

Monocyte Chemotactic Protein-1 Secreted by Primary Breast Tumors Stimulates Migration of Mesenchymal Stem Cells

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Abstract Purpose: Major barriers to effective adenovirus-based gene therapy include induction of an immune response and tumor-specific targeting of vectors. The use of mesenchymal stem cells (MSC) as systemic delivery vehicles for therapeutic genes has been proposed as a result of their combined ability to home in on the tumor site and evade the host immune response. This study is aimed at investigating factors mediating homing of human MSCs to breast cancer primary cultures and cell lines *in vitro* and *in vivo*.

Experimental Design: Fluorescently labeled MSCs were given to mice bearing breast cancer xenografts, and tumor tissue was harvested to detect MSC engraftment. MSC migration in response to primary breast tumors *in vitro* was quantified, and chemokines secreted by tumor cells were identified. The role of monocyte chemotactic protein-1 (MCP-1) in cell migration was investigated using antibodies and standards of the chemokine. Serum MCP-1 was measured in 125 breast cancer patients and 86 healthy controls.

Results: Engrafted MSCs were detected in metastatic breast tumors in mice after systemic administration. There was a significant increase in MSC migration in response to primary breast tumor cells *in vitro* (6-fold to 11-fold increase). Tumor explants secreted a variety of chemokines including GRO α , MCP-1, and stromal cell – derived factor-1 α . An MCP-1 antibody caused a significant decrease (37-42%) in MSC migration to tumors. Serum MCP-1 levels were significantly higher in postmenopausal breast cancer patients than age-matched controls ($P < 0.05$).

Conclusions: These results highlight a role for tumor-secreted MCP-1 in stimulating MSC migration and support the potential of these cells as tumor-targeted delivery vehicles for therapeutic agents.

Despite improved therapy and advances in detection methods, breast cancer remains the most common cause of cancer death in women worldwide (1). Clearly, there remains a need for novel therapeutic strategies for treatment of this disease. A variety of gene therapy approaches for breast cancer treatment have been developed and evaluated, but clinical responses remain poor (2). The majority of these approaches are based on the use of adenoviral vectors to deliver the therapeutic gene or product. Adenoviral vectors can transduce a variety of cell types, infect both proliferating and quiescent cells, and can be

produced in high titers with relative ease. The major barriers to effective therapeutic results are the induction of an immune response (2, 3) and the ability to obtain tumor-specific targeting of the vector (4). The use of mammalian cells as systemic delivery vehicles for therapeutic genes has been proposed as a method to overcome rapid adenovirus degradation by the immune system and provide potential for increased delivery doses (5).

Mesenchymal stem cells (MSC) are a subset of nonhematopoietic stem cells within the bone marrow stroma, characterized by their ability to differentiate into cells of connective tissue lineages and to self-renew. MSCs contribute to the maintenance and regeneration of connective tissues through engraftment induced by signals produced by tissues when there is a demand for new connective tissue cells, such as during wound healing or scar formation (6). Malignant cells also induce formation of connective tissues to support tumor growth and progression. This has led to studies investigating homing of MSCs to tumors to investigate their potential as targeted delivery vehicles for therapeutic genes or their products (7 – 12). MSCs offer a number of advantages as gene delivery vehicles: they are easy to isolate and expand to the numbers required for use, are transducible with viral vectors, will engraft after reintroduction, and can be delivered systemically or locally. Also, perhaps most importantly, they seem to evade the host immune response (13, 14).

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A number of recent studies have reported successful engraftment of *ex vivo* transduced MSCs at the site of lung metastases, gliomas, colorectal tumors, and ovarian tumors in animal models (8–12, 15, 16). However, the signals that mediate migration to specific targets remain unknown and will need to be fully elucidated before translation into clinical practice. Thus far, *in vitro* studies investigating factors controlling MSC migration to breast cancer have been based solely on commercially available breast cancer cell lines. Although an invaluable research tool, the majority are not derived from primary tumors and are prone to genetic drift (17). Primary culture of fresh breast tissue specimens harvested at surgery, although challenging, more closely represents the *in vivo* situation and takes into account reciprocal interactions between cells within the tumor microenvironment.

Demonstration of MSC homing in on breast tumors *in vivo* has been limited to pulmonary metastases of the disease, and although a promising therapeutic effect was shown, there remains a question as to whether engraftment was due to tumor-specific targeting or trapping of the MSCs by the capillary system in the lung (8). In a glioma model, the majority of i.v. given MSCs were reported to be filtered by the lung after systemic injection, with few reaching the tumor target (9). Lung sequestration has also been shown after intravascular delivery for cardiac applications (18).

Chemotactic cytokines (chemokines) have been implicated in controlling MSC migration to tumors (16). Monocyte chemoattractant protein-1 (MCP-1) is a 76-amino acid chemokine that plays a role in recruitment and activation of macrophages during inflammation. It has been shown to play a role in stimulating MSC migration in response to ischemia in the rat brain (19). Recent studies have shown that MCP-1 is secreted by tumor cells. This may activate the cytostatic function of monocytes against tumor cells but has also been reported to enhance tumor invasion and metastasis through increased neovascularization (20–22). There are conflicting reports as to whether increased serum MCP-1 is a positive or negative prognostic indicator in breast cancer patients, and no consensus exists as to whether tumor production of MCP-1 contributes significantly to circulating levels of the chemokine (22–27).

