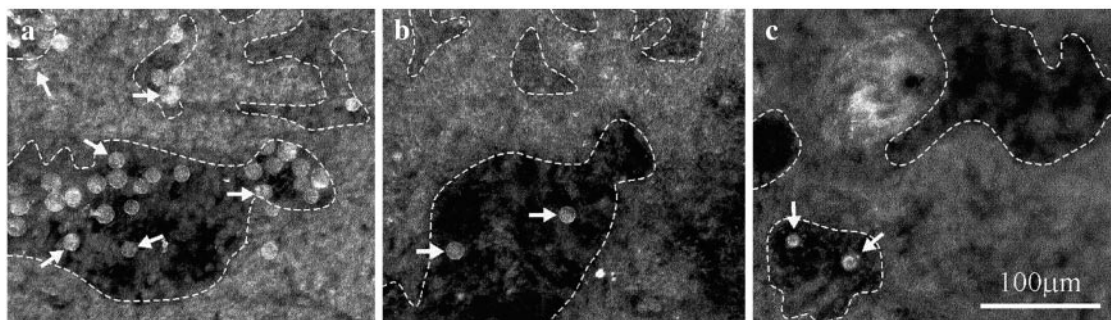


Fig. 1. Adhesion of malignant cells to the lymphatic endothelium vary. Some cell lines bind avidly to the lymphatic sinusoidal endothelium. *a*, 35 rounded UT-SCC-38 cells, of which 6 are pointed out by arrows, adherent to the lymphatic sinusoids. In *b*, the same lymphatic sinusoids do not support UT-SCC-38 cell binding after treatment with anti-MR antibody (only two cells present, pointed out by arrows). UT-SCC-50 cells, in turn, bind very poorly, even without antibody treatment (only 2 adherent cells are present and pointed out by arrows; *c*). The lymphatic sinusoids are outlined with a dashed line. Because the adherent cells are laying on the top of the tissue section, the focus layer of the photograph was chosen as a compromise between the tissue and adherent cells.

also different when the anti-MR and anti-CLEVER-1 immunostains were compared. Some lymphatic vessels expressed the MR but not CLEVER-1 and *vice versa* (Fig. 4). CLEVER-1 stains HEV-like vessels at sites of inflammation (16). Because many tumors share features of inflammatory reactions, some CLEVER-1-positive struc-

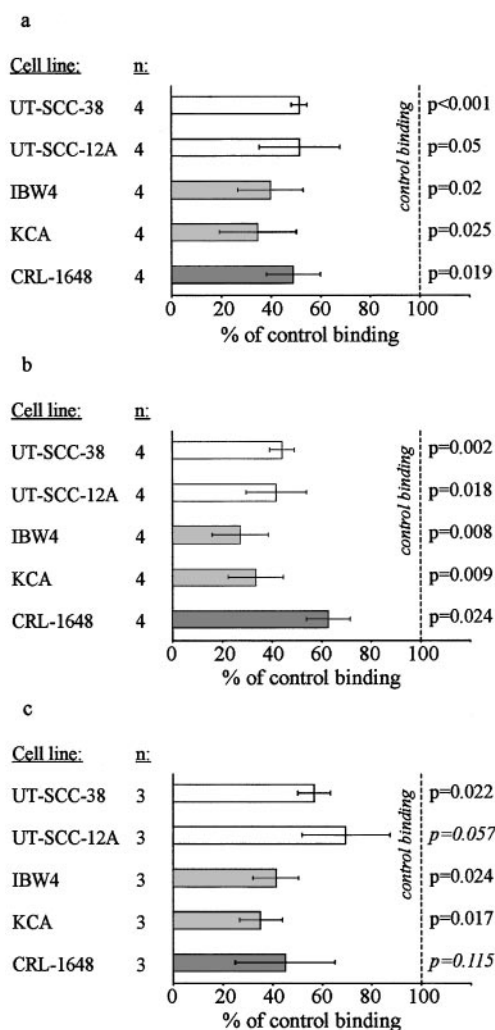


Fig. 2. Binding of malignant cells to the lymphatic endothelium is CLEVER-1 and MR dependent. Binding of head and neck carcinoma cells to the lymphatic endothelium was inhibited with an α -MR antibody as compared with a negative control antibody (*a*). Comparable results were obtained when an α -CLEVER-1 antibody was used to inhibit cancer cell binding to the lymphatic endothelium (*b*) or to the HEVs (*c*).

