Title: Mesenchymal Stem Cells (MSCs) and Cancer: tumour specific delivery vehicles or therapeutic targets?

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Abstract:

Mesenchymal Stem Cells (MSCs) are a subset of non-haematopoietic multipotent cells found primarily within the bone marrow stroma. The ability of MSCs to specifically home to sites of tumours and their metastases, while escaping host immune surveillance holds tremendous promise for tumour targeted delivery of therapeutic agents. Concerns that MSCs may have an inherent capacity for transformation have lead to a number of studies investigating their stability in vitro, as significant ex vivo expansion will be necessary to yield the number of cells required for therapeutic applications. MSCs have also been seen to influence the morphology and proliferation of cells within their vicinity through a combination of cell to cell interactions and the secretion of chemoattractant cytokines. Understanding interactions between MSCs and tumour cells is required to support realisation of their clinical potential. This review will discuss MSCs and cancer in terms of 1) potential for transformation and de novo tumour formation, 2) interactions with epithelial cancer cells in tumour establishment, and 3) potential role following engraftment at the site of an established tumour. Elucidation of any potential negative effect of MSCs in the tumour setting will support development of protocols to minimize these effects while taking full advantage of the remarkable tumour homing capacity of these cells.

Introduction

Mesenchymal Stem Cells (MSCs) are a subset of multipotent non-haematopoietic cells found primarily within the bone marrow, although they can also be isolated from many other sources including umbilical cord and adipose tissue (Digirolamo et al. 1999; Bieback et al. 2008). MSCs represent a minor proportion (<0.1%) of the total cell fraction of nucleated marrow cells but can be isolated and expanded with great efficiency (Pittenger et al. 1999). Although one single marker has not been identified, a panel of specific cell surface antigens for MSCs have been defined, including expression of CD105, CD73 and CD90 in greater than 95% of the culture, and absence of CD14, CD34, CD19, HLA-DR and CD45 expression (Dominici et al. 2006). MSCs possess an innate ability for self renewal and can differentiate into multiple cell types including osteoblasts, chondrocytes and adipocytes (Barry and Murphy 2004). They play an important role in wound healing and tissue regeneration through differentiation and the release of proangiogenic factors (Wu et al. 2007; Wu et al. 2007), and have been shown to selectively home to sites of injury irrespective of tissue or organ. It has been hypothesised that tumors resemble chronic wounds or 'wounds that never heal' (Dvorak 1986) and MSCs have been shown to specifically home to the site of multiple tumour types (Spaeth et al. 2008). Combined with this tumour homing capacity, MSCs also appear to have immunosuppressive capabilities, which would potentially support tumour targeted delivery of therapeutic agents while bypassing host immune surveillance. MSCs are thought to interact with almost all cells of the innate (primarily dendritic cells) and adaptive (primarily T cells) immune systems (Momin et al.) (Ramasamy et al. 2007), with the immunological aspects of MSC transplantation reviewed in detail elsewhere in this issue (Bernard Mahon/Matt Griffin). The combination of these two remarkable traits has prompted researchers to analyse MSCs as possible vectors for the targeted delivery of anti-cancer agents to tumor microenvironments, with very promising results reported in animal models of a range of tumour types (Kumar et al. 2008).

However, MSCs have also been seen to influence the morphology and proliferation of cells within their vicinity through a combination of cell to cell interactions and the secretion of chemoattractant cytokines. Recently the interaction between MSCs and tumour cells has become a focus of research due to the possibility that MSCs may have tumour promoting properties, potentially mediated through their propensity to secrete a variety of tumouirgenic chemokines. Understanding these interactions has

become fundamental to determining whether the homing ability of MSCs can be harnessed for tumor targeted delivery of therapeutic agents.

This review will discuss MSCs and cancer in terms of 1) potential for transformation and de novo tumour formation, 2) interactions with epithelial cancer cells in tumour establishment, and 3) potential role following engraftment at the site of an established tumour.