In the present study, we have shown successful engraftment of MSCs at the site of both primary tumors and nodal breast cancer metastases after systemic administration *in vivo*. We have also examined factors secreted by primary breast tumors *in vitro*, identified MCP-1 as a mediator of MSC migration, and determined systemic levels of the chemokine in a cohort of breast cancer patients and age-matched controls.

Materials and Methods

Primary culture. After ethical approval and written informed consent, fresh specimens of human breast tumors were harvested from patients undergoing surgery. Tissue from patients undergoing reduction mammaplasty served as controls. Primary culture of breast cancer tissue specimens was done based on a method described by Speirs et al. (28). Briefly, tissues were washed, minced finely, and digested overnight with 0.1% collagenase type III. Cells were then separated into organoid, epithelial and stromal fractions using differential centrifugation. Each cell population was then cultured in appropriate selective medium as previously described (28). Cells were further characterized by immu-

nostaining using known cell type-specific surface markers (MNF116, epithelial; CD90, stromal).

MSCs were obtained through the Regenerative Medicine Institute at National University of Ireland Galway. Briefly, after ethical approval and written informed consent, bone marrow was aspirated from the iliac crest of a donor according to an approved clinical protocol. MSCs were then isolated by Percoll gradient centrifugation followed by culture for 12 to 14 days to deplete the nonadherent hematopoietic cell fraction (6). The cells were routinely cultured in DMEM supplemented with preselected 10% fetal bovine serum (FBS) and 2 units/mL penicillin G/100 mg/mL streptomycin sulfate. The ability of cells to differentiate into osteocytes and adipocytes was confirmed before use. Osteogenesis was induced in the presence of dexamethasone (100 nmol/L), ascorbic acid (50 μ mol/L), and β -glycerophosphate (10 nmol/L). Calcium deposition was detected using Von Kossa staining. Adipogenesis was induced in the presence of methylisobutyl-xanthine (0.5 mmol/L), dexamethasone (1 μ mol/L), insulin (10 μ g/mL), and indomethacin (200 μ mol/L). Accumulation of lipid deposits was detected using Oil Red O (6, 29).

Culture of cell lines. The cell lines used in this study included T47D, estrogen and progesterone receptor-positive (ER+, PR+), and MDA-MB-231, estrogen and progesterone receptor-negative (ER-, PR-). The media used for each cell line was as follows: MDA-MB-231, L-15; T47D, RPMI. Both media types were supplemented with 10% FBS and 2 units/mL penicillin G/100 mg/mL streptomycin sulfate. Cells were maintained at 37°C, 5% CO₂ with a media change twice weekly and passage every 7 days.

Collection of conditioned medium. To detect the presence of chemokines potentially mediating any paracrine effects observed, conditioned medium (CM) from breast cancer cells (primary cultures and cell lines) was collected and analyzed. CM refers to medium that has been incubated in the presence of cells for 24 to 48 h, with varying concentrations of FBS (as required for chemokine stability). Cells were seeded at a fixed density (2.5×10^5 per well) in a six-well plate for CM collection. CM was also collected after exposure to whole-tumor explants for 24 h. In this case, the whole fresh breast tissue specimen, as received from theatre, was suspended in a fixed volume of medium for 24 h to detect chemokines secreted by the mixed cell population. This medium was then harvested, sterile filtered, aliquoted, and stored at -20°C until required.

In vivo MSC migration. Female athymic nude mice (B&K Universal, Ltd.) were implanted with 60-day release estradiol pellets (Innovative Research of America) to support breast tumor growth. Mice received a s.c. injection of 2×10^7 T47D cells suspended in 0.2 mL RPMI medium in the right flank. When tumors had reached an appropriate volume (≥ 100 mm³), mice received either an s.c. (adjacent to tumor, $n = 6$) or i.v. (systemic, $n = 5$) injection of 1×10^6 PKH26 (Sigma)-labeled MSCs suspended in 100 μ L DMEM. Cells were labeled with PKH26 according to manufacturers' protocol.

A second group of animals received an i.v. (lateral tail vein) injection of 2×10^5 MDA-MB-231 cells in 0.2-mL L-15 medium to establish systemic metastatic disease ($n = 6$). These animals were not implanted with an estradiol pellet as the cells are estrogen receptor-negative and so do not require the hormone for growth. Eight weeks later, palpable nodal metastases (two to four per animal) were detected, and animals received an i.v. injection (lateral tail vein) of 1×10^6 PKH26-labeled MSCs suspended in 100 μ L DMEM. All s.c. injections were done using a 24-gauge needle, whereas i.v. injections were done with a 27-gauge needle. MSCs from the same donor were used for all *in vivo* studies to reduce variation within the experiment. Three days after MSC injection, mice were sacrificed by i.p. injection of sodium pentobarbital, and tumor tissues were harvested. Tissue was immediately embedded in OCT (Cellpath) embedding matrix, placed on dry ice and stored at -80°C until required.

