SDF1/CXCL12 Is Involved in Recruitment of Stem-like Progenitor Cells to Orthotopic Murine Malignant Mesothelioma Spheroids*

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Abstract. *Background/Aim: Tumor progression is influenced by the microenvironment. We found stem cells are recruited to malignant mesothelioma spheroids. We aimed to determine if stem cell recruitment depends on the chemokine SDF1, and if inhibition of the cognate receptor CXCR4 affects tumor growth. Materials and Methods: The kinetics of stem cell recruitment was determined using immunofluorescence staining, BrdU incorporation and eGFP transgenic mice. Chemokines were identified using PCR array. Inhibitors of CXCR4 were used to determine the effect on cell migration and tumor progression. Results: The increasing number of stem cells found in tumor spheroids over time is attributed to cell recruitment. Stem cell migration in vitro was enhanced by exogenous SDF1 and abrogated by CXCR4 inhibition and. CXCR4 inhibition reduced tumor burden in vivo. Conclusion: SDF1 is a candidate chemokine for recruitment of stem cells to malignant peritoneal mesothelioma and a potential target for therapy.*

Diffuse malignant mesothelioma is an aggressive tumor of the mesothelial lining surrounding pleural, peritoneal, and pericardial cavities and tunica vaginalis of the testis. Malignant mesothelioma is responsible for approximately 15,000-20,000 deaths annually worldwide (1), and most patients die within 18 months of diagnosis, for the tumor is highly resistant to current therapies. Proliferating mesothelioma cells can detach from extracellular matrix and proliferate within the pleural or peritoneal cavities as free-floating tumor spheroids (2). Formation and growth of tumor spheroids along with fluid accumulation in the peritoneal cavity result in ascitic tumors typical of ovarian cancer, colon cancer, gastric cancer and diffuse malignant mesothelioma. Tumor spheroids have been found to be more chemoresistant than primary tumors, potentially explaining tumor recurrence after chemotherapy (3). After tumor spheroids grow to a critical size, they attach to serosal surfaces and are capable of co-option of blood vasculature, tissue invasion, and formation of secondary solid tumors (4).

Stromal cells such as cancer-associated fibroblasts and endothelial cells also contribute to tumor progression (5). Using the stem cell marker stem cell antigen-1 (Sca-1), we identified a novel host stromal cell type, stem-like progenitor cells, in malignant peritoneal mesothelioma spheroids. Sca-1 is used as a marker to enrich for tissue-resident and cancer stem cells. Sca-1 expression occurs in embryonic and fetal cells, T lymphocytes, hematopoietic stem cells, mesenchymal stem cells, rare Sca-1-expressing tumor cells, and often overlaps with the side population (SP) phenotype found in tissues and cancers (6). Sca-1 expression has been correlated with a more malignant phenotype in tumors (7). A variety of murine cancer types, including retinoblastoma, mammary tumors and prostate tumors, exhibit upregulation of Sca-1 (8).

We hypothesized that the Sca-1-expressing cells found in malignant mesothelioma spheroids contain mesenchymal stem cells, a stromal adult stem cell population. Mesenchymal stem cells are recruited to sites of injury as well as to growing tumors and are potential targets for chemotherapeutic therapy or drug delivery. The most commonly expressed factor responsible for mesenchymal stem cell recruitment is the chemokine SDF1/CXCL12. There is a growing list of tumors that overexpress SDF1, including ovarian carcinoma, glioblastoma, pancreatic cancer, prostate cancer and thyroid cancer (9).

SDF1 binds to CXCR4, a 7-transmembrane G-protein coupled receptor expressed by vascular endothelial cells, hematopoietic stem cells and mesenchymal stem cells (10-13). Clinically, high CXCR4 expression correlates with

*This research was supported by: The National Institutes of Health grants RO1 ES03721 and F30 ES013639 with use of a core facility funded by the Superfund Research Program P42 ES013660.

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Key Words: Malignant mesothelioma, animal tumor model, tumor spheroids, mesenchymal stem cell, chemokine.

poorer prognosis in acute myelogenous leukemia and breast carcinoma (14). In epithelial ovarian carcinoma and gastric cancer, CXCR4 expression enhances peritoneal metastasis, a pathologic feature similar to diffuse malignant mesothelioma (15, 16). Therefore, the SDF1/CXCR4 chemotactic axis is a potential therapeutic target to prevent peritoneal tumor seeding and dissemination.

CXCR4 inhibition has been a focus of cancer therapeutics. Classes of drugs include small molecule inhibitors (AMD3100), antagonistic peptides (T22), antibodies, and small interfering RNA (17). Studies show that administration of these drugs decreased tumor growth, angiogenesis and metastasis; however, overall survival was frequently not improved (18). In cancer therapy, AMD3100 synergizes with cytotoxic chemotherapy in gliomas by decreasing activation of the Akt survival pathway (19).