MSCs as Targets for Neoplastic Transformation

Concerns that MSCs may have an inherent capacity for transformation have lead to a number of studies investigating their stability in vitro, as significant ex vivo expansion will be necessary to yield the number of cells required for therapeutic applications.

Telomeres are repetitive genetic material that cap and therefore protect the ends of chromosomes. Telomere length is maintained by telomerase, and shortened during each cycle of cell division (Serakinci et al. 2008). Telomerase is frequently activated in cancer cells providing indefinite proliferative potential. Despite the requirement for rapid proliferation during wound healing and tissue regeneration, MSCs have been shown to express low levels of telomerase (Serakinci et al. 2004) (Jiang et al. 2006). This is considered a target for transformation and a number of studies have analysed telomerase expression following MSC manipulation or potential transformation in vitro. Following transduction with hTERT, and passage to high numbers (P256), MSCs were shown to exhibit loss of contact inhibition, anchorage independent growth and tumour formation in mice (Serakinci et al. 2004). In another study, in the presence of high concentrations of cytokines, embryonic bone MSCs mutated to form a tumour cell population with significantly enhanced telomerase activity, overexpression of BMI-1, and loss of expression of cell cycle regulatory genes p16, p53, p21, pRb (Jiang et al. 2006). Matushansky et al also illustrated, via inhibition of Wnt signaling, that hMSCs can be transformed to form sarcoma-like tumors in nude mice (Matushansky et al. 2007). MSCs carrying a mutation in one allele of the p53 gene completely lost p53 expression after long term in vitro culture, and this was accompanied by a significant increase in growth rate, karyotype instability, loss of p16 expression and lack of senescence response (Rodriguez et al. 2009). Further, these cells were shown to form sarcomas partially differentiated into mesenchymal lineages in immunodeficient mice (Rodriguez et al. 2009).

These studies highlight MSC sensitivity to mutations in genes involved in cell cycle control, and while in the preceding reports cells were specifically manipulated to determine their potential as targets for transformation, studies have also shown spontaneous transformation of human (Rubio et al. 2005) (Wang et al. 2005), rat (Foudah et al. 2009) and particularly mouse (Miura et al. 2006; Zhou et al. 2006; Li et al. 2007; Tolar et al. 2007) MSCs during in vitro culture.

Rubio et al reported spontaneous transformation of adipose derived human MSCs that initially displayed a normal karyotype, and were unable to form tumours in mice (Rubio et al. 2005). The cells bypassed senescence spontaneously and subsequently displayed an accelerated cell cycle rate (Rubio et al. 2005). Cell senescence is defined by moderate telomere shortening, cell cycle arrest and positive β-galactosidase staining at pH6. If cells bypass this stage, they continue to grow until telomeres become critically short and cells enter crisis phase, characterized by generalized chromosome instability that provokes mass apoptosis (Rubio et al. 2005). 30% of post-senescence MSCs presented trisomy of chromosome 8, but remained unable to form tumours when injected into immunodeficient mice. These post-senescence MSCs grew on to crisis phase, after which 50% continued to proliferate spontaneously. At this point, there were extensive changes in the cell genotype and phenotype observed. Cells displayed a small compact morphology, reduced duplication time, decreased expression of MSC-specific markers, loss of contact inhibition and grew in semisolid agar (Rubio et al. 2005). Karyotype analysis revealed trisomy, tetraploidy and/or chromosome rearrangement with c-myc, telomerase, and p16 identified as targets. Following injection into immunodeficient mice, the transformed mesenchymal cells (TMCs) resulted in early signs of illness and tumour formation in multiple organs (Rubio et al. 2005). In contrast, pre or post senescent MSCs caused no tumour formation (Rubio et al. 2005). The authors concluded that although they can be managed safely during the standard ex vivo expansion period of 6-8 weeks, human MSCs can undergo spontaneous transformation following long-term in vitro culture (4-5 months) (Rubio et al. 2005).

