EPIGENETIC TRANSFER OF METASTATIC ACTIVITY BY UPTAKE OF HIGHLY METASTATIC B16 MELANOMA CELL-RELEASED EXOSOMES

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Aim: To investigate potential role of highly metastatic BL6-10 tumor cell-released exosomes (EXO) in transfer of metastatic activity into poorly metastatic tumor cell line F1. Methods: The highly metastatic B16 melanoma cell line (BL6-10) was generated in our laboratory. EXO from this cell line were isolated and amount of exosomal recovered proteins was measured using Bradford assay. For phenotypic analysis BL6-10 and F1 melanoma cells were stained with FITC-conjugated anti-MHC I (H-2Kb), MHC II (Ia) and Met 72 antibodies and analyzed by flow cytometry. C57BL/6 mice (8 per group) were injected (i.v.) with 0.5 × 10⁶ F1, BL6-10 and F1EXO melanoma cells. Lungs were removed 4 weeks after tumor cell injection, fixed in 10% neutral buffered formaldehyde and embedded in paraffin for histological analysis. Results: Data revealed that BL6-10 cells expressed metastasis marker (Met 72 tumor antigen), while F1 cells did not display this cell surface marker. All mice inoculated with BL6-10 melanoma cells had numerous lung tumor colonies, while mice injected with F1 tumor cells were free of lung metastatic colonies. BL6-10 tumor cells-released EXO also expressed Met 72 tumor antigen as BL6-10 tumor cells, but in less amount. F1 tumor cells can uptake EXO from BL6-10 tumor cells and express acquired exosomal Met 72 tumor antigen. Conclusion: The metastatic activity of highly metastatic BL6-10 tumor cells can be transferred to poorly metastatic F1 tumor cells by uptake of highly metastatic BL6-10 tumor-released EXO.

Key Words: exosome, melanoma, lung metastasis.
reported during comparison highly and poorly metastatic cell lines derived from the same parental tumor [20–23]. In addition, it was shown that tumor cells possess some metastasis-associated surface modifications [24, 25] which are believed to aid them in negotiating different steps in metastasis. A number of these tumor-associated antigens were identified as glycolipids [26, 27]. It was reported that neutral glycolipid antigen (isoglobotetraosylceramide) which is cell surface antigenic marker for the metastatic subpopulation in rat mammary adenocarcinoma cell line (R3230AC) is involved in metastatic colonization of lymph nodes by tumor cells [28, 29].

One general characteristic of tumor cells is their ability to release or shed intact, vesicular portions of membrane material (termed membrane vesicles or microvesicles), which were rediscovered in the mid-1980s and termed “exosomes” [30]. EXO include molecules with biological activity such as Fas ligand, mdr 1, CD44, and tumor antigens [31, 32]. Recently, these tumor-derived exosomes (EXO) was gained attentions as source of tumor antigens for vaccines, either directly or as part of processed dendritic cells (DC) [33–35]. However, potential role of these tumor-released EXO in transferring metastatic activity between highly and poorly metastatic tumor variants was not studied yet.

Previously we generated highly metastatic B16 melanoma cell line (BL6-10) and identified metastasis marker Met 72 glycoprotein expressing highly on BL6-10 cells, but not in the parental poorly metastatic B16 melanoma cell line (F1) [36]. In this study, we purified BL6-10 tumor cell-released EXO, assessed the Met 72 expression in EXO, and investigated the potential role of highly metastatic tumor cell-released EXO in transferring metastatic activity into poorly metastatic tumor cell line F1. We demonstrated that highly metastatic activity of BL6-10 tumor cells can be epigenetically transferred into the poorly metastatic F1 tumor cells by uptake of EXO derived from highly metastatic BL6-10 tumor cells.

MATERIALS AND METHODS

Reagents, cell lines and animals. Highly lung metastatic B16 melanoma cell line (BL6-10) was generated in our laboratory [36]. Poorly metastasizing counterpart B16 melanoma cell line (F1) was obtained from American Type of Culture Collection (ATCC) (Rockville, USA). Both cell lines were cultured in α-MEM medium (GIBCO, USA) containing 10% fetal calf serum (FCS). The fluorescence isothiocyanate (FITC)-labeled monoclonal anti-H-2Kb antibody (AP6-88.5) was obtained from Pharmingen Inc (Mississauga, Canada). The FITC-labeled monoclonal anti-Met 72 antibody (K88.151) was available in our laboratory [36]. The 5-carboxy-fluorescein diacetate (CFSE) was obtained from Molecular Probes (Eugene, USA). Female C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, USA) and maintained in the animal facility at the Saskatoon Cancer Center and treated according to the Animal Care Committee guidelines of the University of Saskatchewan.