We therefore hypothesize that mesenchymal stem cells home to transplanted malignant mesothelioma spheroids along a SDF1/CXCL12 chemotactic axis and that targeting this chemotactic axis can abrogate tumor growth and progression.

Materials and Methods

Immunofluorescence staining for Sca-1. C57Bl/6 mice were injected intraperitoneally with 1×10^6 syngeneic malignant mesothelioma cells. The tumor cells aggregate and form spheroids that grow and eventually attach to serosal surfaces in the peritoneum, invade local organs and metastasize to distant organs over a 28-day period (20). Peritoneal lavage cells were collected, immunofluorescently stained for Sca-1 (antibody from BD Pharmingen) and visualized using a Zeiss Axiovert 100 confocal fluorescence microscope (Carl Zeiss, Thornwood, NY, USA) or Nikon Eclipse E800 digital microscope.

Flow cytometry studies to distinguish recruited from proliferating Sca-1-expressing cells. Peritoneal lavage cells from wild-type mice were collected 14 days after malignant mesothelioma cell injection and reinjected into eGFP transgenic mice. Seven days post-injection into eGFP transgenic mice, peritoneal lavage cells were collected, labeled (for eGFP, Sca-1 and pulse-labeled with BrdU) and analyzed on a BD FACS Calibur Flow Cytometer or imaged with confocal or epifluorescence microscopy. Lavage cells from five eGFP mice were evaluated.

Sorting of Sca-1 cells from peritoneal lavage fluid using flow cytometry. Sca-1-labeled peritoneal lavage cells collected 21 days after IP injection of malignant mesothelioma cells were sterilely sorted using a BD FACS Aria flow cytometer. The sorted cells were plated and allowed to adhere to the plastic overnight. Adherent and non-adherent cells were collected separately for immunofluorescence analysis using antibodies against antigens reported to be expressed by various Sca-1$^+$ cells, including CD34, CD45, WT1, CD90, CD4 and CD8 (BD Pharmingen). Cells were pooled from ten C57Bl/6 mice.

RT^2 Profiler™ PCR arrays and ELISA for mouse cytokines and receptors. Total RNA was isolated from peritoneal lavage at 7, 14, 21 and 28 days following intraperitoneal injection of mesothelioma cells or from mesothelioma cells grown in monolayer *in vitro*. Quantitative real-time PCR array was used to screen for expression of inflammatory cytokines, their receptors and angiogenic factors according to the manufacturer's instructions (SuperArray Bioscience. Frederick, MD, USA). A gene-wise, two sample, t-test was performed for each transcript to identify statistically significant differences in expression between solid tumors and the cell line *in vitro*. The results from 3 replicate arrays were analyzed. Quantitative ELISA (R&D Systems) assays in triplicate were used to determine the levels of SDF-1 protein in peritoneal lavage fluid collected from mice 7 and 21 days following injection of malignant mesothelioma cells.

Reverse transcription PCR. Total RNA was isolated using Tri-Reagent (Molecular Research Center Inc. Cincinnati, OH). Total RNA was reverse transcribed and PCR was performed using the following forward and reverse primers: *CXCR4* (345 bp product, 5'-TCTTCCTGCCCACCATCTAC, 3'- TCAGCAGCAGTTTCCTTGG); 18S (106 bp product, 5'-AAACGGCTACCACATCCAAG, 3'-GGCCTCGAAAGAGTCCTGTA); *β-actin* (250 bp product, 5'-GTGGGCCGCTCTAGGCACCA, 3'-TCACGGTTGGCCTTAGGGT TCAGGG). PCR products were separated on 3% agarose gels and visualized using a Bio-Rad Gel Doc Imaging Station (Bio-Rad, Hercules, CA, USA).

Transwell migration assays. Mesenchymal stem cells (from Dr. Darwin Prockop of Tulane University) were plated on transwell filters and placed in wells containing lavage fluid collected 7 or 21 days after injection of malignant mesothelioma cells. After 24 hours, transmigrated mesenchymal stem cells were collected and counted using a Coulter® Cell Counter. In SDF1/CXCR4 studies, MSCs were pretreated for one hour with either T22 (3 μM, Bachem) or AMD3100 (10 μg/mL, Sigma), or 10 ng/ml SDF1 recombinant protein (R&D Systems) was added to the lavage fluid. For transwell migration studies with conditioned media, spheroid cells were plated for five days before collection of conditioned media. Graphical representation shows standard deviation of the mean with *p*-values calculated using Student's *t*-test.