Table 2 Expression of the MR and CLEVER-1 in head and neck and breast cancer

A. Head and neck cancer		
	Peritumoral MR expression	Peritumoral CLEVER-1 expression
Negative (-)	0 ^a (0) ^b	0 (0)
Weak (+/-)	1 (6)	2 (12)
Sparse (+)	6 (35)	6 (35)
Moderate (++)	5 (29)	3 (18)
Strong (+++)	5 (29)	6 (35)
	Intratumoral MR expression	Intratumoral CLEVER-1 expression
Negative (-)	7 (41)	4 (24)
Weak (+/-)	6 (35)	7 (41)
Sparse (+)	3 (18)	6 (35)
Moderate (++)	1 (6)	0 (0)
Strong (+++)	0 (0)	0 (0)
B. Breast cancer		
	Peritumoral MR expression	Peritumoral CLEVER-1 expression
Negative (-)	2 (3)	0 (0)
Weak (+/-)	9 (13)	5 (7)
Sparse (+)	38 (53)	26 (36)
Moderate (++)	22 (31)	32 (44)
Strong (+++)	1 (1)	9 (13)
	Intratumoral MR expression ^c	Intratumoral CLEVER-1 expression ^c
Negative (-)	45 (65)	18 (26)
Weak (+/-)	14 (20)	24 (35)
Sparse (+)	7 (10)	16 (23)
Moderate (++)	3 (4)	11 (16)
Strong (+++)	0 (0)	0 (0)

^a Number of the patients.

^b Percentage of the patients.

^c Only 69 breast cancer samples were available for analysis.

tures in tumors are likely to be venules and not lymphatic vessels. To demonstrate that this indeed is the case, some tumors were double stained with a blood vessel marker (anti-PAL-E; Fig. 4). CLEVER-1- and MR-expressing intratumoral lymphatic vessels were sparse and stained only faintly as compared with the peritumoral vessels. Yet, 10 (59%) and 13 (76%) of the 17 head and neck carcinomas contained at least a single intratumoral lymph vessel that stained for the MR or CLEVER-1, respectively. Neither the peritumoral or intratumoral MR nor CLEVER-1 stainings of the lymphatic vessels was significantly associated with the tumor histological grade ($P > 0.5$ for all analyses).

Similarly, the majority of breast carcinomas ($n = 72$) had peritumoral lymphatic vessels that expressed MR and CLEVER-1 at least weakly (97 and 100%, respectively). The peritumoral MR expression was not associated with the histological grade ($P = 0.2$). However, moderate to strong (++ or +++) peritumoral lymphatic vessel CLEVER-1 expression was more common in poorly differentiated breast carcinomas (grade 3, 26 of 37, 70%) and moderately differen-