Generation and purification of exosomes. BL6-10 tumor cells were labeled with CFSE by incubating tumor cells with CFSE for 2 h followed by PBS washing twice with centrifugation. EXO were isolated as described previously [37, 38]. Briefly, culture supernatants of BL6-10 or CFSE-labeled BL6-10 melanoma cells were subjected to four successive centrifugations at 300 × g for 5 min to remove cells, 1,200 × g for 20 min and 10,000 × g for 30 min to remove cellular debris and 100,000 × g for 1 h to pellet EXO. EXO pellets were washed twice in large amount of PBS and recovered by centrifugation at 100,000 × g for 1 h. The amount of recovered exosomal proteins was measured using Bradford assay (Bio-Rad, CA).

Phenotypic characterization of BL6-10 tumor cells and its released exosomes. For phenotypic analysis, BL6-10 and F1 melanoma cells were stained with FITC-conjugated anti-MHC I (H-2Kb), MHC II (Iab) and Met 72 antibodies, respectively, and analyzed by flow cytometry [39]. For phenotypic analysis of EXO, BL6-10 melanoma cells-derived EXO (20 µg) were incubated with FITC-conjugated antibodies on ice for 30 min, and then analyzed by flow cytometry [40]. To determine the optimal voltage suitable for EXO analysis, Dynal M450 beads with the size of 4.5 µm in diameter (DYNAL Inc, Lake Success, USA) were used as size control for flow cytometric analysis [40] using FACScan (Coulter EPICS XL, Beckman Coulter, San Diego, USA). Isotype-matched FITC-labeled irrelevant antibodies were used as controls.

Exosome uptake by F1 cells. F1 cells were cocultured with BL6-10 melanoma cell-derived EXO (10 µg/1 × 10⁶ cells) in 0.5-1 mL α-MEM medium containing 10% FCS at 37 °C for 4 h and washed twice with PBS by centrifugation, and then termed F1EXO cells.

Animal study. To study lung tumor colonization, naïve C57BL/6 mice (8 per group) were injected i.v. with 0.5 × 10⁶ F1 and BL6-10 melanoma cells, respectively, in one set of experiments. In another set of experiments, naïve C57BL/6 mice (8 per group) were injected i.v. with 0.5 × 10⁶ F1 and F1 melanoma cells. Mice were sacrificed 4 weeks after tumor cell injection, and lung metastatic tumor colonies were counted in blind fashion. Metastases on freshly isolated lungs appeared as discrete black pigmented foci that were easily distinguishable from normal lung tissues and confirmed by histological examination. When count of numerous metastatic foci was impossible this case was assigned as arbitrary value of > 100.

Histology. Lungs were removed from mice after 4 weeks of tumor cell injection, fixed in 10% neutral buffered formaldehyde and embedded in paraffin for histological analysis. Sections (5-6 µm thickness) were stained with hematoxylin-eosin using standard procedures [41].

RESULTS

Phenotypic and functional characterization of highly and poorly metastatic cell lines. Both BL6-10 and F1 cell lines expressed similar amount of cell surface MHC class I molecules (Fig. 1). BL6-10 cells expressed metastasis marker (Met 72 tumor antigen), while F1 cells did not display this cell surface marker. When wild-type C57BL/6 mice were i.v. injected with these tumor cells, all mice inoculated with highly metastatic BL6-10 melanoma cells had numerous lung tumor colonies, while mice...
injected with poorly metastatic F1 tumor cells were free of lung tumor metastatic colonies (Table 1).

**Fig. 1.** Phenotypic analysis of BL6-10 and F1 cells by flow cytometry. BL6-10 and F1 cells (thick solid lines) were stained with FITC-Abs specific for MHC class I and Met 72, and then analyzed by flow cytometry. These cells were also stained with isotype-matched irrelevant FITC-Abs, respectively, and employed as control populations (thin dotted lines)

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<td>BL6-10OVA</td>
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<tr>
<td>F1</td>
<td>0/8 (0)</td>
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<td>PBS</td>
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Notes: C57BL/6 mice (n = 8) were i.v. injected with BL6-10OVA and F1 cells or with PBS as a control. The mice were sacrificed 4 weeks after tumor cell injection and the numbers of lung metastatic tumor colonies were counted. One representative experiment of three is shown.