In vivo studies of malignant mesothelioma progression. C57Bl/6 mice were intraperitoneally injected with syngeneic malignant mesothelioma cells followed by daily intraperitoneal injections of either 5 mg/kg AMD3100 or 1 ml PBS vehicle control. Fourteen and 21-days after tumor cell injection, tumor spheroids were harvested by peritoneal lavage and tissue was dissected for formalin-fixation and paraffin-embedding. The care and study of animals were in accordance with institutional guidelines. Organs harvested included: intestines with attached mesentery, kidneys, liver, spleen, pancreas, pelvic mesentery, diaphragm, lungs and heart. Formalin-fixed tissues were sectioned at 5 μm, and two sections from each tissue were selected for staining with hematoxylin and eosin (H&E; Richard Allan Scientific, Kalamazoo, MI, USA). Tumor number and area were calculated from brightfield images. Statistics were calculated from 6 mice per group. Graphical representation shows standard deviation from the mean with *p*-values calculated using Student's *t*-test.

Results

Spatial and temporal distribution of Sca-1$^+$ cells in mesothelioma spheroids. Z-stack images illustrate that Sca-1$^+$ cells were located towards the center of the tumor spheroids (Figure 1a). The kinetics of Sca-1$^+$ cell recruitment to tumor spheroids after 14-21 days show an increasing number of Sca-

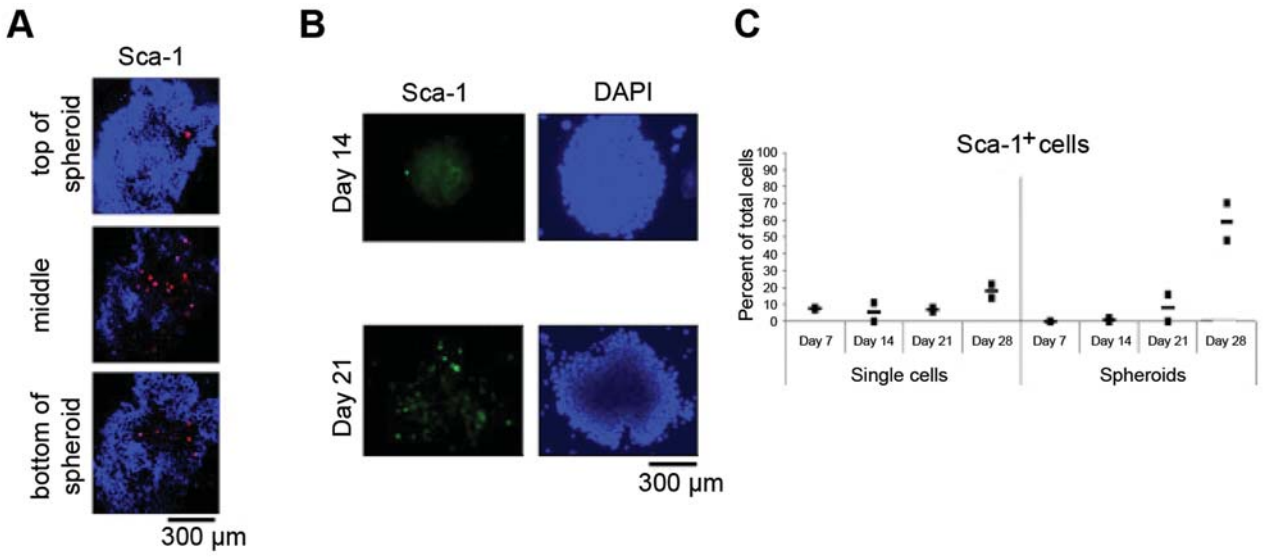

Figure 1. *Spatial and temporal distribution of Sca-1+ cells. A: Confocal Z-stack (60 µm) spheroid showing Sca-1+ cells (red) in the center. B: Increasing number of Sca-1+ cells (green) in spheroids over time. C: Flow cytometry data showing no increase in percentage of Sca-1+ cells amongst single cells but an increasing percentage in spheroids over time.*

