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Cervical Lymph Node Metastasis: Model for Study of Head/Neck Melanoma

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Tumour cells spread from primary tumours to form distant metastatic deposits by both lymphatic and blood routes. Melanomas occurring in the head and neck have an extremely poor prognosis largely in part due to late detection resulting in extensive dissemination by lymphatic metastasis. The purpose of this study was to develop an animal model for the study of head and neck melanoma metastasis. B16-F1 parental cells were injected into the subcutis of the ear mid-lobule of C57BL/6 mice. At selected time periods after inoculation, animals were killed by cervical dislocation and autopsied. In some animals tumours had spread to the cervical lymph nodes. Examination of organ systems revealed no evidence of distant metastases. Histological examination of the cervical lymph nodes revealed tumour invasion, beginning at a subcapsular sinus and progressing into the paracortical sinuses. Cells from these nodes were adapted to cell culture, expanded by passage and reinjected into new mice. Subsequent generations of lymph node-selected B16 cell lines were more metastatic than their parental cell line, as evidenced by a more rapid appearance of cervical lymph node and extensive node invasion. Morphologically, the lymph node-selected B16 cell lines were more dendritic than the original B16-F1 parent line and had a larger number of pseudopodial projections. Perhaps increased expression of pseudopods by the metastatic variants may allow for greater migratory potential and hence increased metastatic ability. These results indicate that highly mobile variant B16 sublines can be selected with an increased capacity for cervical lymphatic metastasis.

Keywords: melanoma, metastasis, neoplastic

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INTRODUCTION

THE INCIDENCE of cutaneous melanoma is increasing in the U.S.A. and throughout the world. Data from the National Cancer Institute's Surveillance Epidemiology and End Results System indicate that between 1973 and 1980, the incidence of melanoma in the U.S.A. increased by 80% [1]. We expect 32 000 new cases of melanoma to occur each year and 20% of these will originate in the head and neck [2]. Melanomas are malignant melanocytic tumours which at first grow superficially with little tendency to metastasise. As the lesion thickens and becomes vertically invasive, chances for metastasis increases sharply [3]. The tumour cells then invade the surrounding tissues and penetrate into blood vessels or the lymphatic system where they may be transported to distant sites. This metastatic process involves multiple interactions between the tumour cells and the extracellular matrix. These interactions are mediated by a variety of cell surface molecules, most notably the integrins [4].

It is now accepted that primary tumours consist of heterogeneous populations of cells and within the tumour exist subpopulations which are able to form metastases [5]. This supports the work of Nowell, who suggested that tumour cell variants arising within developing tumours are subjected to host selection pressures and give rise to new sublines with increased metastatic potential [6]. In addition, it has been demonstrated that dominance by highly metastatic clones in the primary tumour can enhance eventual metastatic spread of the tumour [7].

The purpose of this study was to develop a model for metastatic head and neck melanoma in mice. We sequentially selected *in vivo* a metastatic variant of the B16-F1 murine melanoma cells line which had a high efficiency in developing cervical lymph node metastases. We characterised these site-specific variants with respect to morphology.

MATERIALS AND METHODS

The parental B16-F1 melanoma cell line whose origin and properties have been described previously [8] was obtained from E.G. and E. Mason Laboratories. Parental and lymph node-selected B16-F1 variant cell lines were grown on tissue culture plastic in Dulbecco's modified minimum essential medium, supplemented with 5% fetal bovine serum (Gibco Laboratories, Grand Island, New York, U.S.A.) and 50 μg/ml gentamicin. Cells were passaged at preconfluence after a brief treatment with trypsin–EDTA.

Lymph node-specific variant lines were selected using a modification of the protocol developed by Talmage and Fidler

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Table 1. *Incidence of regional lymph node and pulmonary metastases in BL6 mice with different variants of B16 melanoma*

Variant	Tumour size (mm)	Total no. of mice	No. of positive nodes (%)	Pulmonary metastases
F1	2×3	5	4 (80)	0
F1	3×3	7	4 (57)	0
F1	3×4	8	4 (50)	0
F4	2×3	6	6 (100)	0
F4	3×3	6	6 (100)	0
F4	3×4	6	6 (100)	0

[9]. Tumour cells were injected subcutaneously into the ear lobule of 20 syngeneic 5–10-week-old male C57/BL6 mice. The mice were anaesthetised by measured inhalation of methoxyflurane. For injection, B16-F1 parental cells were harvested at subconfluency and brought up to a final concentration of 1×10^5 tumour cells/ml in Dulbecco's minimum essential medium. A 30-gauge needle was inserted into the subcutis of the ear mid-lobule and 50 μl of the cell suspension was injected.