Detection of fluorescently labeled MSCs. OCT embedded tumor tissues were cryosectioned into 5 μ m thick sections and allowed to air dry while protected from light. Sections were fixed for 15 min in

4% paraformaldehyde, followed by three 5-min washes in PBS. Cells were then stained using 4',6-diamidino-2-phenylindole (DAPI; 1 μ g/mL) for 4 min in foil and washed thrice for 5 min in PBS. Sections were then mounted in DPX mounting medium and examined using a fluorescent microscope (Olympus BX60). The excitation/emission wavelength for PKH26 and DAPI are 551 nm/567 nm and 358 nm/461 nm, respectively. Individual single-channel images were captured using the appropriate filters and merged to create composite images with analySIS software.

In vitro MSC migration. Migration of two individual populations of adult MSCs in response to breast cancer cell lines and primary culture cells/explant CM was investigated using Transwell permeable supports (Corning, Inc.). This permitted separation of two cell populations with a porous membrane (8- μ m pore size). A suspension of MSCs was inoculated into the insert, and their migration in response to the breast cancer cells/explant CM or cells in the well below was quantified. Migration experiments were also done in the presence of a monoclonal antibody to hMCP-1 (40 ng/mL) and in response to recombinant human standards of the protein (150-600 pg/mL). After an 18-h migration incubation, cells remaining above the membrane were mechanically removed by scrubbing twice with a moistened cotton swab. The membrane insert was then transferred to ice-cold methanol to fix cells, followed by staining in hematoxylin. Once dry, the membranes were excised from the insert using a scalpel and mounted on slides for quantification of migrated cells (five fields of view on each membrane). Cell counts were done on an Olympus BX60 microscope using analySIS software. Each experiment was repeated, at least in triplicate, with results expressed as mean \pm SD.

Chemokine detection. Chemokines potentially mediating any change in cell migration were detected using ChemiArrays (Chemicon) and Quantikine ELISA kits (R&D Systems) according to the manufac-

turer's protocol. They are both antibody-based detection methods. ChemiArrays have antibodies targeting a range of different chemokines immobilized on the same membrane to permit detection of a variety of chemokines secreted by one cell population. CM for ChemiArray analysis was serum-free, in accordance with manufacturer's recommendations, and was freshly harvested on the day of analysis. Chemiluminescent images were acquired using a FluorChem imaging system (Alpha Innotech) and analyzed with Alpha Ease FC software. ELISA was used to permit direct quantification of the level (pg/mL) of MCP-1 in tumor cell-CM and patient serum samples, with results read on a platereader (Multiskan RC). CM for ELISA was collected and stored at -20°C to accumulate the required number of samples for each assay and so contained 2% FBS for chemokine stability, as recommended by the manufacturer. Fresh medium containing 2% FBS was included in the assay as a control.

Collection of blood samples. Preoperative serum samples were collected in Vacutainer SST II (serum separator tubes) tubes (Becton Dickinson), mixed by inversion, and allowed to clot for 30 min. All samples were then centrifuged at 2,000 rpm, 4°C for 10 min in a Sorvall RT6000D centrifuge. Samples were then aliquoted into 2-mL tubes and stored at -20°C (short term) and -80°C (long term). Control samples were collected from healthy women attending an outpatient facility with no current or previous malignancy or inflammatory condition.

Results

In vivo MSC migration. The ability of fluorescently labeled MSCs to preferentially home in on the site of a tumor was determined in nude mouse models of both localized (flank tumor) and systemic (metastatic) disease. The tumor type and