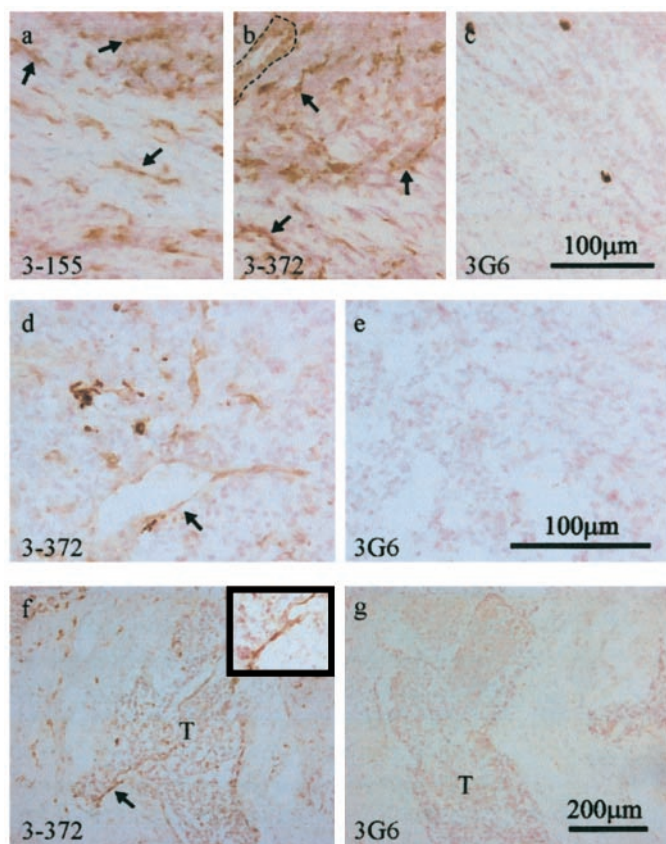


Fig. 3. CLEVER-1- and MR-immunostained vessels are found in the peritumoral and intratumoral areas of HNSCC. Abundant MR (a) and CLEVER-1 (b) expression on the peritumoral lymphatic vessels was present in the tissue samples containing head and neck cancer (arrows). CLEVER-1 is expressed also in HEV-like vessels (b, outlined with a dashed line), which were present in most of the cancer samples. Some enlarged lymphatic lacunas are also seen (d, arrow). In general, the intratumoral lymph vessels were sparse comparing with the peritumoral vessels (f, arrow points to a vessel presented in a higher magnification in the inset). Negative control stainings of the same samples are shown in c, e, and g. T, tumor.

tiated breast carcinomas (grade 2, 13 of 25, 52%) than in the well-differentiated ones (grade 1, 2 of 9, 29%, $P = 0.025$). Most of the breast cancers (74%) had at least weakly CLEVER-1-positive intratumoral vessels, whereas intratumoral lymph vessel MR receptor expression was less common (35% of these gave positive staining for the MR; Table 2). Neither intratumoral MR nor CLEVER-1 expression was associated with the histological grade in breast carcinoma ($P = 0.73$ and 0.94 , respectively).

Intratumoral MR Expression Is Associated with the Frequency Of Regional Lymph Node Metastases in Breast Carcinoma. To evaluate whether CLEVER-1 and MR play a role in metastatic spread of cancer via the lymphatics, we investigated the association between receptor expression and the presence of metastases in regional lymph nodes. Interestingly, intratumoral CLEVER-1-positive lymphatic vessels were present in all 8 (100%) primary head and neck cancers that had given rise to locoregional lymph node metastases at the time of diagnosis as compared with only five of the nine tumors (56%) that did not have cervical lymph node metastases ($P = 0.082$, Fisher's exact test). However, neither peritumoral lymph vessel CLEVER-1 expression, nor intra or peritumoral MR expression showed an association with the presence of cervical lymph node metastases at the time of diagnosis in head and neck cancer ($P > 0.1$ for each analysis). In breast carcinomas, the intratumoral MR expression (+/- or stronger) was associated with the presence of axillary lymph node metastases at the time of diagnosis. Only 8 (22%) of the 36 axillary

node-negative breast carcinomas showed MR staining on intratumoral lymphatic vessels as compared with 16 (50%) of the 32 node-positive carcinomas ($P = 0.017$). In breast carcinomas, neither intratumoral or peritumoral CLEVER-1 expression nor peritumoral MR expression was associated with the presence of axillary lymph node metastases at the time of diagnosis ($P = 0.91$, 0.54 , and 0.4 , respectively).

