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Tail Spontaneous Metastatic Mouse Model: Comparison of Metastatic Potential of Orthotopic and Heterotopic Models Imaged by GFP and RFP Protein

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Abstract. *Studies over the past decade have clearly shown that s.c. implant of primary and cultured tumor cells rarely leads to the occurrence of metastatic disease. Orthotopic transplantation of cell suspensions, surgical orthotopic implantation (SOI) of cancer tissue fragments resulted in metastases in many cancer types reaching 100% successful rate. We compared two metastatic models – heterotopic model of Lewis lung cancer and orthotopic B16 mouse melanoma. Both models were syngeneic with high metastatic ratio in C57BL/6 mice after transplantation of cancer cells, by injection into subcutaneous region of mice tail and without surgical intervention. The conclusion is that the localisation of cancer cell injection is a crucial condition for metastatic potential. The site with 100% haematogenous and lymph metastasis rate, after simple injection of cancer cells only, has been defined in mice, without dependence on the genetically predisposition and tumor cell line.*

Metastasis is the major cause of cancer deaths and there is a need for clinically relevant *in vivo* models. For decades, xenografts using well-established human tumor cell lines have been the most commonly used models to study human cancers in mice. Historically, transplantable tumor models were characterized by and selected for rapid primary tumor growth at subcutaneous (*s.c.* -heterotopic) sites. In this setting, it was uncommon to observe spontaneous metastasis to distant sites (1).

Orthotopic transplantation refers to the delivery of cancer cells to the anatomic location or tissue from which a tumor was derived. Experimental evidence suggests that orthotopically transplanted tumours may be more appropriate models to investigate these physiological strategies than the usual *s.c.* transplanted tumour models (2, 3).

Here we compared two metastatic models – heterotopic model of Lewis lung cancer and orthotopic B16 mouse melanoma. We observed metastases in C57BL/6 mice after transplantation of cancer cells by injection and without surgical intervention.

Materials and Methods

Cell culture. Cell lines used in this study have been described previously (4). Lewis lung cancer cell line was labeled by GFP (green fluorescent protein) and B16 melanoma with GFP and RFP (red fluorescent protein). Except where noted, cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum and gentamicin to 70–80% confluence as described previously (4).

Subcutaneous tumor growth. All of the animals were maintained in a barrier facility. All animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals under assurance of Directive 86/609/EEC on the protection of animals used for scientific purposes in the Czech Republic. Three C57BL/6 mice, 6 weeks of age, were injected *s.c.* with single dose of 2×10^6 Lewis lung cancer cells and three C57BL/6 mice, 6 weeks of age, were injected *s.c.* with single dose of 2×10^6 B16 mouse melanoma. Cells were first harvested by trypsinisation and washed three times with cold serum-free medium and then injected - total volume of 0.2 ml.

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Table I. *Observed micro and macrometastases in relation to tumor weight (× micrometastases, •macrometastases).*

	Heterotopic Model - Lewis Lung Cancer										Orthotopic Model - B16 Mouse Melanoma									
Mice number →	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Time of living	4 weeks										4 weeks									
Volume of primary tumor →	2798	2167	1389	1245	1286	1409	2877	1134	1066	1339	1360	2180	1589	1289	1670	1184	1768	1446	2274	2546
Caudal nodes	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Sciatic nodes	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Lumbar nodes	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Inguinal nodes	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Mesenteric nodes	•	•	•	•	×	•	•	•	•	•	×	•	•	×	•	•	•	•	•	•
Suprarenal nodes	•	•	•	×	×	•	•	•	•	×		•								•
Pyloric or pancreatic nodes	•	•	•	•	•	•	•	•	•	•	×	•		•	×		×		•	•
Axillary nodes	•	×	•	•	×	×	•	•	•	×	•	•	×				×		•	•
Brachial nodes	•		•	•			•	•	•						×					
Mediastinal nodes	•																			
Deep cervical nodes	×	•	×	×			•	×	×				×				×			
Superficial cervical n.	×		×	×			×	×	×				×				×			
Kidney																				
Spleen																				
Liver	•			×		•	•	•				•								
Peritoneum	•	•	•	•		•	•	•	•			•		•			•		•	•
Lung	•	•		×			•	×				•					×		•	•