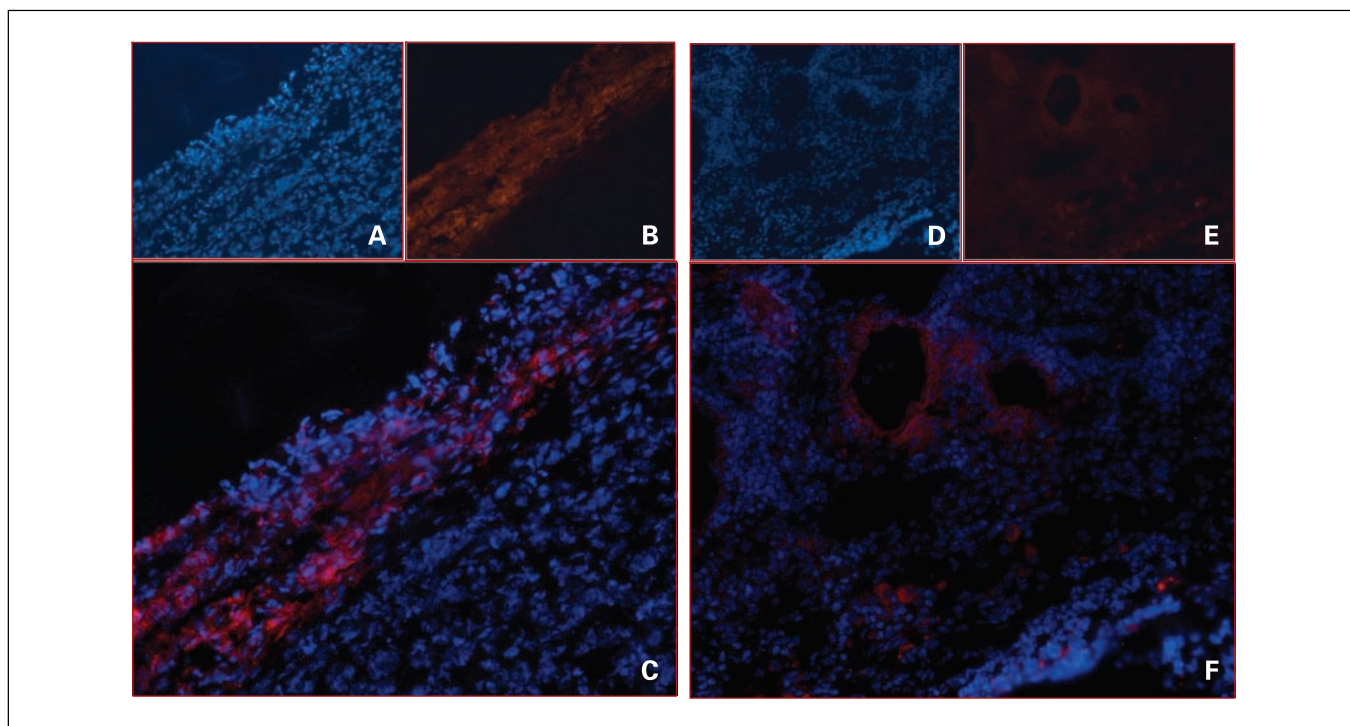


Fig. 1. Images showing examples of patterns of *in vivo* MSC engraftment in breast tumor tissue (5- μ m thick sections). Sections were counterstained with DAPI (blue) to visualize nuclei and PKH26-labeled MSCs engrafted in the tumor tissue (red). A-C are of the same section after injection of MSCs adjacent to a T47D tumor. D-F are of a section from a metastatic deposit of breast cancer after i.v. injection (tail vein) of MSCs. Single-channel images were taken to visualize DAPI-stained nuclei (A, D) and PKH26-labeled cells (B, E), and these images were then merged to form composites using analySIS software (C, F). After injection adjacent to the T47D tumor, engrafted MSCs were localized around the edge of the sections (C) in 6/6 sections examined. After systemic administration, MSCs were detected in multiple sites throughout sections of MDA-MB-231 nodal metastases (6/6 positive) and seem to be lining blood vessels (F). A similar pattern of engraftment throughout the tissue was also seen in animals with an isolated flank tumor that received i.v. injection of MSCs (4/5 positive; results not shown).

route of MSC administration were as follows: (a) T47D flank tumor + local injection of MSCs adjacent to tumor, $n = 6$; (b) T47D flank tumor + i.v. (lateral tail vein) injection of MSCs, $n = 5$; (c) MDA-MB-231 metastatic disease (multiple nodal metastases) + i.v. injection of MSCs, $n = 6$. Tumor tissue sections ($5 \mu\text{m}$) were stained with DAPI (blue) to visualize cell nuclei and confirm that any engrafted MSCs were morphologically intact cells (Fig. 1A and D). Sections were then examined for the presence of engrafted PKH26-labeled (red) MSCs (Fig. 1B and E). Composite images were then created, merging DAPI and PKH26 single-channel images using analySIS software (Fig. 1C and F). Three days after injection of MSCs adjacent to subcutaneous tumors, engrafted MSCs were detected surrounding the border of the tumor tissue (Fig. 1C). In animals with nodal metastases of breast cancer or primary flank tumors that had received i.v. (systemic) injection of MSCs, the fluorescently labeled cells were detected in patches throughout the tumor mass (Fig. 1F) and in some areas seemed to be lining the blood vessels of the tumor. Only one animal had no detectable MSCs engrafted at the site of the tumor (flank tumor + local MSC 6/6 positive, flank tumor + IV MSC 4/5 positive, systemic disease + IV MSC 6/6 positive).

In vitro MSC migration. Migration of MSCs in response to breast tumor cells was tracked using Transwell inserts. MSCs from two individual donors were used for *in vitro* experiments, and both populations showed a significant increase in migration in response to all tumor samples examined. In all migration experiments, MSCs were suspended in serum-free medium, and migration in response to the same medium served as a negative control/baseline, whereas migration in response to serum-rich medium served as a positive control. MSC migration was quantified in five fields of view on each membrane, and all conditions were repeated in triplicate (Fig. 2). There was a significant increase in MSC cell migration in response to all breast tumor samples examined, whereas there was no significant change in response to normal tissue harvested at reduction mammoplasty (Fig. 2). The greatest migration was

seen in response to whole breast tumor explants ($P < 0.001$) and was also significantly increased in response to the stromal population ($n = 6$) of cells from tumors after digestion and separation ($P < 0.01$). Immunostaining revealed that these cells were predominantly CD90^+ fibroblasts. MSC migration in response to epithelial cells (MNF116^+ , $n = 6$), although increased, was not significantly higher than controls ($n = 3$).