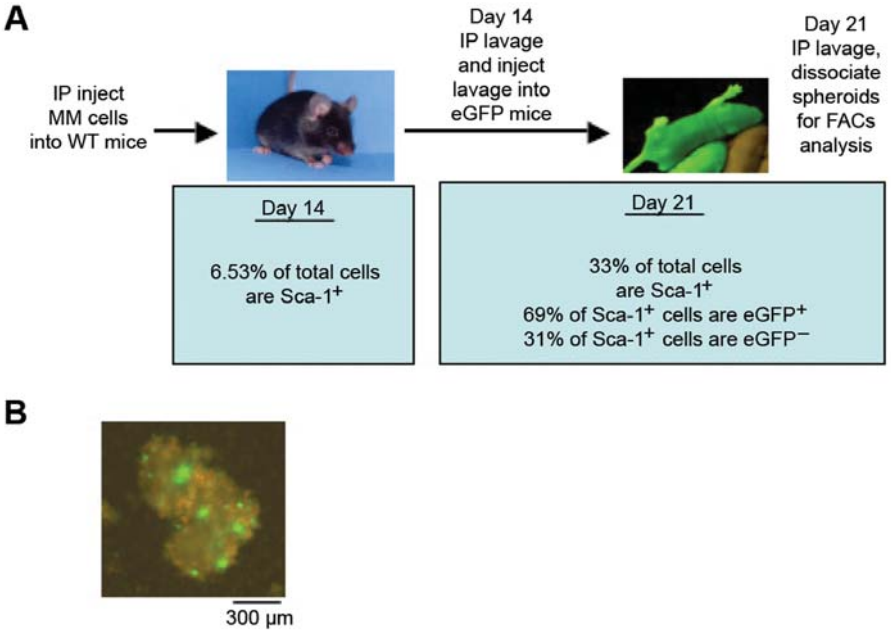

Figure 2. *Sca-1+ cells are recruited to tumor spheroids and are not proliferating. A: Schematic of experimental design. Flow cytometry shows that 69% of Sca-1+ cells are recruited (eGFP+) while 31% are proliferative (eGFP–). B: Day 21 tumor spheroids were pulse-labeled with BrdU and immunostained for BrdU incorporation and Sca-1. BrdU+ cells (green) were not Sca-1+ (red).*

1+ cells (Figure 1b). The single cell population not incorporated into the tumor spheroids consisted of a low percentage of Sca-1+ cells throughout this time course, while tumor spheroids showed an increasing percentage of Sca-1+ cells over time (Figure 1c).

Ongoing cell recruitment rather than cell proliferation accounts for the increasing number of Sca-1+ cells. The percentage and total number of Sca-1+ cells in tumor spheroids increased from 14 to 21 days after injection of malignant mesothelioma cells (Figure 2a). We aimed to

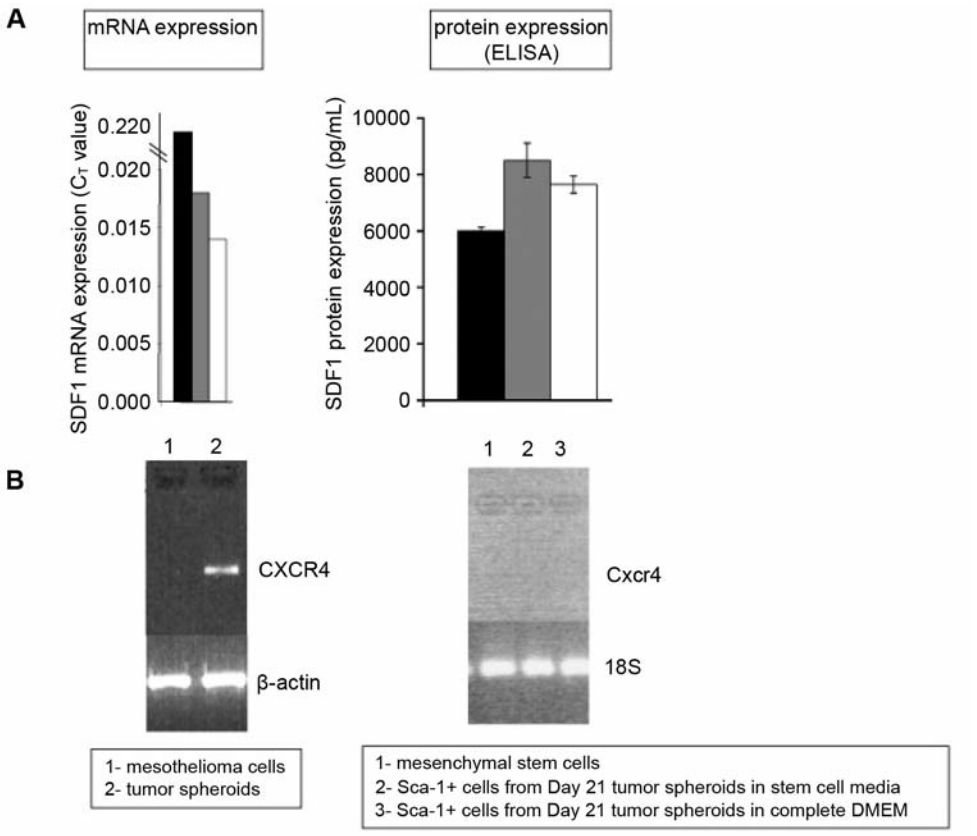

Figure 3. *SDF1 and CXCR4 expression in tumor spheroids. A: SDF1 expression at the mRNA and protein levels. B: Left panel: CXCR4 expression by tumor spheroids but not by mesothelioma cells grown in vitro; β-actin is the loading control. Right panel: No CXCR4 expression by mesenchymal stem cells grown in monolayer or by Sca-1+ cells collected from day 21 lavage; 18S is the loading control.*

