Pathophysiological Characteristics of Melanoma In-Transit Metastasis in a Lymphedema Mouse Model

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In-transit metastasis (ITM) is a unique manifestation of intralymphatic tumor dissemination, characterized by the presence of melanoma cells between the primary lesion and the draining regional lymph node basin that is clinically associated with poor prognosis. In this study, we aimed to establish an experimental animal model of melanoma ITM, as research progress in this field has been hampered by a lack of suitable experimental models. We reproduced melanoma ITM in a mouse hind limb by transplanting melanoma cells into the footpad of a mouse with lymphedema (LE). The tumor cells at the ITM site were highly proliferative, and mice with ITMs were more likely than control mice to develop distant lymph node and lung metastases. Peritumoral lymphatic vessels and tumor-associated blood vessels were increased in the primary tumor site of the LE mice. Our established ITM melanoma mouse model enabled us to clarify the molecular determinants and pathophysiology of ITM. This ITM model is also comparable to the unfavorable clinical behavior of melanoma ITM in humans and, moreover, underlined the importance of lymphangiogenic factors in the tumor dissemination through the lymphatic system.

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INTRODUCTION

Cutaneous melanoma is one of the most aggressive solid tumors, and its incidence and mortality rates are increasing in most countries (Marks, 2000). The aggressiveness of melanoma is characterized by its high metastatic ability and resistance to chemotherapy (Satyamoorthy and Herlyn, 2002; Soengas and Lowe, 2003; Postovit *et al.*, 2006; Gajewski, 2007). Cutaneous melanoma metastasizes frequently via lymphatic systems, which is one of the major prognostic factors for tumor recurrence and survival (Balch *et al.*, 2001). Once the melanoma has spread to the lymphatic systems, only 40–50% of these patients survive for 5 years or more (Tsutsumida *et al.*, 2005). In-transit metastasis (ITM) is a unique pattern of intralymphatic metastasis and is associated with poor prognosis (Pawlik *et al.*, 2005a).

Traditionally, ITM has been regarded as a recurrent loco-regional disease found in the dermis or subcutaneous tissue between the primary melanoma and the regional lymph node basin. This pattern of metastasis has a reported incidence of 5–10%, but is associated with significant morbidity, and may be a source of eventual distant metastasis (Gershenwald and Fidler, 2002; Pawlik *et al.*, 2005a, b). Eighty-six percent of patients have been found to progress to systemic disease ranging from 2 to 244 months (median 16 months) following the development of ITM. The overall 5-year survival and the median survival, from the time of ITM diagnosis, have been reported as 12% and 19 months, respectively (Wong *et al.*, 1990).

The molecular determinants and pathophysiology of ITM are still poorly understood; one of the reasons seems to be a lack of suitable experimental animal models. In this study, we aimed to reproduce ITM of melanoma to clarify the pathophysiology of ITM using mice models. ITM is known to be promoted by disrupted lymph flow resulting from regional lymph node basin intervention (Cascinelli *et al.*, 1986; Calabro *et al.*, 1989; Wong *et al.*, 1990; Zogakis *et al.*,

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Abbreviations: BVA, blood vessel area; FP, footpad; IR (−), surgery without preoperative irradiation; ITM, in-transit metastasis; LE, lymphedema; luc, luciferase; LVA, lymphatic vessel area; LYVE-1, lymphatic vessel endothelial hyaluronan receptor-1; VEGF-C, vascular endothelial growth factor C

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2001; Pawlik *et al.*, 2005b); hence, we hypothesized that ITM could be reproduced when melanoma was transplanted to the hind limb of a mouse with lymphedema (LE; Oashi *et al.*, 2011).

RESULTS

LE mice developed ITM of melanoma

To create ITM of melanoma, we developed a new experimental animal model of acquired LE in the mouse hind limb in animals with LE (see Figure 1; Oashi *et al.*, 2011). Firefly luciferase (luc)–expressing melanoma cells (B16-F10-luc2; see Figure 2a) were transplanted to the LE hind limbs of mice with LE.

At 24 days after tumor transplantation, two out of five LE mice developed ITM in their LE hind limbs (see Figure 2b and c), whereas non-LE mice developed no ITM. So far, we have performed the same procedure in an additional eight LE mice, and all of these mice successfully developed ITM (data not shown).

High expression of the proliferation marker Ki-67 in ITM

A schematic representation of the experimental groups is shown in Figure 3a. The percentage of Ki-67-positive tumor cells was significantly higher in the ITM of LE mice (LE-ITM) compared with either the tumor in the footpad (FP) of non-LE mice (non-LE-FP) or LE mice (LE-FP; $P<0.05$), which indicates the high proliferative activity of LE-ITM (see Figure 3b). There was no significant difference in the expression level of Ki-67 between LE-FP and non-LE-FP.