Animals were inspected daily for general health and for growth of pigmented tumour. At selected time periods after inoculation, beginning at 2 weeks and continuing for 3 months, animals were killed by cervical dislocation and autopsied for the presence of tumours in the regional lymph nodes and organs. This process was repeated four times to obtain the B16-F4 cell line. When metastases were harvested for isolation of tumour cells, the animals were sacrificed, immersed in a disinfectant solution (70% EtOH), and removed in a laminar flow hood. Suspected regional and distant lymph nodes that appeared to be involved with the tumour were placed into culture dishes containing Hank's balanced saline solution (HBSS) and dissociated, as described by Fidler and Nicolson [10]. This process was repeated four times. The cell line obtained after four *in vivo* passages was named the B16-F4. Thirty-eight mice were sacrificed and 38 regional nodes were scored for lymphatic metastasis (Table 1).

RESULTS

Injection of the parental B16-F1 cells into the ear lobule of C57/BL6 syngeneic mice resulted in the isolation of subpopulations with an increased capacity to metastasise to regional lymph nodes. A majority of mice had developed a primary tumour at 6 weeks postinoculation (Fig. 1). Through the selection process described in Materials and Methods, the cells were reinjected into the ear lobules of syngeneic mice and again lymph node-selected variants were obtained. Subsequent generations of cervical lymph node-selected B16-F1 cell lines were more metastatic than their parental cell line, as evidenced by a more rapid appearance of tumours in the lymph node and more extensive nodal invasion (Table 1). Morphologically, the lymph node-selected B16-F1 cell lines were more dendritic than the original parent cells and had a larger number of pseudopodial projections (Fig. 2).

In earlobe-injected animals, tumours had spread to the cervical lymph nodes. Histological examination of the cervical lymph nodes revealed tumour invasion, beginning at a subcapsular sinus and progressing into the paracortial sinus (Fig. 3). Examination of the lungs and heart under a dissecting microscope failed to reveal evidence of tumour metastases.

DISCUSSION

What is known about lymph node metastasis is based on studies with a few animal models [11]. The initial event leading to metastasis to lymph nodes is the entry of tumour cells into lymph capillaries near the edge of the neoplasm. The cells migrate into the lumen by penetrating the cell–cell junctions of the lining lymphatic endothelium. Tumour cell emboli are then carried to the regional lymph nodes, where they are initially trapped in the medullary sinuses and establish an expanding metastatic deposit that frequently invades the

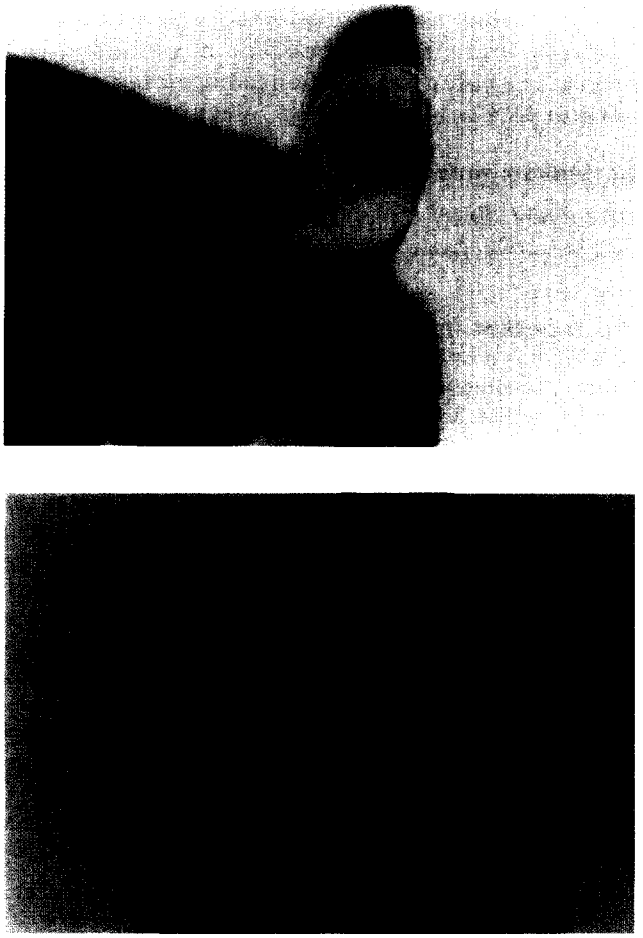

Fig. 1. Parental B16-F1 melanoma cells were injected subcutaneously into the mid-ear lobe of C57BL/6 mouse, at 6 weeks postinoculation the animal was autopsied. At this time, a primary tumour had formed at the site of injection (a) and a large metastases was present in the cervical lymph nodes (b).

adjacent parenchyma. If a secondary tumour is successfully formed, tumour cell proliferation and increased vascularisation occur. Neighbouring nodes may be subsequently seeded and retrograde lymph flow can result in distant deposits of metastases.

Malignant tumours are known to contain subpopulations of cells with differing metastatic abilities. Thus, within the parental B16-F1 tumour a heterogeneous population of melanoma cells exists with differing capacities to metastasise to various target organs. By applying the correct selection pressures, it is possible to amplify a desired subpopulation within the parental tumour. In our studies we have selected and characterised variant tumour cell populations from the parental B16-F1 tumour that are more efficient in metastasising to target cervical lymph nodes.