During this time another group identified a novel subpopulation of human MSCs with a transformed phenotype, displaying chromosome aneuploidy and translocations, with a relatively high level of telomerase activity (Wang et al. 2005). Injection of these cells into NOD/SCID mice resulted in formation of poorly differentiated, aggressive tumours in multiple organs. However this subpopulation of cells was CD105-,

CD133+ve and their origin was not determined, so they may have pre-existed in the bone marrow of the donor, or may have been generated through transformation in vitro. It is also worth noting that this event was observed in one of 40 samples examined (Wang et al. 2005).

In stark contrast to these findings, Bernardo et al (Bernardo et al. 2007) found no evidence of human MSC transformation after long term in vitro culture. In this case ten populations of bone marrow derived MSCs were analysed up to passage 24 where possible. The cells were unable to bypass senescence, and did not develop a crisis phase (Bernardo et al. 2007). Observations included the absence of telomerase activity, evidence of telomere shortening, lack of an ALT phenotype, and no unbalanced chromosomal abnormalities upon molecular karyotyping (Bernardo et al. 2007). The authors concluded that human BM-derived MSCs do not display an aptitude for spontaneous transformation and can be safely expanded in vitro without any sign of immortalization or development of chromosomal abnormalities.

In terms of MSCs from other species, in vitro culture of murine MSCs was shown to give rise to populations of cells capable of forming invasive soft tissue sarcomas after IM injection into nude or SCID mice (Zhou et al. 2006), with IV injection leading to tumour foci in lungs. Direct injection into immunocompetent mice also resulted in tumour formation. Karyotype analysis revealed increased chromosomal number and multiple robertsonian translocations as early as passage 3 coincident with loss of contact inhibition (Zhou et al. 2006). However it is noteworthy that this study did not observe transformation of rat or human MSCs when cultured in the same conditions (Zhou et al. 2006).

Malignant transformation of murine MSCs has also been shown to result in fibrosarcoma formation in vivo (Miura et al. 2006). Yet again, in this study no transformation of human MSCs under the same culture conditions was observed (Miura et al. 2006). With aging, murine MSCs were also shown to spontaneously transform in culture and in a murine model in vivo, recapitulated the naturally occurring fibrosarcomas of the aged mice (Li et al. 2007). Tolar et al also reported transformation of murine MSCs in vitro, that were then capable of forming sarcomas in vivo (Tolar et al. 2007).

Thus is appears that murine MSCs are more prone to undergo immortalization in culture than human cells, although these studies underscore the critical importance of monitoring hMSCs for clinical application (Tolar et al. 2007). Rat MSCs have also

been reported to be unstable in long term culture, with a markedly aneuploid karyotype and progressive chromosomal instability with increasing passage (Foudah et al. 2009). In this study also, human MSC were cultured for comparison, with no evidence of clonal numeric or structural aberration observed up to p16 (Foudah et al. 2009).

Role of MSCs in the tumour microenvironment

The tumour microenvironment contains many distinct cell types, including endothelial cells, pericytes, smooth muscle cells, fibroblasts, carcinoma associated fibroblasts (CAFs), myofibroblasts, neutrophils, eosinophils, basophils, mast cells, T and B lymphocytes, natural killer cells and antigen presenting cells such as macrophages and dendritic cells (Coussens and Werb 2002). Experimental data have demonstrated a role for each of these individual components in promoting tumour growth and progression (Lorusso and Ruegg 2008). Thus understanding the biology of the tumour microenvironment is becoming as important as knowledge of the neoplastic epithelial cells (Albini and Sporn 2007).

Microenvironmental changes sustaining tumour progression include modifications of the extracellular matrix composition, activation of stromal cells, myoepithelial cells, and the recruitment of pericytes or smooth muscle cells and immune and inflammatory cells (Tlsty and Coussens 2006). MSCs are also recruited to the stroma of developing tumours (Hall et al. 2007), and this has prompted many studies investigating their potential role in this environment.

