

Functional CCR9 Expression Is Associated with Small Intestinal Metastasis

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In general, metastases to the small intestine are rare, and mostly occur in melanoma. CCR9 has been shown to be the principal chemokine receptor for the thymus expressed chemokine (TECK), a chemokine selectively expressed in the small intestine and thymus. Here we show that CCR9 is highly expressed on melanoma cells and all melanoma cell lines isolated from small intestinal metastases, and on a proportion of cell lines from other sites. Only melanoma cells and cell lines from small intestinal metastases, however, were responsive to the CCR9 ligand TECK, as assessed by receptor downregulation and by actin polymerization. CCR9 expression was also found on the adenocarcinoma cell line CaCo-2 expressing characteristics of enterocytic differentiation, but not on any other cell line isolated from colorectal, breast, and lung cancer. Our data provide evidence that the aberrant functional cell surface expression of an organ-specific chemokine receptor is associated with metastasis to this site. The regulation of receptor function seems to be a critical step in the metastatic process.

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Neoplasms of the small intestine are rare, representing only 2%–5% of gastrointestinal tumors. Most of them are of metastatic origin and melanoma is the most common tumor metastatic to the small intestine (Bender *et al*, 2001; Gill *et al*, 2001). Approximately 5% of melanoma patients develop clinically overt metastases to the small intestine, but there are reports describing the presence of small intestinal metastases in up to 60% of melanoma patients postmortem (Blecker *et al*, 1999). The small intestine is the most common site of metastatic melanoma in the gastrointestinal tract (Das Gupta *et al*, 1964).

Recent findings indicate that chemokines and their receptors are important in determining the metastatic destination of tumor cells. The expression of CXCR4 and CCR7 was found in a variety of human cancer cell lines, and evidence for their role in organ-specific metastasis comes from various animal models (Muller *et al*, 2001; Wiley *et al*, 2001; Murakami *et al*, 2002; Zeelenberg *et al*, 2003). The ligand of CXCR4, SDF-1, is expressed at high levels in various organs, including the lung, liver, and lymph nodes, which are frequently involved in tumor metastases. Blocking of CXCR4 function was shown to reduce or completely block metastasis of breast and colon cancer cells to the liver, lung, and lymph nodes in murine models (Muller *et al*, 2001; Zeelenberg *et al*, 2003). Conversely, the transfection of CCR7, mediating migration to the lymph node chemokine

CCL21, into tumor cells increased their metastasis to lymph nodes (Wiley *et al*, 2001). An association could be shown for CCR7 expression on the tumor cells and lymph node metastasis in gastric cancer patients (Mashino *et al*, 2002). Histological studies in tumor patients on the association between chemokine receptor expression and organ-specific metastases are, however, hampered, because chemokine receptors expressed on the surface of tumor cells may not be functional (Honczarenko *et al*, 1999; Mitra *et al*, 2001).

CCR9 is an excellent example of an organ-specific chemokine receptor, because its ligand TECK is selectively expressed in the small intestine and thymus (Wurbel *et al*, 2000). CCR9 was identified as the chemokine receptor regulating lymphocyte trafficking during T cell development and in mucosal immunity (Zabel *et al*, 1999). The expression of CCR9 has not been reported on tumor cells so far. In one study, CCR9 expression was analyzed by RT-PCR and no CCR9 mRNA expression was detectable in any of 12 melanoma and seven breast cancer cell lines analyzed (Muller *et al*, 2001).

In this study, we assess whether the expression of CCR9 and the responsiveness of tumor cells to the ligand TECK might be associated with the ability of tumor cells to metastasize to the small intestine. We therefore analyzed CCR9 expression and function on melanoma cells and cell lines generated from metastases to the small intestine and from various other sites.