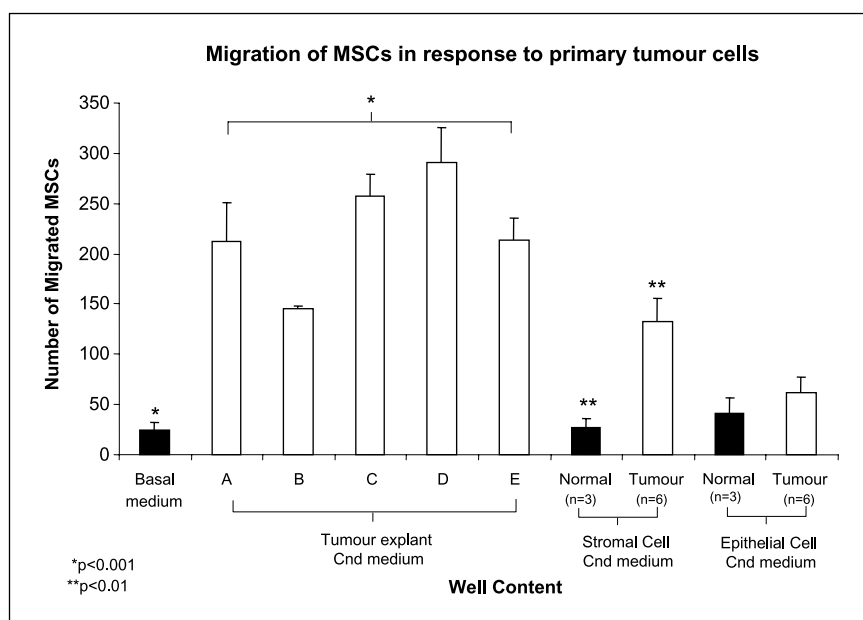
Chemokine detection. ChemiArrays were used for simultaneous detection of the range of chemokines secreted by whole-tumor explants and individual cell populations (Fig. 3). Whole-tumor explants incubated in serum-free conditions were found to secrete a variety of inflammatory chemokines, including GRO, interleukin-6, interleukin-8, and MCP-1 (Fig. 3A). Whereas the level and range of chemokines varied between tumor samples, MCP-1 was detected in serum-free CM from all primary culture samples analyzed (Fig. 3A-C). Chemokines secreted by the epithelial cell line MDA-MB-231 (Fig. 3D) included those listed above, along with granulocyte macrophage colony-stimulating factor, $\text{GRO}\alpha$, and interleukin-7.

The level of MCP-1 secreted by all cell populations in the presence of 2% FBS was quantified using ELISA (Table 1). Whole-tumor explants containing a mixed cell population secreted the highest level of the chemokine, and upon separation into individual cell populations, the stromal fraction consisting predominantly of CD90^+ fibroblasts was found to be responsible for the bulk of this secretion (stromal $668 \pm 101 \text{ pg/mL}$, epithelial $178 \pm 19 \text{ pg/mL}$, $n = 6$ each).

Role of tumor-secreted MCP-1 in MSC migration. Because MCP-1 was secreted by all tumor cells examined, the potential role of the chemokine in mediating breast cancer cell migration was examined using recombinant human standards (rhMCP-1) and a monoclonal antibody to the chemokine (Fig. 4).

MSCs showed a significant increase in migration in response to rhMCP-1 in a dose-dependent manner ($P < 0.001$; Fig. 4). Inclusion of an antibody to MCP-1 in tumor CM caused a significant decrease ($P < 0.05$) in MSC migration in response to the tumor cells (Fig. 4).

Fig. 2. Quantification of MSC migration in response to factors secreted by primary breast cancer cells. The highest level of migration was seen in response to factors secreted by whole breast tumor explants. The stromal subpopulation of cells ($n = 6$) consisting predominantly of CD90^+ fibroblasts seemed to be responsible for the bulk of this migration, whereas migration in response to epithelial population ($n = 6$) was considerably lower. All conditions were repeated in triplicate, with five fields of view counted on each membrane. Columns, mean; bars, SE.



