Mesenchymal Stem Cells Promote Formation of Colorectal Tumors in Mice

KUO–SHU TSAI,* SHUNG–HAUR YANG,[‡,§] YEN–PING LEI,[‖] CHIH–CHIEN TSAI,[¶] HSIN–WEI CHEN,[#] CHIH–YUAN HSU,[#] LING–LAN CHEN,[#] HSEI–WEI WANG,[‖,**] STEPHANIE A. MILLER,[‡‡] SHIH–HWA CHIOU,[‖,¶,#] MIEN–CHIE HUNG,[‡‡,§§,‖‖,¶¶] and SHIH–CHIEH HUNG,[‖,¶,#]

*Institute of Anatomy and Cell Biology, ‖Institute of Clinical Medicine, ¶Institute of Pharmacology, **Institute of Microbiology and Immunology, ‡Department of Surgery, National Yang-Ming University, Taipei, Taiwan; §Division of Colorectal Surgery, Department of Surgery, #Stem Cell Laboratory, Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan; ‡‡Department of Molecular and Cellular Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas; §§Center for Molecular Medicine, ‖‖Graduate Institute of Cancer Biology, China Medical University, Taichung, Taiwan; and ¶¶Department of Biotechnology, Asia University, Taichung, Taiwan

BACKGROUND & AIMS: Tumor-initiating cells are a subset of tumor cells with the ability to form new tumors; however, they account for less than 0.001% of the cells in colorectal or other types of tumors. Mesenchymal stem cells (MSCs) integrate into the colorectal tumor stroma; we investigated their involvement in tumor initiation. **METHODS:** Human colorectal cancer cells, MSCs, and a mixture of both cell types were injected subcutaneously into immunodeficient mice. We compared the ability of each injection to form tumors and investigated the signaling pathway involved in tumor initiation. **RESULTS:** A small number (≤10) of unsorted, $CD133^-$, $CD166^-$, epithelial cell adhesion $molecule^-(EpCAM^-)$, or $CD133^-/CD166^-/EpCAM^-$ colorectal cancer cells, when mixed with otherwise nontumorigenic MSCs, formed tumors in mice. Secretion of interleukin (IL)-6 by MSCs increased the expression of CD133 and activation of Janus kinase 2–signal transducer and activator of transcription 3 (STAT3) in the cancer cells, and promoted sphere and tumor formation. An antibody against IL-6 or lentiviral-mediated transduction of an interfering RNA against *IL-6* in MSCs or *STAT3* in cancer cells prevented the ability of MSCs to promote sphere formation and tumor initiation. **CONCLUSIONS: IL-6, secreted by MSCs, signals through STAT3 to increase the numbers of colorectal tumor-initiating cells and promote tumor formation. Reagents developed to disrupt this process might be developed to treat patients with colorectal cancer.**

Keywords: Marrow Stromal Cells; Cancer Stem Cells; JAK2; Tumor Development.

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Tumor initiating or stem cells (TICs), the initiation cells in tumors, are a minor population of tumor cells that possess the stem cell property of self-renewal and multilineage differentiation. Recently, a subpopulation of TICs were identified in colon cancer.[1,2] They are included in the high-density CD133+ population that accounts for about 2.5% of the tumor cells. Subcutaneous injection of colon cancer CD133+ but not CD133− cells readily reproduced the original tumor in immunodeficient mice. Another study also showed that the ability to engraft in immunodeficient mice was restricted to a minority subpopulation of epithelial cell adhesion molecule $(EpCAM)^{high}/CD44^+$ epithelial cells in colon cancer, and further identified CD166 as an additional differentially expressed marker, useful for TIC isolation in colon cancer.[3] These studies validate the stem cell working model in human colon cancer and provide a highly robust surface marker profile for colon TIC isolation and the small number of undifferentiated tumorigenic cells should be the target of future therapies.