Results and Discussion

CCR9 is expressed on melanoma cell lines derived from the small intestine and other tissues We first analyzed

Abbreviations: CCR, chemokine receptor CC; CXCR, chemokine receptor CXC; IC IFN γ , intracellular interferon gamma; PBMC, peripheral blood mononuclear cells; TECK, thymus expressed chemokine

expression of CCR9 on melanoma cell lines established from small intestinal metastases from three patients. CCR9 was expressed on all three cell lines (UKBF-Mel 12, MA-Mel 16, UKRV-Mel 18B, Fig 1 and Table I). CCR9 expression was in the same range as found on the human T cell leukemia line MOLT-4, which has already been described to express CCR9 (Youn *et al*, 2001, Fig 1). We next analyzed 17 melanoma cell lines originating from the skin, soft tissue, lymph node and CNS metastases, and found CCR9 expression on five of these, including four lymph node (UKRV-Mel 18A, UKRV-Mel 17, SB Mel, MKR) and one skin metastasis-derived cell line (UKBF-Mel 19). In one of these five patients (UKRV-Mel 18A), a small intestinal metastasis had been diagnosed 7 months later, from which the cell line UKRV-Mel 18B had been established. In the remaining four patients, no small intestinal metastases had become clinically evident. Expression of CCR9 on

melanoma cell lines and origin of melanoma metastases from the small intestine showed a significant correlation ($p=0.02$, χ^2 -test).

CCR9 expression was also found on the colonic adenocarcinoma cell line CaCo-2 as shown in Fig 1. This cell line was reported to be isolated from a primary colonic tumor, but the cells express characteristics of enterocytic differentiation (Jumarie and Malo, 1991). In contrast, none out of 29 human tumor cell lines isolated from various malignancies including colorectal cancer ($n=9$), lung cancer ($n=8$), breast cancer ($n=3$), and leukemia ($n=9$) expressed CCR9 (data not shown). This absence of CCR9 is in accordance with the clinical observation that the occurrence of small intestinal metastases was reported in less than 0.5% of patients with lung cancer (Berger *et al*, 1999), and is very rare in patients with colon cancer, breast cancer, and leukemia.