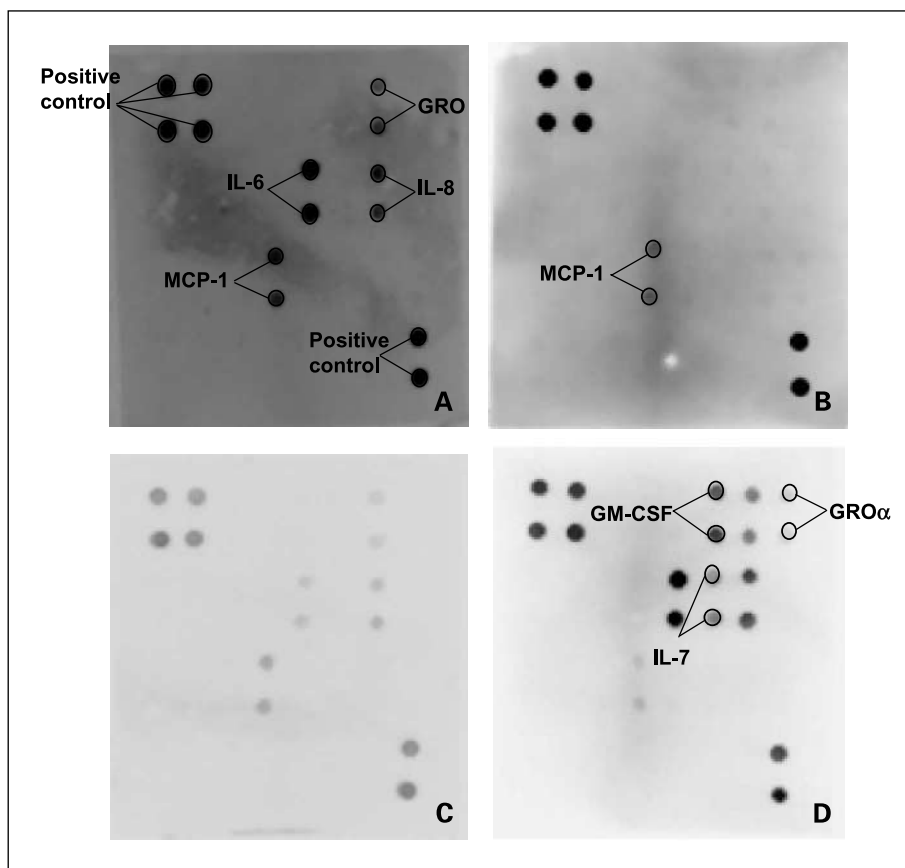


Fig. 3. ChemiArray analysis of serum-free CM from individual whole-tumor explants (A – C) and the breast cancer cell line MDA-MB-231 (D). Each duplicate dot represents detection of a specific chemokine. Chemokines secreted from individual whole tumors (A – C) included GRO α , interleukin-6, interleukin-8, and MCP-1. MCP-1 was detected in all samples analyzed at variable levels. MDA-MB-231 CM also contained granulocyte macrophage colony-stimulating factor (GM-CSF), GRO α , and interleukin-7.

Serum MCP-1 levels. MCP-1 levels were determined in serum collected from 125 breast cancer patients and 86 age-matched controls (Fig. 5). Although not statistically significant, there was a trend toward higher levels in breast cancer patients (332.97 ± 14.43 pg/mL) compared with healthy controls (301.26 ± 13.76 pg/mL). When divided on the basis of menopausal status, MCP-1 levels were found to be significantly higher in postmenopausal patients ($n = 81$) compared with age-matched controls ($n = 53$, $P < 0.05$). Also, within the breast cancer group, MCP-1 was found to be significantly higher in postmenopausal (366 ± 18.86 pg/mL) compared with premenopausal patients (270.88 ± 22.22 pg/mL, $P < 0.001$), whereas there was no such difference in the healthy controls.

MCP-1 levels were found to be independent of commonly used prognostic indicators, including nodal status, tumor stage, and grade (results not shown). Because there was not a balanced representation of different histologic tumor types (majority invasive ductal carcinoma, $n = 71$), it was not possible to reliably correlate serum MCP-1 levels with tumor type.

Discussion

MSCs represent an attractive option as delivery vehicles for therapeutic genes and their products as a result of their apparent ability to both home in on the tumor site and evade the host immune response (7). Studies have shown that i.v. administered MSCs do not engraft in healthy organs, e.g., liver, spleen (5, 11), and, in the case of breast cancer, have been

shown to migrate to pulmonary metastases of the disease in an animal model (11, 12). This permitted targeted delivery of the tumor inhibitor interferon- β (11) or a conditionally replicating adenovirus (12), both of which resulted in improved animal survival. Although the authors reported little or no MSC engraftment in normal areas of the lung, another study reported pooling of MSCs in the healthy lung of an animal after i.v. administration, which raised a question as to whether engraftment had been tumor-targeted or as a result of MSCs getting trapped in the capillary network as a result of their relatively large size (9). In the current study, the ability of fluorescently labeled MSCs to home in on the tumor site

Table 1. Quantification of MCP-1 secretion by cells cultured in the presence of 2% FBS

Cell source	MCP-1, mean \pm SD (pg/mL)
Whole-tumor explant ($n = 10$)	1422 \pm 120
Primary stromal ($n = 6$)	668 \pm 101
Primary epithelial ($n = 6$)	178 \pm 19
MDA-MB-231	555 \pm 27
T47D	97 \pm 4

NOTE: Whole-tumor explants containing a mixed cell population secreted the highest level of the chemokine, with the stromal fraction consisting predominantly of CD90 $^{+}$ fibroblasts responsible for the bulk of this secretion.

was confirmed after systemic administration. Both localized primary tumors and multiple solid tumors that had formed in the nodes of animals showed extensive MSC engraftment throughout the tumor mass that seemed to line blood vessels in many sections. The pattern of engraftment observed in sections harvested after injection of MSCs adjacent to subcutaneous tumors differed considerably, with MSCs localized around the border of the tumor. However in both models, the MSCs were shown to be morphologically intact and to have survived up to 72 h after administration. Further studies will be done to evaluate the differentiation status of engrafted MSCs.