Tumor transplantation of C57BL/6 mice. Tumors derived from the Lewis lung cancer and B16 melanoma *s.c.* tumor growing in the C57BL/6 mice were homogenized and a single cell suspension was prepared. Cells adjusted to appropriate concentration were injected to the subcutaneous space of tail approx. 1 cm from root of the tail. Cells were transplanted in 5×10^6/mL cells in volume 0,1mL into dorsal side of tail. Animals were anesthetized by ketamine and xylazine during transplantation.

Analysis of metastasis. Mice were divided into two groups according to the tumor type. Animals from both groups were sacrificed four weeks after the Lewis lung cancer and B 16 melanoma injection. The size of primary heterotopic tumor was measured. The tumor volume was counted – $Tw=a(b^2)/2$ (where a and b are tumor length and width in (mm), respectively (5). The tissue samples from lymph nodes and organs, were collected and the presence of micro- and macrometastases was assessed. At least three micrometastatic and one macrometastatic lession per organ or lymph node was needed to be present for an organ to be considered positive for metastasis. The total metastases number was correlated to the tumor volume. The origin of a single fluorescent tumor cell was tested in inverted fluorescence microscope. Whole-body images were obtained by placing the mice in a fluorescent light box equipped with a fiberoptic light source of 490 nm (Lighttools Research, Encinitas, CA) and imaged using a Nikon Coolpix 5000 camera.

Results

A high metastatic rate was determined according to the estimated tumor volume in both observed mouse groups. Metastatic process was declared for each mouse in the groups and the mice were sacrificed four weeks after *s.c.* tail injection. The comparison of heterotopic and orthotopic metastasis was assessed – quantity, localization and size of the metastases. The average size of primary tumor was comparable – 167 mm^3 in heterotopic Lewis lung cancer and 173 mm^3 for orthotopic B16 mouse melanoma. The state of detected micro- and macrometastasis is shown in Table I.

Discussion

Studies over the past decade have clearly shown that *s.c.* implant of primary and cultured tumor cells rarely leads to the occurrence of metastatic disease. Injecting tumor cell suspensions into the analogy on or orthotopic mouse sites occasionally allowed relevant metastases.

Orthotopic transplantation of cancer cells may come from direct injection of tumor cells or the surgical implantation of intact fragments of tumor. Orthotopic injection of cell