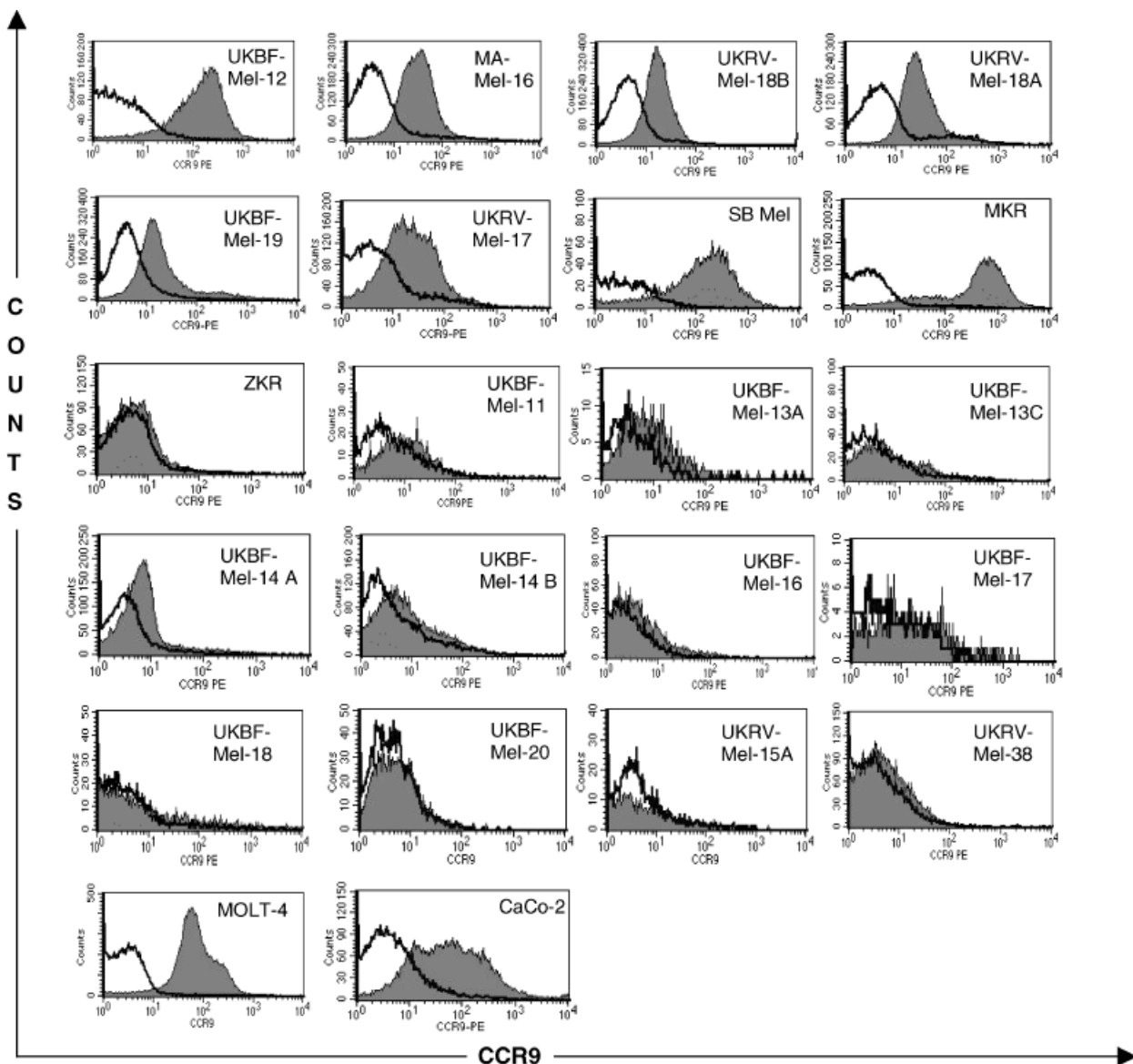


Figure 1

Expression of chemokine receptor CCR9 on cell lines, the T cell leukemia cell line MOLT-4 and the adenocarcinoma cell line CaCo-2 as determined by flow cytometry. Cell lines were stained with CCR9 mAb (shaded) or isotypic control mAb (black line). Results are expressed as fluorescence intensity.

Table I. Characteristics of melanoma cell lines

Melanoma cell line	Site of metastases from which cell line was generated	CCR9 expression	CCR7 expression	CXCR4 expression	Downregulation of CCR9 in response to TECK	Response to TECK in actin polymerization assay	Clinically evident small bowel metastases
UKBF-Mel 12	Small bowel	pos.	neg.	pos.	Yes	Yes	Yes
MA-Mel 16	Small bowel	pos.	neg.	pos.	Yes	Yes	Yes
UKRV-Mel 18B	Small bowel	pos.	neg.	pos.	Yes	Yes	Yes
UKRV-Mel 18A	Lymph node	pos.	neg.	pos.	No	No	Yes, 7 mo after resection of the lymph node metastasis
UKBF-Mel 19	Skin	pos.	neg.	pos.	Yes	No	No
UKRV-Mel 17	Lymph node	pos.	pos.	pos.	No	No	No
SB-Mel	Lymph node	pos.	pos.	pos.	No	No	No
MKR	Lymph node	pos.	pos.	pos.	No	No	No
ZKR	CNS	neg.	neg.	pos.	n.d.	n.d.	No
UKBF-Mel 13A	Skin	neg.	pos.	neg.	n.d.	n.d.	Yes, 2 y after resection of skin metastases when multiple visceral metastases occurred
UKBF-Mel 13C	Skin	neg.	neg.	neg.	n.d.	n.d.	
UKBF-Mel 11	Soft tissue	neg.	pos.	pos.	n.d.	n.d.	no
UKBF-Mel 14A	Skin	neg.	neg.	neg.	n.d.	n.d.	no
UKBF-Mel 14B	Skin	neg.	neg.	pos.	n.d.	n.d.	no
UKBF-Mel 16	Lymph node	neg.	neg.	neg.	n.d.	n.d.	no
UKBF-Mel 17	Skin	neg.	pos.	pos.	n.d.	n.d.	no
UKBF-Mel 18	Skin	neg.	neg.	neg.	n.d.	n.d.	no
UKBF-Mel 20	Skin	neg.	pos.	pos.	n.d.	n.d.	no
UKRV-Mel 15A	Lymph node	neg.	n.d.	n.d.	n.d.	n.d.	no
UKRV-Mel 38	Skin	neg.	pos.	pos.	n.d.	n.d.	no