An initial report using an admix of adherent, non-macrophage bone marrow cells with Lewis lung carcinoma or B16 melanoma (2:1 MSC: tumour cell ratio), showed inhibition of primary tumour growth and metastases formation in mice (Maestroni et al. 1999). In contrast, Djouad et al later reported that subcutaneous injection of B16 melanoma cells led to tumor growth in allogeneic recipients only when MSCs were coinjected (1:1 MSC: tumour cell ratio). This was thought to be mediated through their immunosuppressive effects, protecting tumour cells from host immune surveillance (Djouad et al. 2003). Further study by this group showed that co-injection of MSCs reduced the delay of tumor appearance when Renca cells were implanted, without modifying the kinetics of tumor growth (Djouad et al. 2006). Conversely, an important finding of this study was that a low number of MSCs may induce tumor

rejection, highlighting the importance of the ratio of each cell population employed in these models (Djouad et al. 2006).

Results reported by Spaeth et al also highlighted the importance of the timeline of tumour monitoring. huMSC were admixed at a 1:1 ratio with ovarian tumor cells prior to intravenous injection. Tumors composed of admixed cells displayed a slight relative growth lag until day 80, when rapid growth rates were observed. By day 91, mixed tumors were significantly larger than those containing tumour cells alone (Spaeth et al. 2009). MSC stimulated tumor growth was shown to be mediated primarily through the paracrine production of secreted IL6 (Spaeth et al. 2009).

In another in vitro study, MSCs were shown to stimulate the transient arrest of tumor cell cycle accompanied by a reduction in the apoptotic rate (Ramasamy et al. 2007). However, when tumor cells were injected into immunodeficient mice in conjunction with MSCs, their growth was much faster as compared to the group receiving only tumor cells. To explain the discrepancy between the in vitro and in vivo behaviour, the authors suggest that MSCs have the ability to form a cancer stem cell niche in which tumor cells can preserve the potential to proliferate and sustain the malignant process (Ramasamy et al. 2007).

A number of studies have investigated the interaction between MSCs and tumour cells in the context of breast cancer. In a murine model of breast cancer, when co-injected at the time of tumour establishment, MSCs were shown to integrate into the tumour associated stroma, and without changing the kinetics of primary tumour growth, stimulated increased numbers of micro- and macro-metastases in the lungs of animals (Karnoub et al. 2007). The effects were shown to be reversible, and mediated at least in part through the cytokine CCL5. In the presence of breast cancer cells, MSCs secreted CCL5 which acted in a paracrine fashion on the breast cancer cells, stimulating increased metastasis. MSCs were shown to act locally in the primary tumour, and did not migrate to the lungs in tandem with tumour cells. An excess of MSCs was used for tumour establishment in this model (3:1 MSC: tumour cell ratio). It is also of interest that the observed effects were different depending on the particular breast cancer cell line employed, with varying degrees of metastasis and changes in proliferation observed. MSCs enhanced the growth of MCF-7/Ras tumors without affecting kinetics of MDA-MB-231, MDA-MB-435, or HMLER containing tumors (Karnoub et al. 2007).

Using murine adipose derived MSCs (mASCs), Muelhberg and colleagues found coinjection of these cells with the 4T1 breast cancer cell line caused tumors to grow significantly faster (10:1 mASC: tumour cell ratio) (Muehlberg et al. 2009). In a similar approach to that described by Karnoub et al (Karnoub et al. 2007), breast cancer cells were shown to enhance secretion of stromal cell-derived factor-1α (SDF-1α) from mASCs, which then acted in a paracrine fashion on the cancer cells to enhance their motility, invasion and metastasis. The choice of adipose derived stem cells was to validate that interactions between local tissue resident stem cells and tumour cells play an important role in growth and metastasis. Furthermore, this study demonstrated that mASCs were capable of stimulating tumour growth following intravenous delivery. In this case tumours were not established prior to MSC injection, with just a 12hr time lag between injection of MSCs and tumour cells. mASCs were also shown to incorporate into tumor vessels and differentiate into endothelial cells, demonstrated by positive staining for vWF (Muehlberg et al. 2009).