DISCUSSION

Malignant cells first need to transmigrate through the endothelial cell layer of the afferent lymphatic vessels into the lymph vessel lumen and then escape from the vessel lumen and pass through the structural barriers within the lymph nodes to give rise to metastases via the lymphatic system. Similarly, when circulating within the blood, cancer cells need to get out from the vasculature in a suitable environment to be able to form a hematogenic metastasis. These processes are thought to use similar mechanisms as the lymphocytes use in extravasation (20), although malignant cells may also have their own tissue-specific homing systems (21). The results of the present study show that the ability of cancer cells to adhere to the lymphatic endothelium in lymph nodes varies. Different binding efficiencies may also have consequences in the metastatic potential of cancer cells *in vivo*, although binding capacity alone may not be sufficient, and chemokines and their receptors are needed to guide the cancer cells to draining lymph nodes and further to efferent lymph. Especially CCR7-positive cancers seem to metastasize more frequently than the negative ones (22, 23). However, the human material available for this study did not allow us to directly test the correlation between the binding properties of cancer cells and their capacity to metastasize.

Intratumoral and peritumoral lymphatic vessels provide a route for metastasizing cancer cells to reach first the regional lymph nodes and later the systemic circulation via the thoracic duct. Because CLEVER-1 mediates binding of malignant cells to the lymphatic endothelium and is expressed both on the normal and cancer-associated lymphatic endothelium and HEVs as well, it may have a role not only in cancer cell penetration into the lymphatic vessels but in cancer cell extravasation from the blood circulation to tissues as well. Adhesion of cancer cells to the HEVs via CLEVER-1-mediated mechanisms might also predispose cancer patients to thromboses and tumor emboli, which are commonly found in patients with a malignant disease.

The present analyses on the associations between the expression status of the MR and CLEVER-1 and metastatic spread via the lymphatics had to be performed using frozen tumor material, because the antibodies used against CLEVER-1 and the MR do not stain formalin-fixed and paraffin-embedded tissues, which excludes the possibility to use archival tissues as the starting material. The MR and CLEVER-1 expression on lymphatic vessels was heterogeneous and varied between tumors. The most common staining pattern of lymph vessels was abundant, thin lymphatic vasculature in the peritumoral area with occasional intratumoral branches. Some abnormally enlarged lymphoid lacunas were also seen in the peritumoral area as has been described also with staining for LYVE-1 (10, 12). The MR expression was a more specific tool in measuring the density of lymphatic vessels in tumors than expression of CLEVER-1. As a matter of fact, in genome-wide microarray analyses, MR has turned out to be present in lymphatic endothelial cells and not at all in blood vessel endothelium (24). Intratumoral MR expression was associated with the presence of locoregional lymph node metastases at the time of diagnosis in the present series of breast cancer patients. Most likely, it implicates that MR present on intratumoral lymphatics mediates entrance of tumor cells to lymphatics. Alternatively, it is also theoretically possible that the presence of MR is just a marker for a more metastatic tumor. It is also of interest that all patients with head and

