Malignant Melanoma Induces Migration and Invasion of Adult Mesenchymal Stem Cells

Tammara L. Watts, MD, PhD; Ruwen Cui, BA

Objectives/Hypothesis: To determine if melanoma cells secrete chemotactic factors that result in the migration of multipotent stem cells.

Study Design: In vitro cell culture.

Methods: Chemotaxis and invasion of human mesenchymal stem cells (hMSCs) was determined using the modified Boyden chamber assay. Quantification of growth factors secreted by melanoma cells (A375) was determined using enzyme-linked immunosorbent assay.

Results: Conditioned A375 melanoma media caused significant migration and invasion of hMSCs compared to serum-free controls and conditioned media from normal melanocytes (P < .0001). The migratory effect appeared maximal after the A375 media was conditioned for 48 hours. Physiologically relevant concentrations of fibroblast growth factor-2 (FGF2) (90 pg/mL) secreted by A375 melanoma cells caused significant migration of hMSCs (P < .001) compared to serum-free and normal melanocyte controls. Neutralization of FGF2 inhibited the migration of hMSCs to that of the negative controls (conditioned media from normal melanocytes).

Conclusions: The melanoma tumor microenvironment may be maintained through chemotaxis and invasion of multipotent hMSCs, and this migratory effect appears to be mediated in part through secretion of FGF2 by melanoma cells.

Key Words: Head and neck cancer, mesenchymal stem cells, migration, FGF2.

INTRODUCTION

Although the incidence of many common cancers is declining, the incidence of melanoma is rising at a rate faster than that of any of the seven most common cancers.1 In 2011, an estimated 123,590 cases of melanoma were diagnosed, whereas in 2006 this number totaled ~62,000 cases (www.acs.org).1 More than half of the cases diagnosed in 2011 were invasive melanoma (high vertical growth rate). Survival for invasive melanoma is poor (~15%), especially when compared to noninvasive melanoma (radial growth phase), which has a cure rate of 99%.2 Transition from the radial growth phase to the vertical growth phase is the hallmark of invasive disease and associated with poor clinical response. Tumor invasion and metastasis require tumor cells to breach the basement membrane and surrounding connective stroma, creating a biologically active tumor microenvironment. The molecular pathways governing this response in melanoma and many other cancers are incompletely understood. Current hypotheses suggest complex signaling pathways involving cross-talk between matrix proteins, fibroblasts, and microvascular endothelial cells within the tumor–stromal microenvironment are critical for maintenance of the malignant phenotype.3

Understanding how the tumor microenvironment supports the growth and propagation of cancer is an active area of investigation. The process of carcinogenesis results in a tumor–stromal environment where inflammatory cells and activated fibroblasts express extracellular matrix proteins and secrete growth factors that potentiate malignant phenotype in a paracrine fashion.4 In vitro models of melanoma have shown that growth and proliferation of melanoma is basic fibroblast growth factor (bFGF, also known as FGF2) dependent.3 Other growth factors have been implicated in the pathogenesis of melanoma including stromal-derived growth factor-1α (SDF-1α).5 Melanoma cells that express the SDF-1α receptor, CXCR4, are associated with the presence of ulceration, increased tumor thickness, and higher rates of local and distant metastatic disease.5,6

Li et al. postulate that tumor cells and their stroma simultaneously evolve.7 Tumor stromal cells are thought to secrete chemokines and growth factors that initiate and maintain the malignant phenotype. The complex cellular interactions between fibroblasts/myofibroblasts, immune, endothelial, epithelial, and fat cells, along with extracellular cellular matrix proteins within the tumor stromal environment, either directly and/or indirectly with cancer cells, result in an acquired altered phenotype.7 Furthermore, these altered cells respond to growth factors and chemokines that stimulate tumor cell growth and recruitment of precursor cells.7

From the Department of Otolaryngology–Head and Neck Surgery, University of Texas Medical Branch, Galveston, Texas, U.S.A.

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Send correspondence to Tammara L. Watts, MD, PhD., 301 University Blvd., Galveston, TX 77555. E-mail: tlwatts@utmb.edu

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of these precursor cells is an area of active cancer investigation, and several reports suggest these cancer precursor cells may be cancer-associated fibroblasts, myofibroblasts, and/or an as yet unidentified cellular constituent.

Multipotent hMSCs responding to chemotactic stimuli from melanoma may suggest that the presence of cells within the tumor microenvironment is important for tumor growth and differentiation. However, to date studies specifically examining the physiology of hMSCs in melanoma are lacking. Therefore, we sought to understand whether invasive melanoma causes migration of hMSCs, and to further elucidate whether growth factor(s) secreted by melanoma may underlie this migration of hMSCs to the tumor microenvironment.