Quantification of vessel area in tumor sections

The tumors were harvested and immunostained using anti-CD31 antibody and anti-lymphatic vessel endothelial hyaluronan receptor (LYVE)-1 antibody for the histopathological examinations (see Supplementary Materials online). The quantitative results of tumor-associated blood vessel area (BVA) are shown in Figure 3c. Tumor-associated blood vessels were defined as CD31-positive/LYVE-1-negative vessels located within the tumor mass and within an area of 100 μm from the tumor border. Tumor-associated blood vessels were homogeneously distributed throughout the

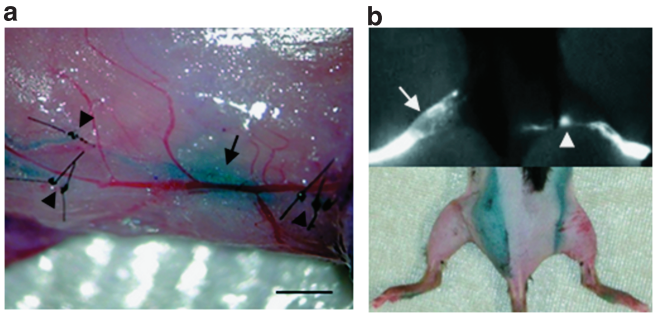

Figure 1. The lymphedema (LE) mouse. (**a**) After injecting patent blue dye into the left paw, the left side inguinal skin was circumferentially incised. The stained lymphatic vessels were carefully tied at three points with a 10-0 nylon suture, and the subiliac and popliteal lymph nodes were resected. The skin edges were sutured to underlying muscle, leaving a gap of 1–2 mm between the skin edges. Black arrow, popliteal lymph node; black arrowheads, ligations of lymphatic vessels. Bar = 1 mm. (**b**) Fluorescent lymphangiography of LE mouse 8 weeks after the lymph node resection demonstrates disappearance of major lymphatic trunks on the treated side. Normal, distinct vessel structure was replaced by a bright, punctuate fluorescence pattern over a foggy background. The bottom row represents the visual image. White arrow, treated limb; white arrowhead, popliteal lymph node of the untreated limb.

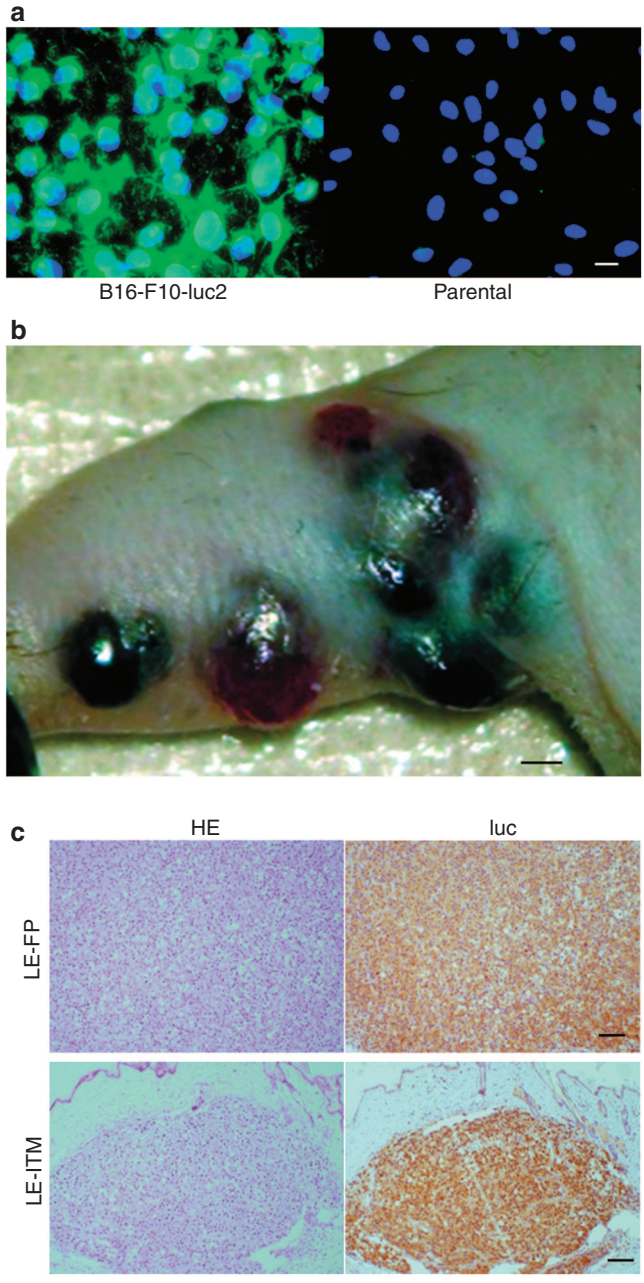

Figure 2. Luciferase (luc)-expressing B16-F10-luc2 cells cause in-transit metastasis (ITM) in the lymphedema (LE) hind limb of a mouse. (**a**) Cultured B16-F10-luc2 melanoma cells and B16-F10 parental melanoma cells were immunostained with antibodies against luc (green). Nuclear DNA was labeled with 4,6-diamidino-2-phenylindole (DAPI; blue). Bar = 100 μm. (**b**) Forty-two days after tumor transplantation into the LE hind limb of a mouse, ITMs were seen. Bar = 1 mm. (**c**) Histology of melanoma at the site of transplantation (top row) and ITM (bottom row), both of which express luc. HE, hematoxylin and eosin stains; LE-FP, LE-footpad; bar = 100 μm.