CNS, central nervous system; pos., positive; neg., negative; n.d., not done.

CCR9 expression on uncultured melanoma cells isolated from a small intestinal metastases To show that CCR9 is expressed on tumor cells *in vivo*, we analyzed CCR9 expression on a single-cell suspension, which was generated from a melanoma metastasis resected from the small intestine. The melanoma cells were stained with the melanoma marker S100 and CCR9 and analyzed by flow cytometry. The S100-positive melanoma cells expressed all CCR9 comparable to the levels seen in the CCR9-positive melanoma cell lines (Fig 2a).

Expression of CCR7 and CXCR4 on CCR9-positive and CCR9-negative melanoma cell lines Next we analyzed the expression of the chemokine receptors CCR7 and CXCR4 on the melanoma cell lines by flow cytometry. Both chemokines had been shown to be expressed by a substantial proportion of melanoma cell lines as detected by quantitative polymerase chain reaction (Muller *et al*, 2001). Interestingly, all eight CCR9-positive melanoma cell lines also expressed CXCR4, while only six of 11 CCR9-negative melanoma cell lines analyzed were CXCR4-positive. Tumor cells expressing CXCR4 have an increased potential to metastasize to various organs and to proliferate and grow (Muller *et al*, 2001; Murakami *et al*, 2002; Zeelenberg *et al*, 2003). Thus, the co-expression of CXCR4 found on the CCR9-positive melanoma cells may facilitate their potential to metastasize. It would be of interest to investigate whether there is a molecular link between the acquisition of CXCR4 and CCR9 expression in tumor cells. In contrast, the lymph node-associated chemokine receptor CCR7 was not expressed on the three CCR9-positive small intestinal melanoma cell lines or the one isolated from the skin, but on three of four CCR9-positive melanoma cell lines derived from lymph node metastases (Table I).

Melanoma cells express functional CCR9 Chemokine receptors expressed on normal and malignant cells may not be functional in response to their specific ligands (Honczarenko *et al*, 1999; Mitra *et al*, 2001). To determine whether CCR9 expressed on melanoma cells is functional, the effect of the ligand TECK was assessed in two independent assays, namely CCR9 internalization and actin polymerization. As shown in Fig 3a and b, the CCR9 ligand TECK is able to induce a downregulation of CCR9 expression on all three small intestinal melanoma cell lines (UKBF-Mel 12, MA-Mel 16, UKRV-Mel 18B) and on the one CCR9-positive melanoma cell line from a skin metastasis (UKBF-Mel 19), but not on the other four CCR9-positive melanoma cell lines derived from lymph node metastases (UKRV-Mel 17, UKRV-Mel 18A, MKR, SB-Mel). Remarkably, UKRV-Mel 18A originated from the patient from whom we could establish the TECK-responsive small intestinal line UKRV-Mel 18B 7 mo later.