determine whether the increasing number and percentage was due to cell recruitment or proliferation. Double positive ($eGFP^+/Sca\text{-}1^+$) cells are indicative of $Sca\text{-}1^+$ host cells recruited to the tumor spheroids. In contrast, $eGFP^-/Sca\text{-}1^+$ cells are the original $Sca\text{-}1^+$ cells injected into the transgenic mice. Flow cytometry results show the majority of $Sca\text{-}1^+$ cells are recruited (69% $eGFP^+$) *versus* proliferating (31% $eGFP^-$) (Figure 2a).

Furthermore, $BrdU^+$ cells visualized in the tumor spheroids did not co-localize with $Sca\text{-}1^+$ cells (Figure 2b). Therefore $Sca\text{-}1^+$ cells in tumor spheroids are not proliferating in S phase of the cell cycle at day 21.

$Sca\text{-}1^+$ cells sorted from tumor spheroids are a heterogenous cell population. Cells expressing T lymphocyte markers comprised the majority of the $Sca\text{-}1^+$ cell population (94%), followed by mesenchymal stem cells (4.5%), malignant mesothelioma cells (1%) and hematopoietic stem cells (0.5%) (Table I). T lymphocytes and hematopoietic stem cells are nonadherent in cell culture and together comprised 94.5% of the $Sca\text{-}1^+$ cell population. The adherent cells, mesenchymal stem cells and mesothelioma cells, comprised the other 5.5% of the $Sca\text{-}1^+$ cell population.

Table I. *The $Sca\text{-}1^+$ cell population contains multiple cell types.*

Antigen expression (putative cell type)	% Total $Sca\text{-}1^+$ cells	Adherence after 48 h
$CD34^+CD45^+$ (hematopoietic stem cell)	0.5%	Non-adherent
$CD34^+$ $WT1^+$ (mesothelioma cell)	1%	Adherent
$CD34^-$ $CD45^-CD90^+$ (mesenchymal stem cell)	4.5%	Adherent
$CD34^-CD45^-/CD4^+$ or $CD8^+$ (T lymphocytes)	94%	Non-adherent

Gene and protein expression of the SDF1/CXCR4 chemotactic axis in the tumor spheroid microenvironment. Previously reported factors for mesenchymal stem cell recruitment to tumors include the chemokines stromal-

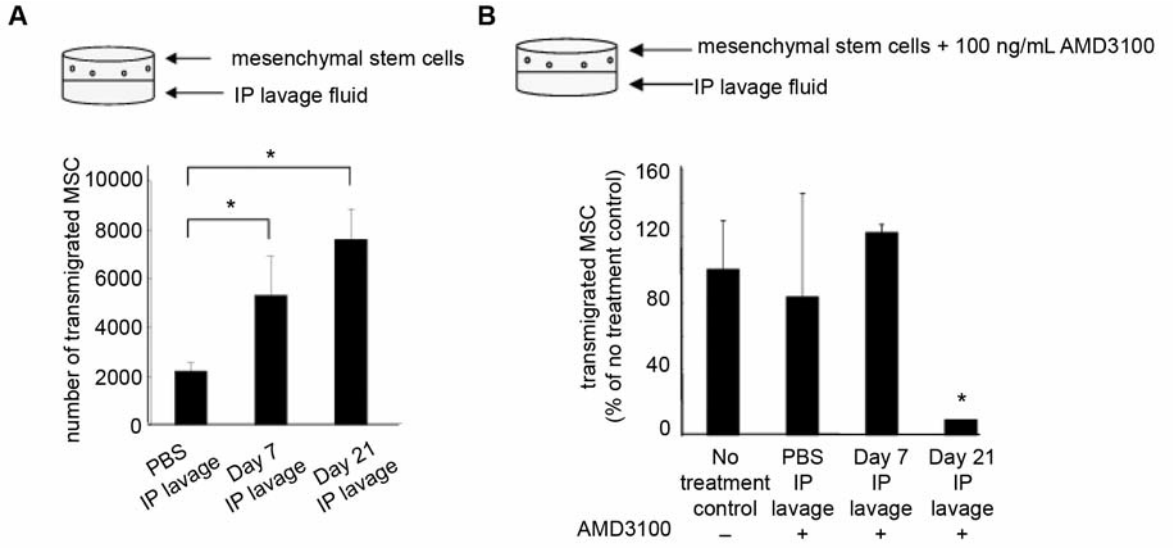

Figure 4. *A: Migration of mesenchymal stem cells to lavage fluid collected from PBS-injected mice and tumor-bearing mice 7 and 21 days following injection of mesothelioma cells. B: AMD3100 inhibits transmigration of mesenchymal stem cells to lavage fluid. (*p-value <0.05 compared to no treatment control unless otherwise noted, Student's t-test).*

