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The regulation of cancer cell migration by lung cancer cell-derived exosomes through TGF-β and IL-10

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Abstract. Tumorigenesis has been considered to be as a result of abnormal cell-cell communication. It has been proposed that exosomes act as communicators between tumors and their microenvironment and have been demonstrated to be involved in tumorigenesis and subsequent metastasis. However, the mechanisms underlying the role of exosomes in these processes remains elusive. The present study sought to determine the underlying mechanisms. Using two lung cancer cell lines, it was demonstrated that exosomes derived from metastatic small cell lung cancer cells (NCI-H1688) have greater effects on cancer cell migration, compared with exosomes derived from primary non-small cell lung cancer cells (NCI-H2228). Further characterization of the contents of the exosomes demonstrated that there were increased levels of TGF-β and IL-10 in exosomes from NCI-H1688 cells compared with exosomes derived from NCI-H2228 cells, in particular under hypoxia. Blockade of TGF-β and IL-10 with antibodies confirmed that these cytokines were essential for the regulation of cancer cell migration. Taken together, the results of the present study indicated that exosomes derived from cancer cells regulated the cellular migration of tumor cells through TGF-β and IL-10, which may provide a novel approach for developing therapeutic methods against cancer.

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Introduction

Cell-cell communication is critical in the development and function of an organism, in addition to cellular proliferation, differentiation, and apoptosis. Tumorigenesis is considered to be a result of abnormal cell-cell communication (1,2). The microenvironment of solid tumors is essential for their rapid growth and metastasis to other sites. As angiogenesis is required for all stages of tumor growth, proangiogenic factors such as TGF-β and VEGF are required for tumor formation and development (3-6). Previous studies have proposed that exosomes are potent communicators between tumors and their microenvironment, and potentially contribute to tumorigenesis and subsequent metastasis (7,8).

With a size of 50-100 nm, exosomes are tiny vesicles from endosomes or multivesicular bodies, and are present within the extracellular microenvironment and biological fluids, such as the blood and urine (9,10). A previous study presented evidence that exosomes have numerous functions and are key in an array of biological events, including coagulation and intercellular signaling (11). The contents of exosomes includes proteins, nuclear acids, and lipids, which usually vary with the cellular and tissue origins of the exosomes and are adapted to their functions (12,13). Indeed, the source of exosomes defines their function. Antigen-presenting cell-derived exosomes induce an immune response and tumor-derived exosomes suppress it. Exosomes contain selectively enriched mRNA and miRNA that regulate gene expression in target cells (14).

Lung cancer is the most common cause of cancer-associated mortality globally (15). The majority of lung cancer cases are carcinomas derived from epithelial cells in the lung, and may be divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Smoking has been demonstrated to be a major contributor to developing lung cancer (16). The prognosis for lung cancer is poor, with <15% of the inflicted population surviving for 5-years following diagnosis (17). Elucidation of the mechanisms underlying the tumorigenesis of lung cancer may aid the diagnosis, therapy and prevention. Although certain studies have demonstrated that cancer cell-derived exosomes are involved in tumor growth, to the best of our knowledge, there are no previous studies that investigate the roles of exosomes in the development of lung cancer.

In the present study, 2 lung cancer cell lines were used to investigate the exosome functions in the processes of migration and immunoregulation by cancer cells.

Materials and methods

Materials and reagents. TGF-β, IL-10 and MCP-1 were obtained from Peprotech (Rocky Hill, NJ, USA). Monoclonal mouse anti-human TGF-β (cat. no. 16-9243-85; 1:1,000) and rat anti-human IL-10 antibodies (cat. no. 16-7108-85; 1:1,000) were purchased from eBioscience (San Diego, CA, USA). The materials for cell culture were obtained from Thermo Fisher Scientific, Inc., (Carlsbad, CA, USA).