Although these results are promising and support previously reported findings, the factors mediating MSC migration and engraftment need to be fully elucidated to realize any potential clinical application. This information could also be used to enhance engraftment in the tumor with resulting improved therapeutic effects. A recent study reported increased stromal cell-derived factor-1 α expression in MSCs after exposure to CM from breast cancer cell lines, which acted in an autocrine manner and induced migration to the tumor cells (16).

In the present study, we have shown migration of human MSCs in response to factors secreted by whole-breast tumor explants and primary cultures of tumor-derived cell populations. There was no significant increase in MSC migration in response to factors secreted by normal breast stromal cells harvested at reduction mammoplasty. The use of fresh tissue specimens from patients lends increased relevance to this study, as it takes into account the heterogeneous population of cells within a tumor mass *in vivo*, which is not captured in purely epithelial breast cancer cell lines. The greatest level of MSC migration was seen in response to the whole-tumor explants, which were found to secrete an array of chemokines at varying levels.

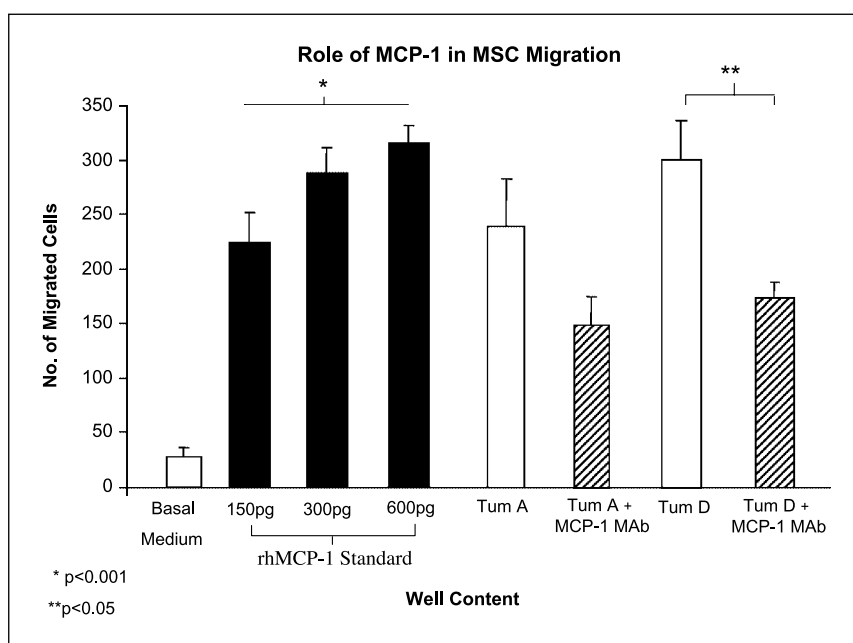
MCP-1 was detected in CM from all breast tumor explants and cells examined and, through the use of an antibody specific to the chemokine, was shown to play a significant role in mediating migration of MSCs. It is worth noting that the

stromal population of cells, consisting predominantly of CD90⁺ fibroblasts, seemed to be the main source of MCP-1 secretion, highlighting the influence of these cells within the tumor microenvironment.

Previous reports have shown expression of the MCP-1 receptor CCR2 in MSCs, but there have been conflicting reports on MSC migration in response to the chemokine (19, 30). Rat MSCs have been reported to migrate in response to MCP-1 secreted by ischemic brain tissue (19), but a recent study reported no migration of human MSCs in response to rhMCP-1 standards (30). The discrepancy between the latter report and the results presented here may be as a result of variations between cell donors, which is a factor in primary culture.

Because MCP-1 secretion was detected in all breast cancer populations, we proceeded to investigate whether systemic MCP-1 levels in breast cancer patients were significantly different to those of healthy age-matched controls. In recent years there have been a number of studies investigating a potential link between MCP-1 expression levels and breast cancer progression, however a consensus has yet to be reached. Results have been conflicting, with some groups reporting an association of MCP-1 with tumor neovascularization and progression (22), early disease relapse (26), advanced tumor stage, and lymph node involvement (25). Conversely, Dehqanzada et al. (23) reported a correlation with favorable prognostic variables, such as earlier stage and fewer nodal metastases. Yet another study found no correlation between MCP-1 levels and tumor size, grade, or lymph node status (27). Some of these variations may be accounted for, at least, in part by the methods used for MCP-1 detection — immunohistochemistry (22, 27) and ELISA on tissue extracts (26) or serum samples (23–25). In the current study, serum MCP-1 levels were measured by ELISA on 125 breast cancer patients and 86 age-matched controls. There was a trend toward higher levels in breast cancer patients overall, but the difference was not statistically significant. However, when divided on the basis of menopausal status, MCP-1 levels in postmenopausal patients