MATERIALS AND METHODS

Cell Lines

Adult human malignant melanoma cell line designation A375 was obtained from America Type Culture Collection (ATCC, Bethesda, MD), and is an invasive melanoma with a high vertical growth phase. A375 cells were cultivated in ATCC modified Dulbecco’s modified Eagle’s medium with 4 mM L-glutamine, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10% fetal bovine serum (FBS), penicillin, and streptomycin. hMSCs were obtained from Lonza (Walkersville, MD) and maintained in mesenchymal stem cell media (Lonza) supplemented with 10% FBS, 4 mM L-glutamine, penicillin, and streptomycin. A375 and hMSCs were grown at 37°C in an atmosphere of 5% CO2 in air.

hMSC Chemotaxis

hMSC chemotaxis was determined using the modified Boyden chamber assay, as previously described. Briefly, 24-well transwell, 6.5-mm-diameter, polycarbonate filter supports with an 8 μm pore size were precoated with collagen (50 μg/mL). hMSCs media was exchanged for serum-free media 24 hours prior to seeding the transwell filter supports. A total of 2.5 × 10^5 hMSCs in serum-free media were seeded onto the upper chamber of the transwell in a final volume of 250 μL. The upper chamber was placed into the lower chamber, which contained serum-free control media, conditioned A375 media, or experimental media with purified growth factors. Boyden chambers were incubated overnight at 37°C. Following a 24-hour incubation period, the media from the upper chamber was removed and the membranes fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). The membranes were washed in PBS and incubated with crystal violet. Nonmigrating hMSCs were removed from the upper chamber with a cotton-tip applicator. Cells that had migrated through to the bottom surface were then counted as described above. Migration was expressed as cells per high-powered field (HPF). Migration experiments were performed in triplicate (n ≥ 3).

hMSC Chemoinvasion

To determine chemoinvasion of hMSCs in response to conditioned melanoma media, transwells were prepared as described above and plated on Matrigel (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer’s protocol. The Matrigel was diluted in coating buffer (0.01M Tris Ph 8, 0.7% sodium chloride) final concentration at 200 μg to 300 μg/mL with precooled pipettes. hMSCs were resuspended in the media containing 1% penicillin-streptomycin at a density of 2.5 × 10^5 cells/mL. Transwells were incubate at 37°C overnight, and prepared as described above for staining and counting.

Enzyme-Linked Immunoassays for FGF2 Quantification

FGF2 secretion by A375 cells in culture medium was determined using a FGF2-specific enzyme-linked immunosorbent assay (ELISA) kit (Human FGF2 Immunoassay; Invitrogen, Grand Island, NY) according to the manufacturer’s instructions. All experiments were repeated three times in duplicate.

RESULTS

Melanoma Cells Cause Migration and Invasion of hMSCs

To condition the A375 melanoma media, monolayers were grown to ~80% confluent state prior to placing cells in serum-free media for 24 to 48 hours. This conditioned melanoma media was then placed into the lower chamber to determine if chemoattractants secreted by melanoma cells caused migration of hMSCs. A375 melanoma cells induced a >2-fold increase in the migration of hMSCs when compared to serum-free controls. A375 melanoma cells caused migration of hMSCs. A375 melanoma cells induced a >2-fold increase in the migration of hMSCs compared to cells exposed to conditioned media (CM) from normal melanocytes (P < 0.00001) (Fig. 1). This significant migration was maximal following melanoma media that was conditioned for 48 hours (P < 0.00001) (Fig. 1). As a further control, the media from primary normal melanocytes was conditioned in a manner similar to that for A375 melanoma cells. Conditioned media from primary melanocytes did not cause significant migration of hMSCs at 24 or 48 hours, suggesting the migration of hMSCs is induced by chemoattractants that are uniquely secreted by melanoma cells compared to primary melanocytes.

Fig. 1. Melanoma-induced migration of human mesenchymal stem cells (hMSCs). Modified Boyden chamber chemotaxis assay demonstrates a significant increase in migration of hMSCs compared to cells exposed to conditioned media (CM) from normal melanocytes (P < .000001). The migratory effects appeared maximal when the melanoma media was conditioned for 48 hours. Experiments were performed in triplicate (n = 3). HPF = high-powered field.
Invasion is one hallmark of the malignant phenotype. In vitro invasion was determined using the modified Boyden chamber assay with hMSCs grown on a Matrigel surface. Matrigel consists of type IV basement membrane collagen. hMSCs exposed to conditioned media from melanoma cells caused significant invasion of hMSCs through the basement membrane compared to hMSCs exposed to CM from normal melanocytes, serum-free Dulbecco’s modified Eagle’s medium (DMEM), and serum-free melanocyte basal media (MBM) \((P < .0001)\). Experiments were performed in triplicate \((n = 3)\). HPF = high-powered field.

**Physiologically Relevant Concentrations of FGF2 Secreted by Melanoma Cells Causes Migration of hMSCs**

Preliminary data from our labs suggest that several growth factors are chemoattractants for hMSCs (data not shown). Using an ELISA approach, A375 melanoma media conditioned for 48 hours was found to secrete significant levels of FGF2 (~90 pg/mL), whereas the concentration of FGF2 secreted from the conditioned media from normal melanocytes was comparable to serum-free media controls. Significant migration of hMSCs was observed when cells were treated with purified FGF2 at similar concentrations \((P < .0001)\) (Fig. 3B).