Actin polymerization, which is an early event in the migratory response to chemokines, was studied by staining of intracellular filamentous actin by FITC-phalloidin. TECK induced a 1.2–1.6-fold increase in intracellular F-actin in all three CCR9-positive melanoma cell lines isolated from the small intestine (UKBF-Mel 12, MA-Mel 16, UKRV-Mel 18B) (Fig 3a and Table I). Responsiveness to TECK was also shown for the CCR9-positive melanoma cell suspension

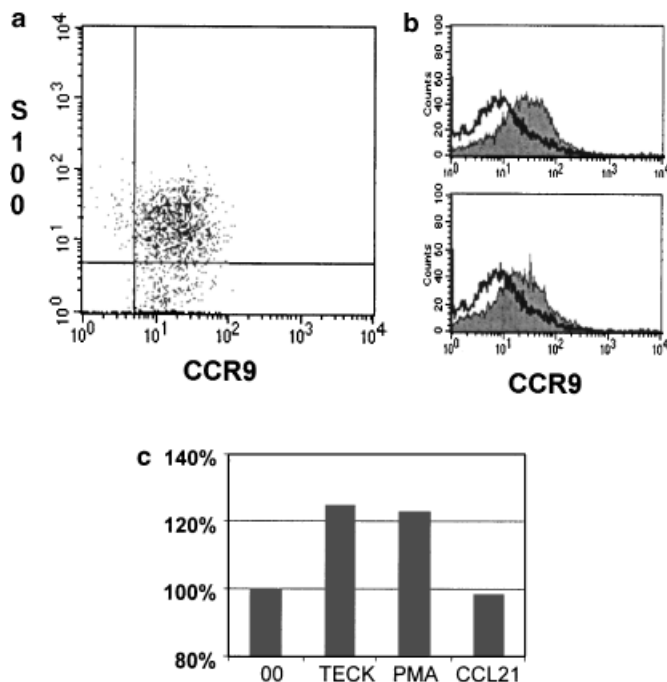


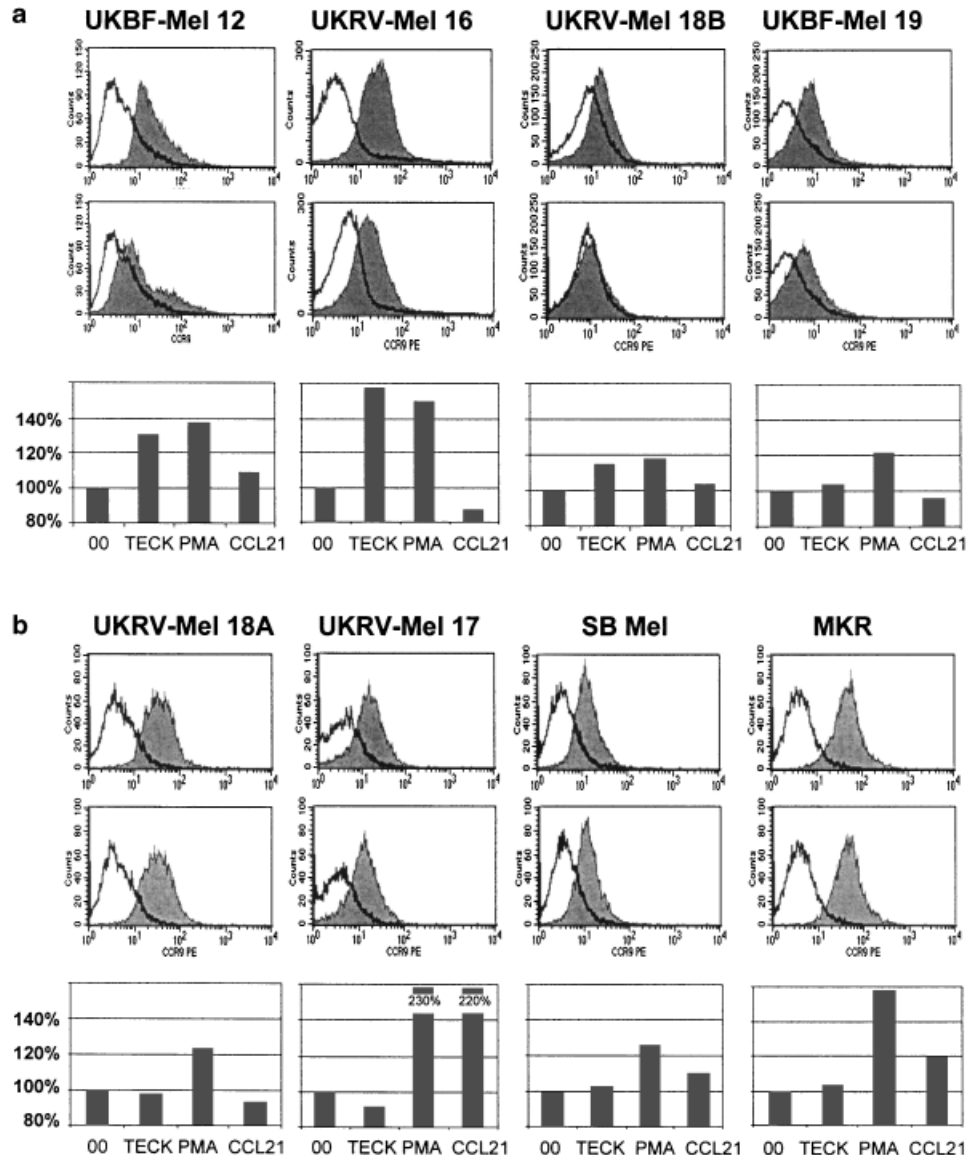
Figure 2
CCRS expression and function on freshly isolated melanoma cells. (a) Expression of chemokine receptor CCR9 on a single cell suspension that was generated from a melanoma metastasis resected from the small intestine as determined by flow cytometry. Melanoma cells were identified by staining with the melanoma marker S100 and costained with CCR9 mAb. (b): CCR9 expression was determined by flow cytometry after incubation with medium alone (control, upper histogram) or after 45 min of incubation with 1 μ g per mL of human TECK (lower histogram). Melanoma cell lines were extensively washed before staining with CCR9 mAb (shaded) or isotypic control mAb (black line). Results are expressed as fluorescence intensity. (c) The relative F-actin content was determined by flow cytometry in the melanoma cells without stimulation or after 1 min of stimulation with the CCR9-ligand TECK, and the CCR7-ligand CCL21/6Ckine and PMA as negative and positive controls, respectively. Results are expressed relative to the mean fluorescence intensity of the unstimulated sample.