Normal stem cells are controlled by a mechanism that allows them to proliferate or adapt to the microenvironment or niche of stem cells. The tumor microenvironment is composed of altered extracellular matrix and various nontransformed cells (eg, fibroblast, myofibroblast, myoepithelial, and endothelial cells). The orchestra interaction between microenvironmental components and tumor cells is bidirectional. Microenvironmental components regulate gene expression in tumor cells, thereby directing the tumor into one or several possible molecular evolution pathways, some of which may lead to tumor formation, progression,[4,5] metastasis,[6] and drug resistance[7] of neoplasms.

Among the microenvironment components of tumor, mesenchymal stem cells (MSCs) recently have attracted great interest because of their ability to migrate and engraft to areas of tumor development.[8] MSCs reside in the stroma of breast cancer and enhance tumor metastases via the Chemokine (C-X-C motif) ligand 5 (CCL5)–Chemokine (C-C motif) receptor (CCR) signaling pathways.[6] Cancer development involves a series of oncogenic transformations that may be endowed by tumor microenvironment. We have shown the integration of MSCs into tumor-associated stroma of colorectal cancer.[8] However, the involvement of MSCs (or their

Abbreviations used in this paper: αSMA, α-smooth muscle actin; CAF, cancer-associated fibroblasts; CCCs, colorectal cancer cells; CDX2, caudal-type homeobox transcription factor 2; DF, dermal fibroblast; EpCAM, epithelial cell adhesion molecule; GE, gingival epithelial; GFP, green fluorescent protein; IL, interleukin; MSC, mesenchymal stem cell; MSC-CM, MSC-derived conditioned medium; NTCs, non-transformed cells; TICs, tumor initiating or stem cells; TSM, tumor sphere medium.

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associated tumor stroma) in tumor initiation has not been addressed.

In the current study, we found that a very small number (≤10) of unsorted, CD133−, CD166−, EpCAM−, or triple-negative CD133-CD166-EpCAM- human colorectal cancer cells, when mixed with otherwise nontumorigenic bone marrow–derived human MSCs, obtain their de novo tumorigenicity when this cell mixture is introduced into a subcutaneous site and allowed to form a tumor xenograft. Moreover, the signaling pathway involved in MSC-mediated enrichment of TICs was identified.

Materials and Methods

Primary Cells and Cell Lines

The human colorectal cancer cell line HT-29 was obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY) containing 100 U/mL penicillin, 100 μg/mL streptomycin, 4 mmol/L glutamine, and 10% fetal bovine serum (Gibco). The MSC cell line was immortalized by retroviral transduction of HPV16 E6E7 and grown in Dulbecco's modified Eagle medium–low glucose (Gibco) supplemented with 10% fetal bovine serum.[8] Primary MSCs from different normal human volunteers were obtained from the Tulane Center for Distribution of Adult Stem Cells and were prepared and grown as described previously.[9] For preparation of fresh tumor cells, excised tumor tissue samples were digested for 4 hours with 3 mg/mL collagenase I (Sigma-Aldrich, St Louis, MO) in phosphate-buffered saline (PBS)/3% fetal calf serum at 37°C. Single-cell suspensions were obtained by repeated pipetting of cells followed by passage through a 40-μm strainer. MSC-like tumor stromal cells were isolated from colon cancer cells via their preference for migration by culturing primary tumor cells in the upper well of a Transwell (Costar, Cambridge, MA) containing 5-μm pores, where MSC-like cells passed through the base of the upper well and attached to the lower well. The details about MSCs, WI38, dermal fibroblasts (DFs), gingival epithelial (GE) cells, 293 cells, HT-29, and other tumor cells are listed in Supplementary Table 1. All cells were kept in a 37°C humidified atmosphere with 5% CO_2.