**Phenotypic characterization of BL6-10 tumor-released exosomes.** Exosomes (EXO) were purified from BL6-10 tumor culture supernatants by ultracentrifugation, and analyzed by flow cytometry. As shown in Figure 2, tumor-released EXO also expressed MHC class I molecules and Met 72 tumor antigen as BL6-10 tumor cells, but in less amount than BL6-10 tumor cells.

**Fig. 2.** Phenotypic analysis of BL6-10 and BL6-10-derived exosomes by flow cytometry. BL6-10 cells and BL6-10-derived exosomes (thick solid lines) were stained with FITC-Abs specific for MHC class I and Met 72, and then analyzed by flow cytometry. These cells were also stained with isotype-matched irrelevant FITC-Abs, respectively, and employed as control populations (thin dotted lines)

**Uptake of BL6-10 tumor-released exosomes by F1 tumor cells.** To assess the uptake of EXO, we incubated F1 tumor cells with CFSE-labeled EXO released from highly metastatic BL6-10 tumor cells. As shown in Fig. 3A, original F1 tumor cells did not express any CFSE. However, they expressed some amount of CFSE after F1 tumor cells were incubated with CFSE-labeled EXO, indicating that F1 tumor cells can uptake EXO. To assess whether F1 tumor cells can also uptake the exosomal molecules, F1EXO tumor cells derived from incubation of F1 tumor cells with the highly metastatic BL6-10 tumor-released EXO were analyzed by flow cytometry. As shown in Figure 3B, Met 72-negative F1 tumor cells became Met 72 positive, indicating that F1 tumor cells can uptake EXO and express acquired exosomal Met 72 tumor antigen.

**Fig. 3.** Exosome uptake by F1 cells. (A). F1 cells with (thick solid lines) and without (thin dotted lines) uptake of EXO were analyzed for CFSE expression by flow cytometry. (B). F1 cells with (thick solid lines) and without (thick dotted lines) uptake of BL6-10-derived EXO were analyzed for expression of MHC I and Met 72 using FITC-antibodies by flow cytometry. Irrelevant isotype-matched FITC-Abs was used as controls (thin dotted lines). One representative experiment of two is displayed

**The poorly metastatic F1 tumor cells become the highly metastatic Met 72-positive tumor cells after uptake of the highly metastatic BL6-10 tumor-released exosomes.** To further assess metastasis activity, we conducted animal studies by i.v. injection of F1 tumor cell with or without EXO incubation. As shown in Table 2, original F1 tumor cells did not induce any lung tumor colonization. However, the Met 72-positive EXO-uptaken F1EXO tumor cells induced numerous lung tumor colonies after i.v. tumor cell injec-
tion (Fig. 4), indicating that metastasis activity of highly metastatic BL6-10 tumor cells can be transferred to the poorly metastatic F1 tumor cells by uptake of highly metastatic BL6-10 tumor-released EXO.

Fig. 4. Lungs from mice injected with F1, BL6-10 and F1_10 EXO cells, respective. The lungs were fixed in 10% formalin and embedded in paraffin. The tissue sections were stained with hematoxylin and eosin. There is no metastatic tumor nodule in the group of mice with i. v. injection of F1 tumor cells, whereas there are large numbers of lung metastasis (arrows) in groups of mice with i. v. injection of BL6-10 and F1_10 EXO cells.

Table 2. Tumor incidence in mice inoculated with F1 cells and F1_10 EXO cells

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Notes: C57BL/6 mice (n = 8) were i.v. injected with F1 and F1_10 EXO cells or with PBS as a control. The mice were sacrificed 4 weeks after tumor cell injection, and the numbers of lung metastatic tumor colonies were counted. One representative experiment of three is shown.

**DISCUSSION**

Malignant melanoma is the leading cause of death from diseases of skin. Its incidence is increasing in various parts of the world [42, 43]. Despite improvements in diagnosis of locally invasive or disseminated malignant melanoma, curative therapy is not available for these patients [44]. A better understanding of molecular mechanisms involved in the process of metastasis could lead to development of new therapeutic strategies.