from a small intestinal metastasis (Fig 2b and c). In none of the five CCR9-positive melanoma cell lines generated from other sites (UKRV-Mel 18A, UKRV-Mel 17, SB-Mel, MKR, UKBF-Mel 19) actin rearrangement in response to TECK could be observed (Fig 3a and b and Table I).

In the CCR9-positive/CCR7-negative small intestinal melanoma cell lines (UKBF-Mel 12, MA-Mel 16, UKRV-Mel 18B), no alteration of the actin rearrangement could be observed in response to the CCR7 ligand CCL21/6Ckine, which was included as negative control. In contrast, CCL21 induced a more than 2-fold and a 1.2-fold increase in intracellular F-actin, respectively, in the two CCR7-positive lymph node-derived cell lines UKRV-Mel 17 and MKR, showing the general ability of these cell lines to exert a migratory response after chemokine stimulation. All our cell lines responded to PMA, which was used as positive control. The responsiveness towards TECK was not correlated with the expression level of CCR9 on melanoma cell lines. The results obtained with the actin polymerization assay confirm those obtained with the CCR9 downregulation assay showing responsiveness to TECK in all three CCR9-positive small intestinal melanoma cell lines, but not

Figure 3

Functionality of CCR9. (a and b) Functionality of CCR9 cell surface expression as determined by TECK-induced receptor downregulation or actin polymerization on the eight CCR9-positive melanoma cell lines. CCR9 expression was determined by flow cytometry after incubation with medium alone (first line of A and B) or with 1 μ g per mL of the human CCR9 ligand TECK (second line of A and B). Melanoma cell lines were extensively washed before staining with CCR9 mAb (shaded) or isotypic control mAb (black line). Results are expressed as fluorescence intensity. In the third line of A and B, the relative F-actin content in CCR9-positive melanoma cell lines without stimulation (00) and after 1 min of stimulation with the CCR9-ligand TECK, the CCR7-ligand CCL21/6Ckine, and PMA as positive control was measured after permeabilization and intracellular staining with FITC-phalloidin. Results are expressed relative to the mean fluorescence intensity of the unstimulated sample.



in those derived from other sites. Discordant results were obtained only for the CCR9-positive lymph node cell line UKBF-Mel 19, which responds to TECK by CCR9 downregulation but not by actin polymerization. This shows that the TECK/CCR9 interaction is intact and results in receptor internalization, and suggests a block in the downstream signaling machinery. The unresponsiveness to TECK observed in the other four CCR9-positive cell lines may result from the inability of TECK to bind to CCR9 due to possible receptor polymorphisms or mutations or due to an internalization defect. The lack of ligand-mediated chemokine receptor internalization has also been reported for CXCR4 expressed on the hepatoma cell line HepG2, in which the existence of a blocking molecule was hypothesized (Mitra *et al*, 2001).

Taken together, we provide here a first clinical example of a strong association between expression of a chemokine receptor and organ-specific metastasis, but only in case the intracellular signaling in response to the specific ligand is functional. The demonstration of non-functional chemokine receptor CCR9 expression on melanoma cells derived from

metastatic sites other than the small intestine suggests that expression and regulation of chemokine receptor functionality is a two-step process in the development of organ-specific metastasis.

Materials and Methods

Patients and cell lines Melanoma cell lines had previously been established in our laboratories from metastatic tissue as described elsewhere (Schadendorf *et al*, 1994). MOLT-4 and Caco-2 were obtained from ATCC (American Type Culture Collection, Rockville, Maryland) and all cell lines were cultured in RPMI-1640 medium (Biochrom, Berlin, Germany) supplemented with 10% FCS and antibiotics. In one patient, a melanoma cell suspension was analyzed obtained by mechanical disruption and direct freezing of a resected small intestinal metastasis. The study has been approved by the Institutional Ethics Committee and informed consent was obtained from all patients. The study has been performed in compliance with the Declaration of Helsinki.

Flow cytometry analyses Melanoma cells were detached by a cell scraper and incubated for 10 min in ice-cold phosphate-buffered saline (PBS) supplemented with human IgG before

staining for 15 min with phycoerythrin-conjugated antibodies against CCR9 and the corresponding isotypic control (R&D Systems, Wiesbaden, Germany) on ice in the dark. Analysis of TECK-induced CCR9 downregulation was performed after incubation of melanoma cells for 40 min with 1 μ g per mL TECK (R&D Systems). Melanoma cells were then extensively washed prior to staining with CCR9 mAb as previously described (Wiley *et al*, 2001).

Actin polymerization assay Actin polymerization assay was performed as previously described (Bleul *et al*, 1996). Melanoma cells (1×10^6 per mL) were detached by a cell scraper and resuspended in RPMI-1640 medium with 0.5% BSA at 37°C and incubated with either 1 μ g per mL of TECK, 200 nM CCL21/6CKine (R&D Systems), or 100 ng per mL phorbol myristate acetate (PMA) (Sigma, Deisenhofen, Germany). After 1 min of incubation, 50 μ L of a solution containing 4×10^7 mol per liter FITC-labeled phalloidin, 0.5 mg per mL 1-lysophosphatidylcholine (both from Sigma), and 18% formaldehyde in PBS were added to the cell suspension. The fixed cells were analyzed by flow cytometry on a FACSCalibur, and all results are plotted relative to the mean fluorescence of the sample without the addition of chemokines or PMA.

Statistical analysis To test whether there is a significant difference in the CCR9 expression between melanoma cell lines generated from the small intestine or other sites, data were analyzed using χ^2 -test statistics.