derived factor 1 (SDF1/CXCL12) and macrophage inhibitory protein 3β (MIP3β/CCL19); and the growth factors platelet/derived growth factor (PDGF), insulin-like growth factor (IGF) and hepatocyte growth factor (HGF) (21). Transplanted malignant mesothelioma cells and tumor spheroids express all of these factors, as determined using real-time PCR array analysis in our laboratory. Of note is significant and stable expression of the chemokine SDF1/CXCL12 at both the RNA and protein levels at 7, 14 and 21 days following injection (Figure 3a).

The cognate chemokine receptor for SDF1/CXCL12 is CXCR4. Mesenchymal stem cells have been reported to express CXCR4 (10-12). Using RT-PCR, CXCR4 expression was absent from mesothelioma tumor cells and mesenchymal stem cells cultured *in vitro*, but present in multicellular tumor spheroids isolated *ex vivo*.

In vitro migration of mesenchymal stem cells is dependent on the CXCR4/SDF1 chemotactic axis. A significantly greater number of mesenchymal stem cells transmigrated to the lower chamber containing lavage fluid from tumor cell-injected mice than from PBS-injected mice. There was also more robust transmigration to lavage fluid collected 21 days after injection of malignant mesothelioma cells than to lavage fluid collected after 7 days (Figure 4a).

CXCR4 inhibition with AMD3100 resulted in no significant abrogation of transmigration of mesenchymal stem cells to PBS lavage fluid or to lavage fluid collected 7 days after injection of malignant mesothelioma cells; but there was significant abrogation of transmigration to lavage fluid collected 21 days after injection (Figure 4b). These findings suggest that migration of mesenchymal stem cells to malignant mesothelioma spheroids is not dependent on the SDF1/CXCR4 chemotactic axis early after tumor cell injection, but is increasingly dependent at 21 days.

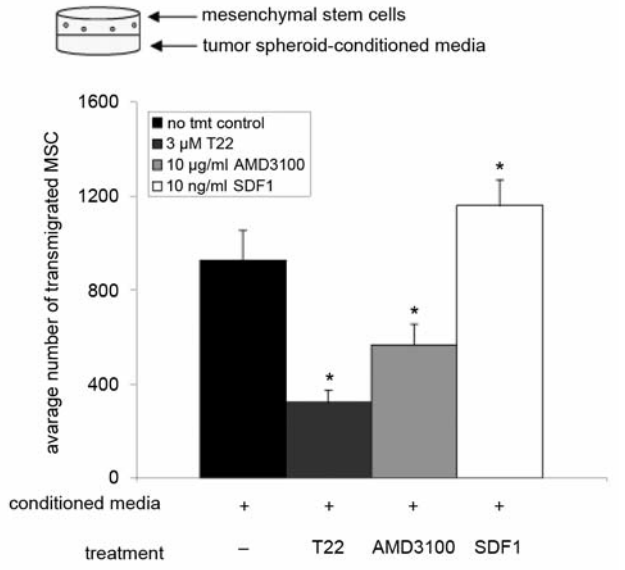

Figure 5. *Transmigration of mesenchymal stem cells to conditioned media from tumor spheroids is dependent on the SDF1/CXCR4 chemotactic axis. MSC transmigration to conditioned media is abrogated after CXCR4 inhibition with T22 or AMD3100. Transmigration is enhanced by addition of exogenous SDF1. (*p-value <0.05 compared to no treatment control, Student's t-test).*

We hypothesized that tumor spheroids are a significant source of SDF1 in the malignant mesothelioma microenvironment. There was significant abrogation of transmigration of mesenchymal stem cells pretreated with CXCR4 inhibitors to conditioned media from tumor spheroids (Figure 5). Also, addition of SDF1 recombinant protein to the lavage fluid significantly increased transmigration of mesenchymal stem cells (Figure 5).