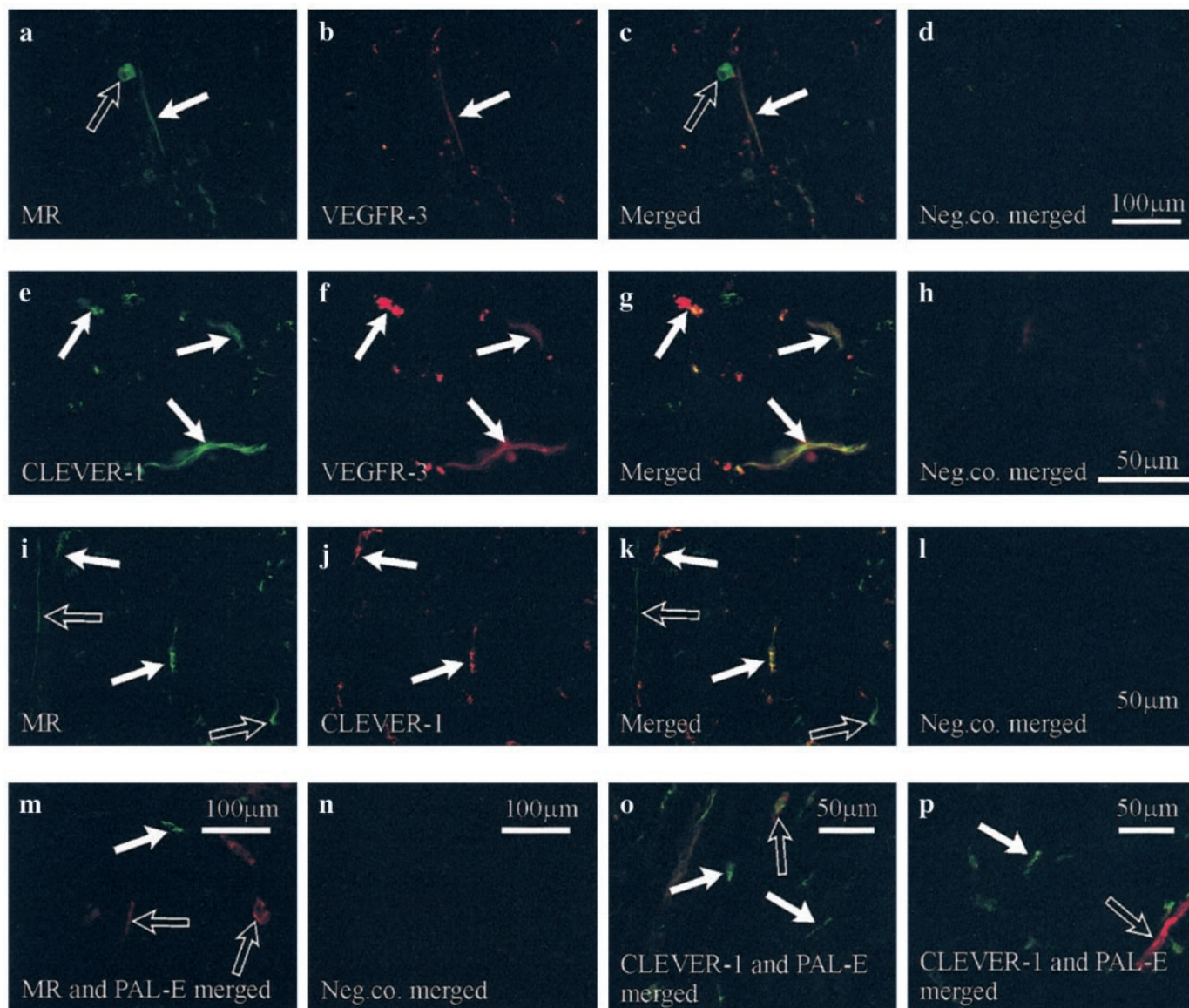


Fig. 4. Lymphatic vessels show heterogeneity in the expression of MR and CLEVER-1. Both the MR and CLEVER-1 colocalized with VEGFR-3 on most of the lymphatic vessels (solid arrows in a-c and e-g). The MR expression was also seen on the tissue macrophages (a and c, open arrows). The MR and CLEVER-1 usually colocalized on the same lymphatic vessels (i-k, solid arrows). Some lymphatic vessels expressed only the MR (open arrows in i and k) but not CLEVER-1 and vice versa (data not shown). MR (arrow) is not detected in PAL-E-positive blood vessels (open arrows, m), but CLEVER-1 is expressed in some PAL-E-positive blood vessels (open arrow, o). CLEVER-1-positive lymphatic vessels were PAL-E negative (arrows), and most PAL-E-positive vessels were CLEVER-1 negative (open arrow, p).

neck cancer and who presented with lymph node metastases had intratumoral CLEVER-1-positive vessels. Unfortunately, the small number of such patients and borderline P obtained ($P = 0.08$) prevents us from drawing definite conclusions on the role of CLEVER-1 expression in the genesis of lymph node metastases in head and neck cancer. The lack of correlation between intratumoral CLEVER-1 expression and presence of nodal metastases at the time of diagnosis in breast cancer may be attributable to the fact that not only lymphatic vessels but also some small intratumoral venules up-regulate CLEVER-1, making immunostaining for CLEVER-1 unspecific. On the other hand, different histological tumor types may also have distinct antigen presentation profiles on their lymphatic vessels. Hence, the lack of association between CLEVER-1 intratumoral expression and the axillary nodal metastasis status does not necessarily mean that CLEVER-1-expressing intratumoral lymphatics are functionally incompetent to promote metastatic spread in breast cancer.