Fig. 4. Graph showing migration of MSCs in response to recombinant human MCP-1 (150–600 pg/mL). Cell migration increased in a dose-dependent manner in response to the chemokine ($P < 0.001$). MSC migration in response to tumor CM was also shown to be significantly decreased in the presence of a monoclonal antibody (MCP-1 MAb) to the chemokine ($P < 0.05$). All experiments were repeated at least in triplicate with five fields of view counted in each membrane. Columns, mean; bars, SE.



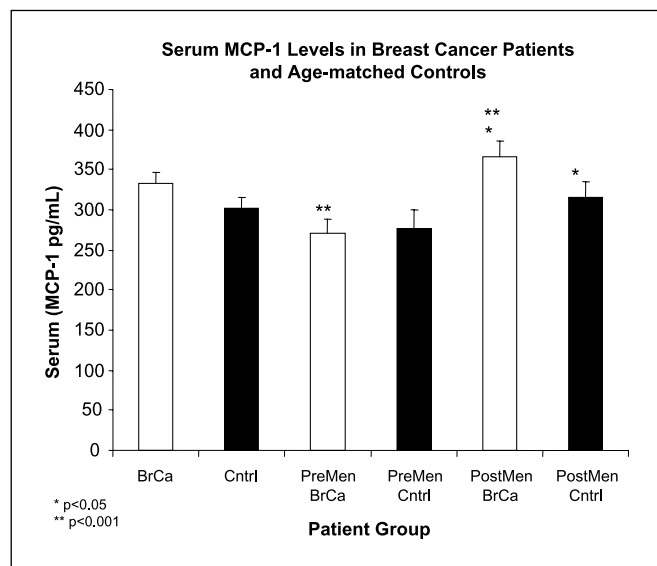


Fig. 5. Graph showing serum MCP-1 levels in 125 breast cancer patients (*BrCa*) and 86 controls (*Cntrl*). There was a trend toward higher MCP-1 levels in breast cancer patients, although this was not significantly different to controls. MCP-1 levels in postmenopausal (*PostMen*) breast cancer patients ($n = 81$) were significantly higher than that of age-matched controls ($n = 53$, $P < 0.05$), and within the breast cancer cohort, MCP-1 levels were significantly higher in postmenopausal patients compared with premenopausal (*PreMen*) patients ($P < 0.001$).

were significantly higher than those of age-matched controls. Also, within the breast cancer cohort, there was a significant difference between postmenopausal and premenopausal levels that was not mirrored in the control group. Thus MCP-1 levels in breast cancer patients may also be a function of menopausal status, which may partly explain the conflicting studies to date. Premenopausal levels may correlate with stage of menstrual cycle as has been previously reported with other cytokines, such as vascular endothelial growth factor (31). One previous study divided breast cancer patients on the basis of "high"

(>250 pg/mL) and "low" (<250 pg/mL) MCP-1 levels, and although the groups were significantly smaller ($n = 10$ and $n = 22$ respectively), patients with high levels of the chemokine were in average 10 years older than their counterparts (23). There have been studies reporting hormonal control of serum MCP-1 in healthy women and those at increased risk for cardiovascular events (32, 33). Both studies reported reduced MCP-1 levels in postmenopausal patients in response to hormone replacement therapy and suggested this may play a part in the reduced incidence of atherosclerosis in postmenopausal women on hormone replacement therapy. One previous study investigated MCP-1 levels through the menstrual cycle in healthy premenopausal subjects who were not on oral contraceptives ($n = 18$; ref. 34). A significant decrease in MCP-1 levels from the follicular to the luteal phase of the cycle was observed, although the number of women in each phase of the cycle was not reported. In the current study, upon review of charts, menstrual status of patients was available on 18 of the 34 premenopausal subjects, and the trend of serum MCP-1 matched that previous report (mean \pm SD, n): follicular 306 ± 50 pg/mL, $n = 6$; midcycle 318 ± 47 pg/mL, $n = 8$; luteal 225 ± 45 pg/mL, $n = 4$. Numbers in each subgroup were low but indicate that the relationship between MCP-1 and menopausal status in breast cancer patients warrants further investigation. Analysis of clinical data on patients showed that MCP-1 levels were independent of tumor stage, grade, or nodal status. There was no correlation found between tumor type and MCP-1 level, as the majority of tumors were invasive ductal carcinoma ($n = 71$), and so without equivalent numbers of other types, a reliable correlation between histologic subtype and serum MCP-1 could not be established.

The promising data presented here support a role for MSCs as delivery agents for therapeutic genes or their products to breast tumors *in vivo*. Increased understanding of the pathways involved in stimulating tumor-specific MSC migration and engraftment will potentially provide for translation of this novel therapeutic approach to the clinical setting.

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