CXCR4 inhibition and tumor growth and progression in vivo. From the studies using CXCR4 inhibition *in vivo*, the total number of tumors in PBS-injected control mice significantly increased from 14 days to 21 days post-injection of malignant mesothelioma cells (Figure 6a). The total number of tumors in AMD3100-treated mice also increased up to 14 days, but then the number significantly decreased in the treatment group. These results are consistent with the kinetics of the *in vitro* findings that AMD3100 treatment abrogates stem cell migration to lavage fluid collected from mice 21 days, but not 14 days, after injection of malignant mesothelioma cells.

The percentage of tumor spheroids in both treatment groups was determined after 14 and 21 days (Figure 6b). The percentage was consistently around 40% in PBS-injected mice at both time points. In AMD3100-treated mice, the percentage significantly decreased from 60% at 14 days following injection to an average of 20% at 21 days (Figure 6b). At 14 days after injection of malignant mesothelioma cells, AMD3100-treated mice had a significantly higher percentage of tumor spheroids than PBS-injected control mice.

Total tumor area was also assessed as a measure of tumor burden. In both PBS-injected mice and AMD3100-injected mice, the total tumor area increased from 14 days to 21 days post-tumor cell injection (Figure 6c). However, the increase in total tumor area was greater in the PBS-injected mice. In summary, CXCR4 inhibition with the small molecule inhibitor AMD3100 *in vivo* reduced tumor burden in this orthotopic murine model of malignant mesothelioma, especially between 14 and 21 days after injection of tumor cells.

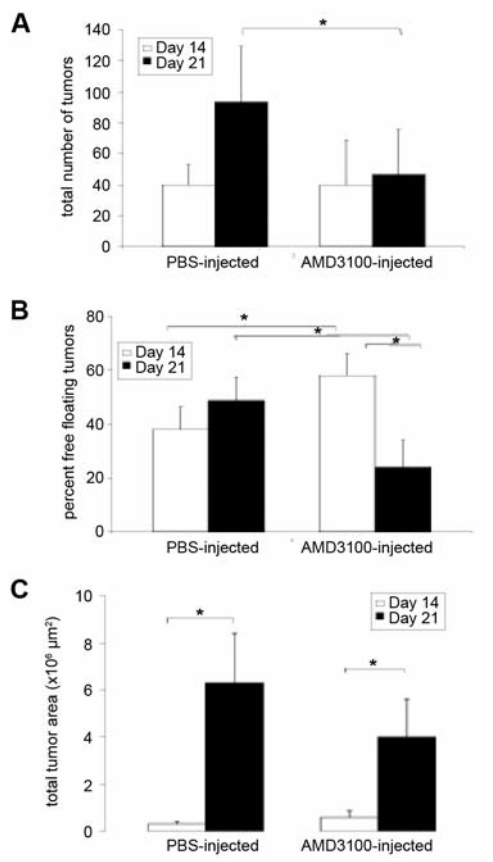

Figure 6. *Tumor burden in mice after mesothelioma cell injection followed by daily injection of PBS or AMD3100. A: Total tumor number significantly increased in PBS-injected but not in AMD3100-injected mice. B: Initial higher percentage of tumor spheroids in AMD3100-injected mice compared to control. C: Greater increasing trend in total tumor area in control than in AMD3100-injected mice. (*p-value ≤0.05, Student's t-test).*

Discussion

The work presented here describes the recruitment of a putative stem cell population to malignant mesothelioma spheroids in a transplantable murine tumor model.

Stem cells are slowly proliferating cells whose proliferation status is niche dependent (22). We found that the increasing number of Sca-1+ cells found in tumor spheroids over time is due to cell recruitment rather than cell proliferation, and was also confirmed by BrdU incorporation studies It is possible there is a number of proliferative Sca1+ cells cycling but not in the S-phase during this BrdU pulse labeling. Alternatively, recruited Sca-1+ cells may undergo cell proliferation at a slow rate.

The Sca-1+ cells consist of adherent mesenchymal stem cells. Recruitment of mesenchymal stem cells to malignant mesothelioma tumors is a novel finding. Mesenchymal stem cells comprised 82% of the total adherent Sca-1+ cell population. We hypothesized that the malignant mesothelioma tumor spheroid microenvironment is conducive to recruitment of mesenchymal stem cells. While many chemokines and growth factors are reported to be involved in recruitment of mesenchymal stem cells (11, 12) the highest expression levels were found for the chemokine SDF1/CXCL12, at both the mRNA and protein levels. Trends in mRNA expression were not paralleled by protein expression in these studies; this could be explained by the fact that mRNA expression was measured using tumor spheroid cells alone while protein expression was

measured using cell culture media samples containing secreted SDF1 protein. However, there is slightly higher expression of SDF1 after 7 days compared to 21 days after injection of malignant mesothelioma cells. This supports the hypothesis that the malignant mesothelioma microenvironment is conducive to ongoing recruitment of mesenchymal stem cells.