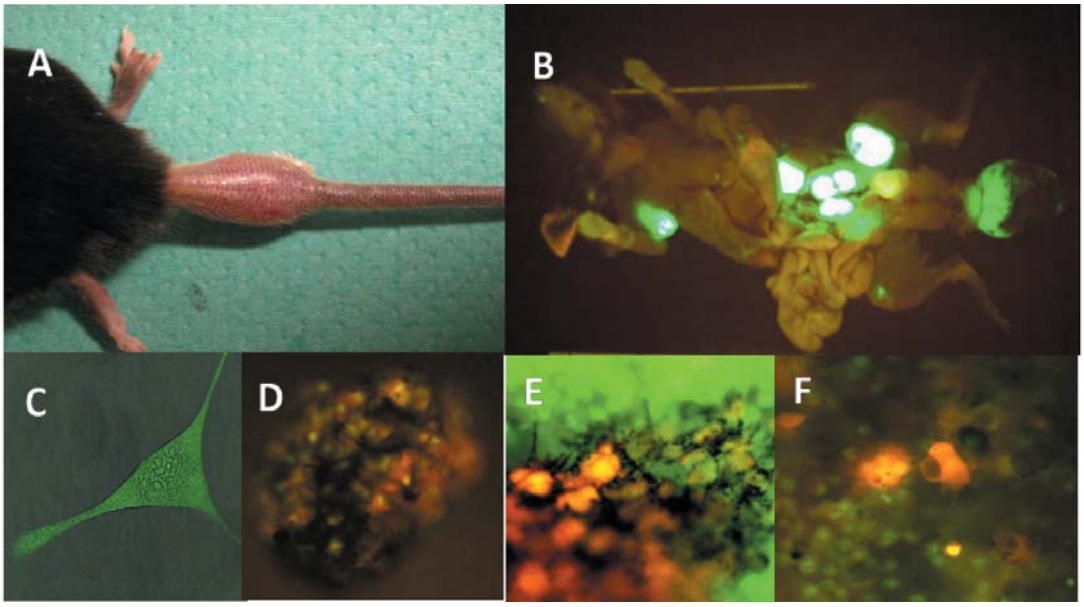

Figure 1. *A. view of cancer cell injection site (a root of the tail) with a growing Lewis Lung –GFP primary tumor; B. A whole body open image of the Lewis Lung metastasing tumor vizualized by GFP-fluorescence, with a focus on intraperitoenal dissemination; C. Disseminated Tumor Cell isolated from peritoneal PBS-washing, after direct cultivation. The proof of cancer origin has been obtained by fluorescent light. The enourmous size of the cell floating in peritoneum and multinuclear stage is interesting; D. Primary tumor of B16-GFP/RFP melanoma imagined by inverted microscopy. We may observe that the melanin produced by tumor mass may block fluorescence under the Illumina Tool Light box and it is not possible to observe metastasis as seen for the Lewis Lung –GFP model on the Figure 1B; E. B16-GFP/RFP melanoma infiltration in the retroperitoneal adipose tissue. We may identify a high proliferative cells with green nucleus and more differentiated cells with RFP-cytoplasma producing melanin; F. Similarly to the Fig.1E, we may also observe a changed cell morphology of B16 cells infiltration in adipose tissue (AT) reminding us on the morphology of macrophage like cells. The abundance of cancer cells in AT has not been unfortunatelly checked in all of the animals, that is why we do not comment on in the text.*

suspensions is an improvement over simple subcutaneous implantation but the technique has several major disadvantages. On the other side for many orthotopic models the use of surgical orthotopic implantation (SOI) of fragments improves the reproducibility and metastatic outcome within the model (6, 7).

In a comparison of SOI with orthotopic transplantation of cell suspensions, SOI of cancer tissue fragments resulted in metastases in many cancer types in 100% of the nude mice with extensive primary tumor growth (8). The primary tumors resulting from SOI were larger and much more locally invasive than primary tumors resulting from orthotopic transplantation of cell suspension. SOI generated higher metastatic rates than orthotopic transplantation of cell suspensions. Median survival time in the SOI model was significantly shorter than that of orthotopic transplantation of cell suspensions. Histological observation of the primary tumors from the SOI model demonstrated a much more rich vascular network than the orthotopic transplantation of cell suspension. Lymph node and lung metastases were larger and more frequent in the SOI model compared to the orthotopic transplantation of cell suspension models.

In our study, we observed a high metastatic rate of lymph and visceral metastasis after injecting of the tumor cell suspension in C57BL/6 mice in both tumor syngeneic versions – between the orthotopic and heterotopic tumors no significant differences in metastatic ratio, in metastases localisation and numbers have been observed. Without any surgical intervention a high metastatic rate of lymph and visceral metastases has been declared only after injection of tumor cells.

We declare that the most important for the metastatic potential are local conditions which can probably be more important that histological specification of primary tumor. Transplantation of cancer cells into region with limited growth and expansion possibility leads to the early metastatic process into lymphatics and visceral organs. If the space for the primary tumor growth is limited, the tumor proliferation potential prevail above the genetically predisposition of tumor to grow only in predisposed sites. The lymph and hematogenous metastatic ratio is 100% after inject transplantation for both cancer cell lines. Similar aspects were observed in our earlier studies where the primary tumor has been localized between skin and cartilage of mouse ear (9, 10). The metastatic ratio B16 and Lewis lung cancer has been similar in these studies.

The conclusion is a that the localisation of primary tumor in mouse metastatic model is crucial condition for metastasis development. There are sites on mouse body, where after injection of cancer cells 100%-rate of haematogenous and lymph metastasis occurs thanks to the limited space for primary tumor growth.

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