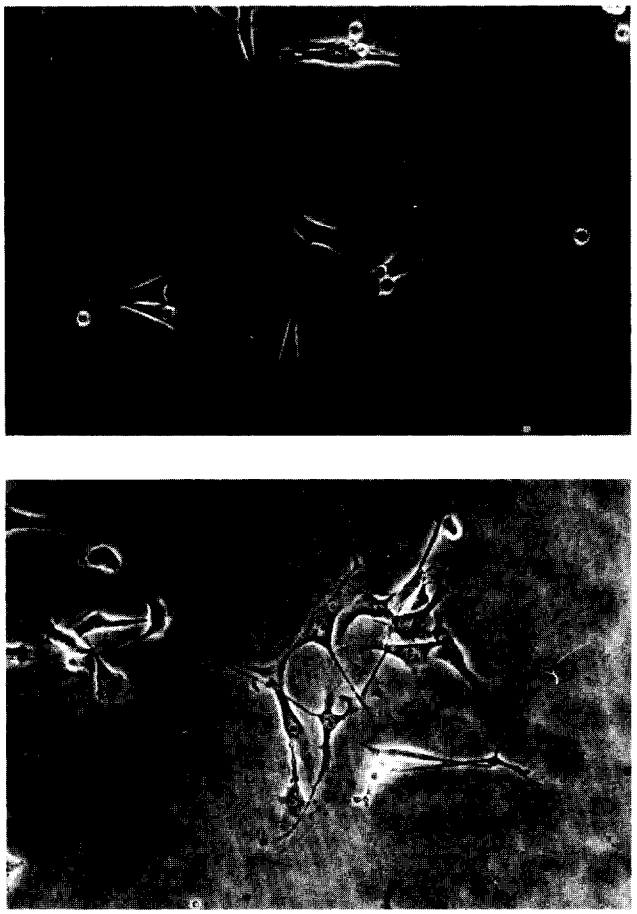

Fig. 2. Phase contrast, photomicrographs of the B16-F1 parent cells (a). The lymph node-selected B16 are more dendritic than the original B16-F1 parent cell line (b).

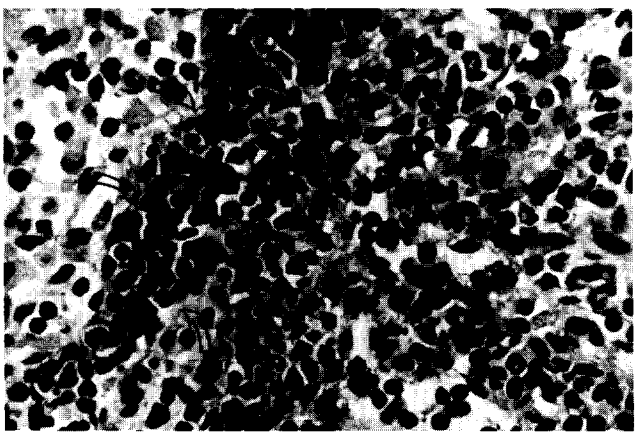

Fig. 3. Invasion of lymph node by B16 melanoma cells. This cervical lymph node from the mouse in Fig. 1 was processed for standard paraffin sectioning and stained with haematoxylin and eosin. Original magnification ×200.

The resulting cell line obtained after four *in vivo* passages was named B16-F4. This cell line demonstrated an increased capacity for cervical lymph node metastasis compared with the parental B16-F1. The results indicate that within the B16-F1 cell line exist unique subpopulations of cells that are efficient in forming cervical lymph node metastases. Previous work by Kramer *et al.* [12] demonstrates that tumour cells interact with the basement membrane-rich reticular fibres of the lymph node. Ramos *et al.* [13] showed that the B16 cell line expresses cell surface receptors specific for the basement membrane components laminin and collagen type IV, so it is possible that the metastatic variants generated in this study have increased expression of cell surface molecules specific for these basement membrane components. Another possible explanation for the increased lymph node metastases in the variants is that the tumour cells may be responding to a variety of cytokines produced by macrophages or other nodal cells during the metastatic process, resulting in enhanced tumour growth in the lymph node environment. It has been established that molecules contained within an organ's extracellular matrix may influence tumour cell attachment and growth properties [14]. It is possible that the lymph node extracellular matrix contains unique adhesive ligands that play a role in organ-specific metastasis. When grown in culture, the B16-F1 cells formed epithelioid clusters with many cell–cell contacts. In contrast, the metastatic variants appeared more dispersed with many pseudopodial projections resembling dendritic processes. Perhaps the apparent loss of cell–cell contacts in the B16-F4 cell line facilitates escape from the primary, which results in increased metastatic efficiency.

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