Cell culture and transfection. HEK 293T, NCI-H2228, NCI-H1688 and HMEC-1 cells were purchased from ATCC (Manassas, VA, USA), and cultured as to the manufacturer's instructions. Briefly, the cells were cultured in DMEM supplemented with 10% FBS and 50 U penicillin/50 μg streptomycin. Prior to exosomes preparation, the serum in the medium was replaced with 10% Serum Replacement reagent (Thermo Fisher Scientific, Inc.). For hypoxia exposure, the cells were cultured in an O_2/CO_2 incubator at either 1% O_2 at 37˚C in a 5% CO_2 humidified condition.

Exosomes preparation. Before the medium was collected for exosomes preparation, the HEK 293T, NCI-H2228 and NCI-H1688 cells were cultured in serum-free medium. The exosomes preparation was performed as previously described (18). Briefly, following 4 days in culture, 100 ml of medium was collected. Exosomes were harvested through subsequential centrifugation of supernatants (2x10 min, 500 x g; 1x20 min, 2,000 x g; 1x30 min, 10,000 x g), followed by centrifugation (90 min, 100,000 x g) and wash (PBS, 90 min, 100,000 x g). The pellet was resuspended in 8 ml phosphate buffered saline (PBS), and pelleted again at 100,000 x g, 60 min. The final pellet was resuspended in a small volume of PBS and was quantified using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Exosome preparations were stored at 4˚C until use.

Migration assay. Migration was assessed using a Transwell migration assay with HMEC-1 cells. The migration assay was performed as previous described (19). Briefly, cells were added to the upper ($1x10^6$ cells/ml) chamber, and to the lower chamber DMEM/10% FCS with 10 μg/ml exosomes. Following 12 h incubation, cells on the lower membrane side were fixed and stained with crystal violet stained and counted using a microscope (Eclipse TS100; Nikon Corporation, Tokyo, Japan).

ELISA. To analyze cytokines presence in exosomes by enzyme-linked immunoassay (ELISA) was performed according to the kit manufacturer's instructions. The ELISA kits for TGF-β, MCP-1 and IL-10 were purchased from R&D system (Minneapolis, MN, USA).

Statistical analysis. Statistical analysis was performed using SPSS software, version 20 (IBM, Armonk, NY, USA). Student *t*-test or one-way analysis of variance were used for comparisons to determine statistical significance, and P<0.05 was considered to indicate a statistically significant difference.

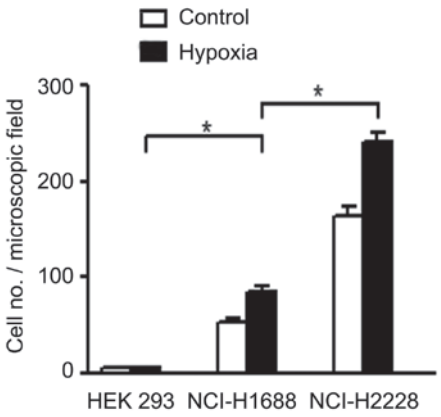

Figure 1. The regulation of cancer cell migration by exosomes. Transwell chemoattractant cell migration assay was performed. A total of $1x10^5$ HMEC-1 cells/well were added to the upper chamber, and DMEM/10% FCS with 10 μg/ml exosomes from different cells under regular or hypoxia to the lower chamber. Cells were allowed to migrate at 37˚C for 12 h in the medium prior to quantitation. Bars represent mean ± SEM number of transmigrated cells from 3 independent experiments. *P<0.05.

Results

The exosomes derived from cancer cells are required for migration. The contents of exosomes include various biologically active materials, including proteins and RNA. As cancer cells grows very rapidly, they are often under hypoxic conditions. As such, the functional differences were assessed in exosomes derived from cancer cells under hypoxic conditions. The present study used 2 cells lines representing classic small cell lung cancer (NCI-H1688) and non-small cell lung cancer (NCI-H2228), respectively. Exosomes were collected from these 2 cell lines cultured with regular oxygen levels (21%) or 1% oxygen. Human non-small lung cancer cells, NCI-H2228, were then used to detect whether the exosomes influence the migration of HMEC-1. The exosomes from 293T cells were used as a control. As presented in Fig. 1, the migration of HMEC-1 cells through Transwell filters was considerably increased by exosomes from lung cancer cells NCI-H1688 (P<0.05) and NCI-H2228 (P<0.05). These results indicate that hypoxia leads to exosomes promoting the migration of cancer cells. In addition, the exosomes from non-small cell lung cancer cell NCI-H2228 had more marked effects on cells migration compared with exosomes from small cell lung cancer cell NCI-H1688.