In summary, we demonstrate in the present study that HNSCCs and

breast carcinomas express almost invariably both CLEVER-1 and the MR on the peritumoral lymphatic vessels and that these molecules are often expressed on the intratumoral lymphatic vessels as well. CLEVER-1 and MR constitute a novel mechanism for cancer cell binding to the lymphatic endothelium in lymph nodes, and CLEVER-1 mediates cancer cell adhesion also to the HEVs. The relative importance of peri and intratumoral lymphatic vessels in lymphatic spread of cancer has been a subject of controversy (14, 25). The present results that the expression of MR on the intratumoral lymphatics is associated with the presence of regional lymph node metastases at the time of diagnosis in breast cancer together with recently published data regarding the role of intratumoral and peritumoral LYVE-1-positive lymphatics in HNSCC metastases (11) suggest that the intratumoral lymph vessels may be of greater importance than the peritumoral vessels in the metastatic process of head and neck and breast carcinomas. The role of intratumoral CLEVER-1 in the metastatic process of HNSCCC needs to be confirmed in a larger patient material with a follow-up.

Cancer cell adhesion mediated by CLEVER-1 and the MR may allow design of novel targeted anticancer therapies directed to control the malignant cell traffic within the lymphatic system and blood circulation.

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REFERENCES

- Folkman, J., Watson, K., Ingber, D., and Hanahan, D. Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature (Lond.)*, 339: 58–61, 1989.
- Karkkainen, M. J., Makinen, T., and Alitalo, K. Lymphatic endothelium: a new frontier of metastasis research. *Nat. Cell Biol.*, 4: E2–E5, 2002.
- Kriehuber, E., Breiteneder-Geleff, S., Groeger, M., Soleiman, A., Schoppmann, S. F., Stingl, G., Kerjaschki, D., and Maurer, D. Isolation and characterization of dermal lymphatic and blood endothelial cells reveal stable and functionally specialized cell lineages. *J. Exp. Med.*, 194: 797–808, 2001.
- Weidner, N., Semple, J. P., Welch, W. R., and Folkman, J. Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. *N. Engl. J. Med.*, 324: 1–8, 1991.
- Nibbs, R. J., Kriehuber, E., Ponath, P. D., Parent, D., Qin, S., Campbell, J. D., Henderson, A., Kerjaschki, D., Maurer, D., Graham, G. J., and Rot, A. The beta-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors. *Am. J. Pathol.*, 158: 867–877, 2001.
- Banerji, S., Ni, J., Wang, S. X., Clasper, S., Su, J., Tammi, R., Jones, M., and Jackson, D. G. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J. Cell Biol.*, 144: 789–801, 1999.
- Mäkinen, T., Jussila, L., Veikkola, T., Karpanen, T., Kettunen, M. I., Pulkkanen, K. J., Kauppinen, R., Jackson, D. G., Kubo, H., Nishikawa, S.-I., Ylä-Herttuala, S., and Alitalo, K. Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nat. Med.*, 7: 199–205, 2001.
- Breiteneder-Geleff, S., Soleiman, A., Kowalski, H., Horvat, R., Amann, G., Kriehuber, E., Diem, K., Weninger, W., Tschachler, E., Alitalo, K., and Kerjaschki, D. Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. *Am. J. Pathol.*, 154: 385–394, 1999.
- Wiltung, J., Papoutsis, M., Christ, B., Nicolaides, K. H., on Kaisenberg, C. S., Borges, J., Stark, G. B., Alitalo, K., Tomarev, S. I., Niemeier, C., and Rossler, J. The transcription factor Prox1 is a marker for lymphatic endothelial cells in normal and diseased human tissues. *FASEB J.*, 16: 1271–1273, 2002.