RT-PCR studies showed no expression of CXCR4 by malignant mesothelioma cells grown in monolayer *in vitro*, but significant CXCR4 expression was found in *ex vivo* malignant mesothelioma spheroids. These findings suggest that malignant mesothelioma cells do not express CXCR4, rather, malignant mesothelioma cells injected *in vivo* recruit CXCR4-expressing host cells that are integrated into tumor spheroids. In addition to a CXCR4-expressing mesenchymal stem population, there may be recruitment of $CXCR4^+$ lymphocyte population similar to the lymphocyte population found in human malignant pleural and peritoneal mesothelioma (23).

CXCR4 expression may be dependent on cell culture conditions. Cells grown in monolayer in culture have different gene expression profiles than cells grown as spheroids in culture (24). It is possible that the mesenchymal stem cells and adherent Sca-1^+ cells used in our studies would up-regulate CXCR4 expression if grown as spheroids *in vitro* or immediately used *ex vivo*.

Transwell migration studies showed increased mesenchymal stem cell recruitment to lavage fluid collected from mice 21 days after malignant mesothelioma cell injection compared to lavage fluid from mice after 7 days, and there was greater mesenchymal stem cell recruitment at both time points compared to lavage fluid from PBS-injected mice. This trend of increasing Sca-1^+ cell recruitment over time is consistent with the *ex vivo* experiments described earlier.

Mesenchymal stem cell migration to lavage fluid from PBS-injected mice and to lavage fluid collected 7 days after injection of malignant mesothelioma cells was unaffected by AMD3100 pretreatment. It is possible that other chemotactic factors play a more significant role for mesenchymal stem cell recruitment at this early time point in tumor growth and progression. However, at 21 days following injection of mesothelioma cells, there is significant inhibition of mesenchymal stem cell recruitment. This result suggests that the SDF1/CXCR4 chemotactic axis plays a significant role in recruitment of mesenchymal stem cells at the later stages of malignant mesothelioma growth and progression.

The peritoneal fluid can be a depot for secreted molecules from multiple cell types found in the peritoneal cavity, including tumor cells, recruited host cells, and resident cells of the intestinal mesentery including milky spots. In experiments where the only source of SDF1 would be from cells of the tumor spheroids, pretreatment of mesenchymal stem cells with CXCR4 inhibitors significantly abrogated mesenchymal stem cell recruitment. Taken together, these findings demonstrate the dependence of mesenchymal stem cell transmigration on the SDF1/CXCR4 chemotactic axis in this murine tumor model, and that tumor spheroids are a significant source of SDF1.

CXCR4 inhibition *in vivo* reduced tumor burden in our murine tumor model. Interestingly, the percentage of total tumor consisting of free-floating tumor spheroids was significantly different between the two treatment groups. By 21 days after tumor cell injection, the percentage of tumor spheroids significantly decreased in AMD3100-injected mice but not in PBS-injected mice. This decrease may be attributed to tumor cell death due to apoptosis in AMD3100-injected mice during this time frame resulting in a decreased percentage of tumor spheroids. When total tumor area is divided into solid tumor area *versus* tumor spheroid area, it is clear that AMD3100-injected mice have less tumor area than PBS-injected mice, and that tumor area in AMD3100-injected mice is comprised mostly of tumor spheroids and not solid tumor. Taken together, there is a trend towards decreased tumor burden in mice treated with AMD3100. This may correlate with decreased Sca1^+ cell recruitment and incorporation into tumor spheroids. It is also possible that AMD3100 inhibits other stages in malignant mesothelioma progression, such as angiogenesis.

In conclusion, tumor spheroids from this orthotopic murine mesothelioma model represent a dynamic state of mesenchymal stem cell recruitment during tumor growth and progression. The SDF1/CXCR4 chemotactic axis plays a significant role in mesenchymal stem cell recruitment to malignant mesothelioma spheroids and could be a novel therapeutic target for this aggressive tumor, especially as part of a multimodal therapeutic approach following surgical debulking.

Acknowledgements

We thank Dr. Darwin Prockop of the Center of Gene Therapy at Tulane University for the mesenchymal stem cells used these studies. We also thank Paula Weston from Brown University's Molecular Pathology Core Facility for her contributions to the histology work.

Bonnie Lau, Ph.D.: Conception and design, financial support, collection and assembly of data, data analysis and interpretation, manuscript writing.

Agnes Kane, MD, Ph.D.: Conception and design, financial support, administrative support, data analysis and interpretation, final approval of manuscript.

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Received March 5, 2010
Accepted March 24, 2010