The exosomes derived from cancer cells contain various migration-associated factors. Since the exosomes derived from lung cancer cells increased the migration of endothelial cells and cancer cells, the present study then sought to further characterize the contents of exosomes involved in the regulation of migration. As TGF-β, MCP-1 and IL-10 have been previously been demonstrated to regulate the cellular migration of A375 human melanoma cells (20), these factors may also affect the migration of lung cancer cells. The Transwell assay demonstrated that TGF-β, MCP-1 and IL-10 all promoted

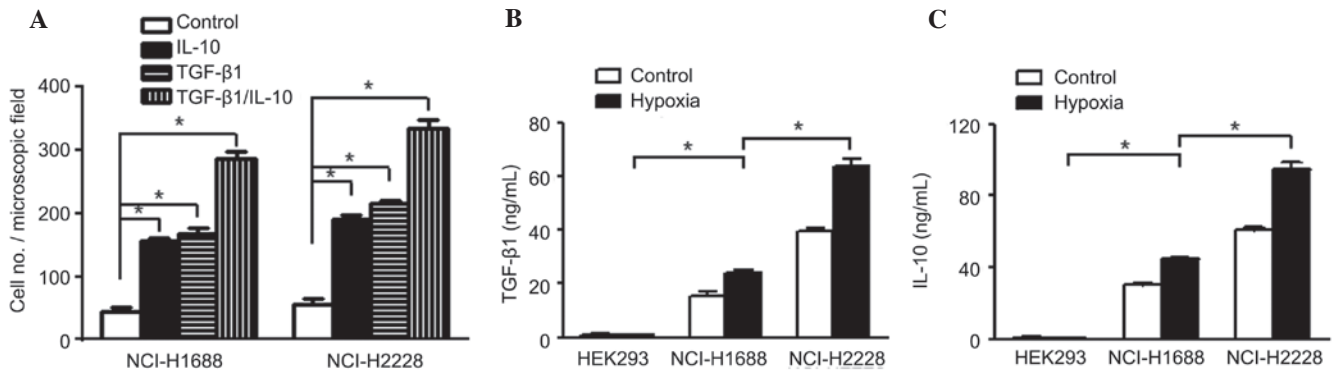

Figure 2. The exosomes derived from cancer cells contain a number of migration-associated factors. (A) 1x10^5 NCI-H1688 or NCI-H2228 cells/well were added to the upper chamber, and DMEM/10% FCS with TGF-β and/or IL-10 (10 ng/ml) to the lower chamber. Cells were allowed to migrate at 37˚C for 12 h in the medium before quantification (B). The exosomes collected from HEK-293, NCI-H1668 and NCI-H2228 under normaxia or hypoxia were freeze-thaw, and subjected to ELISA assay for (B) TGF-β and (C) IL-10. Bars represent mean ± SEM numbers of transmigrated cells from 3 independent experiments. *P<0.05.

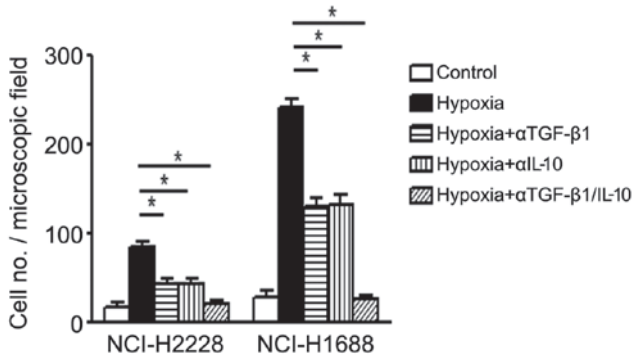

Figure 3. The TGF-β and IL-10 in exosomes are responsible for cellular migration. Transwell chemohaptotactic cell migration assay was performed. 1x10^5 NCI-H1688 or NCI-H2228 cells/well were added to the upper chamber, and DMEM/10% FCS with 10 μg/ml exosomes plus TGF-β and/or IL-10 antibody (5 μg/ml) were added to the lower chamber. Cells were allowed to migrate at 37˚C for 12 h in the medium prior to quantification. Bars represent mean ± SEM numbers of transmigrated cells from 3 independent experiments. *P<0.05.