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References

Bender GN, Maglinte DD, McLarney JH, Rex D, Kelvin FM: Malignant melanoma: Patterns of metastasis to the small bowel, reliability of imaging studies, and clinical relevance. *Am J Gastroenterol* 96:2392–2400, 2001

- Berger A, Cellier C, Daniel C, *et al*: Small bowel metastases from primary carcinoma of the lung: Clinical findings and outcome. *Am J Gastroenterol* 94:1884–1887, 1999
- Blecker D, Abraham S, Furth EE, Kochman ML: Melanoma in the gastrointestinal tract. *Am J Gastroenterol* 94:3427–3433, 1999
- Bleul CC, Fuhlbridge RC, Casanovas JM, Aiuti A, Springer TA: A highly efficient lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J Exp Med* 184:1101–1109, 1996
- Das Gupta TK, Brasfield RD: Metastatic melanoma of the gastrointestinal tract. *Arch Surg* 88:969–973, 1964
- Gill SS, Heuman DM, Mihas AA: Small intestinal neoplasms. *J Clin Gastroenterol* 33:267–282, 2001
- Honczarenko M, Douglas RS, Mathias C, Lee B, Ratajczak MZ, Silberstein LE: SDF-1 responsiveness does not correlate with CXCR4 expression levels of developing human bone marrow cells. *Blood* 94:2990–2998, 1999
- Jumarie C, Malo C: Caco-2 cells cultured in serum-free medium as a model for the study of enterocytic differentiation *in vitro*. *J Cell Physiol* 149:24–33, 1991
- Mashino K, Sadanaga N, Yamaguchi H, *et al*: Expression of chemokine receptor CCR7 is associated with lymph node metastasis of gastric carcinoma. *Cancer Res* 62:2937–2941, 2002
- Mitra P, De A, Ethier MF, *et al*: Loss of chemokine SDF-1 α -mediated CXCR4 signalling and receptor internalization in human hepatoma cell line HepG2. *Cell Signal* 13:311–319, 2001
- Muller A, Homey B, Soto H, *et al*: Involvement of chemokine receptors in breast cancer metastasis. *Nature* 410:50–56, 2001
- Murakami T, Maki W, Cardones AR, Fang H, Tun Kyi A, Nestle FO, Hwang ST: Expression of CXC chemokine receptor-4 enhances the pulmonary metastatic potential of murine B16 melanoma cells. *Cancer Res* 62:7328–7334, 2002
- Schadendorf D, Bohm M, Moller P, Grunewald T, Czarnetzki BM: Interleukin-7 induces differential lymphokine-activated killer cell activity against human melanoma cells, keratinocytes, and endothelial cells. *J Invest Dermatol* 102:838–842, 1994
- Wiley HE, Gonzalez EB, Maki W, Wu MT, Hwang ST: Expression of CC chemokine receptor-7 and regional lymph node metastasis of B16 murine melanoma. *J Natl Cancer Inst* 93:1638–1643, 2001
- Wurbel MA, Philippe JM, Nguyen C, *et al*: The chemokine TECK is expressed by thymic and intestinal epithelial cells and attracts double- and single-positive thymocytes expressing the TECK receptor CCR9. *Eur J Immunol* 30:262–271, 2000
- Youn BS, Kim YJ, Mantel C, Yu KY, Broxmeyer HE: Blocking of c-FLIP(L)—independent cycloheximide-induced apoptosis or Fas-mediated apoptosis by the CC chemokine receptor 9/TECK interaction. *Blood* 98:925–98933, 2001
- Zabel BA, Agace WW, Campbell JJ, *et al*: Human G protein-coupled receptor GPR-9-6/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis. *J Exp Med* 190:1241–1256, 1999
- Zeelenberg IS, Ruuls-Van Stalle L, Roos E: The chemokine receptor CXCR4 is required for outgrowth of colon carcinoma micrometastases. *Cancer Res* 63:3833–3839, 2003