**Neutralization of FGF2 Results in Decreased Migration of hMSCs**

To determine the importance of FGF2 secretion as the primary chemoattractant responsible for the migration of hMSCs in melanoma, a neutralizing antibody approach was used in the modified Boyden chamber assay. A total of 0.2 \(\mu g/mL\) of FGF2 neutralizing antibody reduced the migration of hMSCs to the level of the negative control, conditioned media from normal melanocytes (Fig. 4). Both conditioned media from melanoma as well as isotype controls were able to cause significant migration \((P < .00001)\) of hMSCs, as previously observed.

![Fig. 2. Matrigel invasion assay. Human mesenchymal stem cells (hMSCs) were plated on a collagen type IV basement membrane (Matrigel). hMSCs exposed to conditioned media (CM) from melanoma cells caused significant invasion of hMSCs through the basement membrane compared to hMSCs exposed to CM from normal melanocytes, serum-free Dulbecco’s modified Eagle’s medium (DMEM), and serum-free melanocyte basal media (MBM) \((P < .00001)\). Experiments were performed in triplicate \((n = 3)\). HPF = high-powered field.](image1)

![Fig. 3. (A) Enzyme-linked immunosorbent assays were used to determine the physiologically relevant concentration of fibroblast growth factor-2 (FGF2) secreted by melanoma A375 cells. The media from both normal melanocytes and melanoma was conditioned for 24 hours and 48 hours. When compared to conditioned media from normal melanocytes at both 24 and 48 hours, melanoma cells secrete significant amounts of FGF2 when the media is conditioned for 48 hours \((P < .00001)\). (B) FGF2 dose response. Purified FGF2 resulted in significant migration of human mesenchymal stem cells at physiologically relevant concentrations \((P < .001)\). Experiments were performed in duplicate \((n = 3)\). SF = serum-free; HPF = high-powered field.](image2)

![Fig. 4. Fibroblast growth factor-2 (FGF2) neutralizing antibody blocks migration of human mesenchymal stem cells (hMSCs) in response to conditioned media (CM) from melanoma cells. Addition of 0.2 \(\mu g/mL\) FGF2 antibody resulted in a significant reduction in the migration of hMSCs compared to isotype controls \((P < .00001)\). Experiments were performed in triplicate \((n = 3)\). HPF = high-powered field.](image3)
DISCUSSION

Adult hMSCs are bone marrow-derived multipotent mesenchymal stromal cells capable of undergoing differentiation along the mesenchymal lineage to include osteocytes, adipocytes, connective tissue stroma (fibroblasts and myofibroblasts), and chondrocytes. hMSCs localize to sites of hematopoiesis, inflammation, injury, and solid tumors, suggesting that these stem cells may play an important role in the melanoma tumor microenvironment. In addition, the specific homing of hMSCs to solid tumors raises the hypothesis that hMSCs might be a novel delivery vehicle for targeted cancer therapy. Intravenous injection of hMSCs has been used for treatment of graft versus host disease, osteogenesis imperfecta, hematologic malignancies, and myocardial infarction in both human and animal models, and has thus proved to be an effective therapeutic target for the treatment of disease. There are little published data on the use of hMSCs as potential therapeutic targets in solid tumors. Only recently have reports in solid tumors shown that hMSCs may contribute to the tumor microenvironment.

We report here novel findings that conditioned melanoma media induces significant recruitment to and invasion of hMSCs compared to normal melanocyte controls (Figs. 1 and 2). Moreover, this migratory effect appears to be mediated in part through secretion of FGF2 (Fig. 3A,B). Neutralization of FGF2 inhibits of the migratory effect of hMCs in response to conditioned melanoma media. The FGF family is one of the largest chemokine families that modulate growth and differentiation for cells of mesenchymal origin, and four distinct receptors have been cloned to date. Increased FGF2 expression has been shown to clinically correlate with poorer tumor differentiation. FGF2 secreted by breast cancer cells have been shown induce migration of hMSCs. Future studies will seek to characterize the FGF receptor distribution on hMSCs, A375 cells, and normal melanocytes. The different migratory and invasive properties between normal melanocytes and hMSCs observed in this study may be due in part to varied FGF receptor profiles among these cells.

hMSCs have shown promise in the treatment of osteogenesis imperfecta, myocardial infarction, and acute graft versus host disease. Epithelial tumors, like melanoma, are pathologically characterized as displaying a desmoplastic reaction (fibroblast, myofibroblast, immune cells, and blood vessels embedded in connective tissue). The heterogeneous composition of tumors, in keeping with new concepts of the tumor microenvironment, suggests that several mesenchymal cells, including hMSCs, may be responsible for maintaining the malignant phenotype on the epithelium in vivo. Understanding how hMSCs are recruited by tumor cells to the microenvironment may offer novel therapeutic targets for melanoma and other solid tumors.

CONCLUSION

Melanoma causes significant migration and invasion of hMSCs compared to normal melanocytes. Recruitment to and invasion of hMSCs by melanoma, in part, is mediated by secretion of FGF2 by melanoma. Understanding the physiology of hMSCs within the melanoma tumor microenvironment, and which chemokines are important for their migration, may allow for the development of novel therapeutics for melanoma and other cancers.

BIBLIOGRAPHY