Tumor-derived EXO have gained attention as source of tumor antigens for vaccines [33–35]. The ability of these shed membrane vesicles to modulate lymphocyte and monocyte functions was analyzed in several tumor models. EXO released by metastatic tumor cells suppressed the expression of MHC class II antigens by monocytes and macrophages in dose-dependent manner, while similar ones from early stage tumor cells did not [45, 46]. It was shown that EXO also suppressed lymphocyte activation [47]. These tumor-derived EXO also inhibited activation of lymphocytes with interleukin-2 [48]. Recently, Taylor et al. [49] have demonstrated that these membrane vesicles can also suppress expression of CD3-ζ, subsequent activation signalling, inhibiting proliferation and cytokine production. However, potential role of these tumor-released EXO in transferring metastatic activity between highly and poorly metastatic tumor cell variants has not been studied.

Membrane transfer was abundantly reported in systems requiring or not requiring cell-cell contact [50]. Recently, we demonstrated that EXO were absorbed into mature and immature DC. The expression of MHC class II, CD40, CD54, CD80 was enhanced on DC after EXO uptake. The uptake is mainly mediated by non-specific CD28/CD80 and DEC205/C-type lectin receptor (CTLR) interactions between EXO and DC [51]. In this study, for the first time, we demonstrated that (i) EXO released from highly metastatic B16 melanoma cell line BL6-10 also expressed tumor metastasis marker (Met 72 tumor antigen), (ii) EXO can be uptaken by poorly metastatic B16 melanoma cell line F1, and (iii) the poorly metastatic F1 cells, after uptake of BL6-10 released EXO, can become highly metastatic F1_10 EXO tumor cells with the capacity to form large numbers of metastatic tumor lung colonies.

Tumor cells are genetically unstable, resulting in heterogeneity such as clonal subpopulations of tumor cells with highly and poorly metastatic capacities [11] within the histologically indistinguishable tumors. This study discloses new epigenetic pathway of the highly metastatic tumor cell formation. Our results thus provide first evidence that poorly metastatic tumor cells can epigenetically become highly metastatic tumor cells by uptake of highly metastatic tumor cell-released EXO. Therefore, this study may have great impact in better understanding of molecular mechanisms involved in the process of metastasis, which could lead to the development of new therapeutic strategies.
ACKNOWLEDGEMENT

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ЭПИГЕНЕТИЧЕСКИЙ ПЕРЕНОС МЕТАСТАТИЧЕСКОЙ АКТИВНОСТИ С ПОМОЩЬЮ ЭКЗОСОМ ИЗ КЛЕТОК ВЫСОКОМЕТАСТАЗИРУЮЩЕЙ МЕЛАНОМЫ В16

Цель: изучить возможную роль экзосом (EXO) из линии клеток BL6-10 с высокой метастатической активностью в передаче этого свойства линий клеток F1 с низкими показателями метастазирования. Методы: линия клеток B16 меланомы с высокой метастатической активностью (BL6-10) была создана в нашей лаборатории. EXO из клеток этой линии были выделены и количество экзосомальных белков определяли методом Бредфорда. Для фенотипического анализа клеток линий BL6-10 и F1 инкубировали с FITC-конъюгированными антителами против MHC I (H-2Kb), MHC II (Iab) и Met 72 и анализировали методом проточной цитометрии. Мышам линии C57BL/6 (n = 8 на группу) в/в вводили по 0,5 × 10⁶ клеток линий F1, BL6-10 и F1EXO. Через 4 нед после инъекции легкие животных подвергали гистологическому анализу и подсчитывали число метастазов. Результаты: показано, что клетки BL6-10 в отличие от клеток F1 экспрессируют маркер метастазирования Met 72. Экспрессия антитела Met 72 также была выявлена в EXO, выделенных из клеток BL6-10, но на более низком уровне. Показано, что клетки F1 способны поглощать EXO из BL6-10 клеток и экспрессировать приобретенный экзосомальный Met 72. У всех животных, которым в/в вводили клетки BL6-10 или F1EXO, развивались множественные опухоли легких, в то время как у мышей, инкубированных клетками F1, метастазы не были выявлены. Выводы: метастатическая активность клеток BL6-10 может быть передана неметастазирующим клеткам с помощью экзосом.

Ключевые слова: экзосомы, меланома, метастазы легких.