the migration of NCI-H1688 and NCI-H2228 cells (Fig. 2A; P<0.05), supporting the hypothesis that TGF-β, MCP-1 and IL-10 contribute to lung cancer cell migration. Next, it was examined whether TGF-β, MCP-1 and IL-10 are present in exosomes. Following concentration of the exosome cell fraction by ultracentrifugation, repeated freeze-thaw of pellets was performed, and it was demonstrated that the concentration of TGF-β and IL-10 were increased in exosomes derived from non-small cell lung cancer cells NCI-H2288 and small cell lung cancer cells NCI-H1688, and particularly in hypoxic conditions (Fig. 2B and C; P<0.05). MCP-1, however, was not detected in exosomes from NCI-H1688 and NCI-H2228, under hypoxic conditions or not (data not shown). Thus, the data demonstrated that hypoxia enhanced the TGF-β and IL-10 contents in exosomes released from lung cancer cells, which promoted the migration of cancer cells.

The TGF-β and IL-10 in exosomes are responsible for cellular migration. As the exosome released from cells contains different components, which potentially work with or against each other, the next experiment specifically blocked the effects of TGF-β and IL-10 using neutrolizing antibodies individually or together. The results demonstrated that both TGF-β and IL-10 are essential for lung cancer cells migration, as blockade of TGF-β or IL-10 alone only partially (about 40%) reduced the effects of exosomes, while combined blockade of both TGF-β and IL-10 resulted in marked reduction (>80%) (Fig. 3; P<0.05), supporting the idea that the release of TGF-β or IL-10 from lung cancer cells mediate the enhanced cellular migration, which is essential for tumor metastasis.

Discussion

The contents of exosomes may have important effects on tumor cell malignancy. Previous studies have revealed that tumor cells are a dynamic resource for exosomes, such as melanoma (20), prostate cancer (21) and gliobastoma (22). Exosomes have been detected in human malignant effusions (12). Exosomes derived from different types of cancer contain different contents, therefore they may function distinctively on tumor behavior, indicating the significance of exosomes in prognosis, diagnosis, and therapy for cancer (21,23,24).

The contents of exosomes are heterogeneous, including diverse RNA, protein including cytokines, growth factors, and lipids (13,21,25). A number of miRs have been demonstrated to be present in exosomes from various cells (including cancer cells), which serve essential roles in the regulation of tumor cell migration (21-27). Other miRs may be involved in other steps of tumor development: miR-31, -185, and -34b are involved in melanoma invasion (25).

Proteomic analysis contributes much to the efforts of investigating exosomes, as exosomes contain bulky proteins, including growth factors such as VEGF, EGF, and various cytokines such as MCP-1, IL-4, which serve essential roles in tumor cell survival, proliferation and migration. TGF-β may induce expression of MCP-1 and IL-10 expression, which are involved in melanoma tumor progression (20). The present study investigated the effects of TGF-β and IL-10 on lung cancer cells, and attained similar data on migration of NCI-H2228 and NCI-H1688. TGF-β signaling has widespread crosstalk with multiple signaling pathways, including SMAD, PI3K (phosphoinositide 3-kinase)/AKT, and BRAF-MAPK (mitogen activated protein kinase) and therefore contributes

to the expression of MCP-1 and IL-10 (28). However, no MCP-1 expression was detected in lung cancer cells, both in NCI-H2228 and NCI-H1688 cells.

The present study revealed a novel aspect of exosomes derived from lung cancer cells, indicating that the contents of exosomes may promote migration. This finding could be potentially significant, leading to novel therapeutic methods. Of course, further detailed information into exosomes contents should be obtained in order to further evaluate the therapeutic role of exosomes on anti-tumor effects.

Acknowledgements

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