Characterization of MSCs

These immortalized or primary MSCs have been characterized to meet the definition of MSCs: plastic adherence; expression of MSC surface proteins such as CD29, CD44, CD90, CD73, CD105, and CD166; and possession of differentiation potential into osteoblast, adipocyte, and chondrocyte.[10]

Xenograft Transplantation

Study protocols involving mice were approved by the Institutional Animal Committee of Taipei Veterans General Hospital. Nonobese diabetic/severe combined immunodeficient mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained as a colony at the National Taiwan University Animal Facility in specific pathogen-free conditions. The mice were used for experiments at 6–8 weeks of age. Tumor cells admixed without/with MSCs were injected subcutaneously without Matrigel (BD Biosciences, San Diego, CA) or other extracellular matrix. Some mice transplanted with tumor cells admixed with MSCs were injected intraperitoneally with 10 μL PBS containing 5 μg anti–IL-6 antibodies (MAB206; R&D Systems, Minneapolis, MN) every 3 days until death.

Sphere Culture Conditions and Sphere Counting Assay

Primary colorectal cancer cells or colorectal cell line were resuspended in a modified tumor sphere medium[11] (TSM) (Dulbecco's modified Eagle medium/F12 medium consisting of a chemically defined serum-free medium with N2 supplement, recombinant human [20 ng/mL; PeproTech, Rocky Hill, NJ], epidermal growth factor [20 ng/mL; PeproTech], and fibroblast growth factor [10 ng/mL; PeproTech]), and plated at a density of 10^4 cells/well of a 6-well plate. Spheres were recognized as 3-dimensional cell colonies with a blurred cell margin in Ultra-Low Attachment Microplates (Corning, Lowell, MA). For cells treated with recombinant human IL-6 (R&D) or indirect co-culture with MSCs, spheres were recognized as cell colonies with more than 50% of the area showing a 3-dimensional structure and blurred cell margin. The ratio of the sphere was calculated as the percentage of sphere number to the total colony number.

Results

Nontransformed Cells Derived From Various Tissues Promote Colorectal Tumor Initiation and Tumor Sphere Formation

To investigate the functional consequences of the heterotypic interactions between HT-29 colorectal cancer cells (CCCs) and nontransformed cells (NTCs) derived from various tissues, the growth kinetics of the nontransformed cells containing tumors (CCCs plus NTCs) was compared with those of CCCs or NTCs alone in a xenograft model of immunocompromised mice. We found that co-injection with MSCs, WI38 lung fibroblasts, primary DFs, or primary GE, but not with 293 adenoviral-transfected human embryonic kidney epithelial cells, formed tumors with a smaller amount of tumor cells, 10^4 cells compared with 10^6 cells when injected with tumor cells alone, whereas 10^6 of NTC cells did not form tumors 3 months after transplantation alone (Supplementary Figure 1*A*). To further characterize the effect of NTCs on CCCs, we first compared the tumor sphere formation ability in TSM, a property of TICs in vitro, of HT-29 cells and HT-29 cells admixed with each type of NTC cells. We observed that HT-29 cells cultured alone had a minimal ability to form 3-dimensional tumor spheres and, as expected, MSCs or other NTC cells cultured alone were not able to form tumor spheres either (Figure 1*A* and Supplementary Figure 1*B*). Although co-culture of HT-29 cells with 293 cells did not change the tumor sphere formation (determined by sphere number and sphere ratio), the sphere formation ability of HT-29 increased when directly or indirectly co-cultured with MSCs (Figure 1*A* and *B*), WI38, and DF cells, and directly co-cultured with GE (indirect co-culture with GE only slightly increased sphere formation) (Supplementary Figure 1*C* and *D*). Because MSCs have been reported to migrate and incorporate into colorectal tumor development,[8] we therefore compared the effect of MSCs on tumor initiation and sphere formation with normal or tumor colonic fibroblasts. Interestingly, the ability of MSCs to enhance tumor initiation or sphere formation was significantly higher than normal or tumor colonic myofibroblasts (Supplementary Figure 1*E* and *F*).