- Beasley, N. J., Prevo, R., Banerji, S., Leek, R. D., Moore, J., van Trappen, P., Cox, G., Harris, A. L., and Jackson, D. G. Intratumoral lymphangiogenesis and lymph node metastasis in head and neck cancer. *Cancer Res.*, 62: 1315–1320, 2002.
- Maula, S. M., Luukka, M., Grenman, R., Jackson, D., Jalkanen, S., and Ristamäki, R. Intratumoral lymphatics are essential for the metastatic spread and prognosis in squamous cell carcinomas of the head and neck region. *Cancer Res.*, 63: 1920–1926, 2003.
- Skobe, M., Hawighorst, T., Jackson, D. G., Prevo, R., Janes, L., Velasco, P., Riccardi, L., Alitalo, K., Claffey, K., and Detmar, M. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat. Med.*, 7: 192–198, 2001.
- Mattila, M. M., Ruohola, J. K., Karpanen, T., Jackson, D. G., Alitalo, K., and Harkonen, P. L. VEGF-C induced lymphangiogenesis is associated with lymph node metastasis in orthotopic MCF-7 tumors. *Int. J. Cancer*, 98: 946–951, 2002.
- Padera, T. P., Kadambi, A., Di Tomaso, E., Mouta Carreira, C., Brown, E. B., Boucher, Y., Choi, N. C., Mathisen, D., Wain, J., Mark, E. J., Munn, L. L., and Jain, R. K. Lymphatic metastasis in the absence of functional intratumor lymphatics. *Science (Wash. DC)*, 296: 1883–1886, 2002.
- Irjala, H., Johansson, E. L., Grenman, R., Alanen, K., Salmi, M., and Jalkanen, S. Mannose receptor is a novel ligand for L-selectin and mediates lymphocyte binding to lymphatic endothelium. *J. Exp. Med.*, 194: 1033–1042, 2001.
- Irjala, H., Elima, K., Johansson, E.-L., Merinen, M., Kontula, K., Alanen, K., Grenman, R., Salmi, M., and Jalkanen, S. The same endothelial receptor controls lymphocyte traffic both in vascular and lymphatic vessels. *Eur. J. Immunol.*, 33: 815–824, 2003.
- Lansford, C. D., Grenman, R., Bier, H., Somers, K. D., Kim, S. Y., Whiteside, T. L., Clayman, G. L., Welkoborsky, H.-J., and Carey, T. E. Head and neck cancers. *In: J. Masters and B. Palsson (eds.), Human Cell Culture, Cancer Cell Lines Part 2, Vol. 2*, pp. 185–255. Dordrecht, Holland: Kluwer Academic Publishers, 1999.
- Salmi, M., and Jalkanen, S. A 90-kilodalton endothelial cell molecule mediating lymphocyte binding in humans. *Science (Wash. DC)*, 257: 1407–1409, 1992.
- Jalkanen, S. T., and Butcher, E. C. In vitro analysis of the homing properties of human lymphocytes: developmental regulation of functional receptors for high endothelial venules. *Blood*, 66: 577–582, 1985.
- Hart, I. R., and Saini, A. Biology of tumour metastasis. *Lancet*, 339: 1453–1457, 1992.
- Ruoslahti, E., and Rajotte, D. An address system in the vasculature of normal tissues and tumors. *Annu. Rev. Immunol.*, 18: 813–827, 2000.
- Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verastegui, E., and Zlotnik, A. Involvement of chemokine receptors in breast cancer metastasis. *Nature (Lond.)*, 410: 50–56, 2001.
- Mashino, K., Sadanaga, N., Yamaguchi, H., Tanaka, F., Ohta, M., Shibuta, K., Inoue, H., and Mori, M. Expression of chemokine receptor CCR7 is associated with lymph node metastasis of gastric carcinoma. *Cancer Res.*, 62: 1937–1941, 2002.
- Petrova, T. V., Makinen, T., Makela, T. P., Saarela, J., Virtanen, I., Ferrell, R. E., Finegold, D. N., Kerjaschki, D., Ylä-Herttuala, S., and Alitalo, K. Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. *EMBO J.*, 21: 4593–4599, 2002.
- Karpanen, T., and Alitalo, K. Lymphatic vessels as targets of tumor therapy? *J. Exp. Med.*, 194: F37–F42, 2001.