Using equal numbers of MSCs and breast cancer cells, Rhodes et al recently reported increased tumour growth in the presence of MSCs, but of greater interest was the ability of the hormone dependent cell line to form tumours in the absence of estrogen or matrigel only when admixed with MSCs (Rhodes et al.). This indicated a shift to hormone independence and has potentially important implications in the clinical setting (Rhodes et al.). Carcinoma associated fibroblast (CAF) like differentiation of hMSCs following exposure to conditioned medium from breast tumour cells has also been reported, with increased expression of vimentin, α-SMA and Fibroblast Surface Protein (Mishra et al. 2008).

Galie and colleagues observed no change in breast tumour kinetics when established with an admix of MSCs, but an earlier onset of tumours was observed, suggesting a role for MSCs in prompting the angiogenic switch (Galie et al. 2008). A CAF phenotype was also observed in MSCs following interaction with ovarian cancer cells, characterised by the presence of fibroblast specific protein, fibroblast activated protein, tenascin-c, thrombospondin-1, and stromelysin-1 (Spaeth et al. 2009). The cells were shown to produce protumorigenic growth factors including hepatocyte growth factor, epidermal growth factor, and interleukin-6, and factors associated with vascularization including α-SMA, desmin, and vascular endothelial growth factor were also present (Spaeth et al. 2009). A variety of other tumour types were also

employed in this study, and although a role for MSCs as CAFs was shown in each model, there was a marked difference between the response of each cell line to MSCs. Also human, but not murine MSCs were found to stimulate ovarian tumour growth (Spaeth et al. 2009).

A recent study reported similar results using a colon cancer cell line (Shinagawa et al.). MSCs were shown to enhance tumour growth and lung metastases, and analysis of tumour tissue revealed an increased microvessel density and decreased apoptotic index. Following integration into the tumour, MSCs were shown to express markers associated with carcinoma associated fibroblasts, including α-SMA and PDGFβ. In this case also, an excess of MSCs: tumour cells (2:1) was employed in tumour establishment (Shinagawa et al. ; Shinagawa et al. 2010). Zhu et al also reported that co-injection of MSCs in a 10:1 or 1:1 ratio with colon cancer cells resulted in tumours with elevated proliferation capacity, rich angiogenesis and increased metastatic ability (Zhu et al. 2006). However, an earlier study using an immortalized MSC line co-cultured with rat colon carcinoma cells in vivo showed inhibition of tumour growth, with complete inhibition seen when the number of mesenchymal progenitor cells was at least equal to the number of tumor cells (Ohlsson et al. 2003).

In a model of rat malignant glioma, MSCs were also shown to integrate into tumour vessel walls and express pericyte markers (alpha-SMA, neuron-glia 2, PDGF-β), but not EC markers, suggesting that MSCs act as pericytes within tumours. Further, the antiangiogenic drug Sunitinib markedly reduced the number of grafted MSCs migrating within tumours (Bexell et al. 2009). In contrast, Yu et al reported that co-injection of MSCs derived from human adipose tissues (hASCs) with glioma cells promoted tumor growth in nude mice, but had no influence on the formation of intratumoral vessels (Yu et al. 2008).

Based on observations that MSCs promoted growth of lymphoma xenografts in vivo (1:10 ratio MSC: tumour cells), Roorda and colleagues used a small molecule tyrosine kinase inhibitor PTK, to specifically inhibit MSC proliferation and induce apoptosis. This was found to reduce tumor growth-promoting effects of MSCs both in vitro and in vivo, highlighting the potential to target tumour resident MSCs as an anti-cancer strategy (Roorda et al.). Bagley et al also highlighted MSCs as a potential target for therapeutic intervention, based on expression of Endosialin, which is also expressed at high levels by CAFs (Bagley et al. 2009). In vitro studies showed that MSCs can form

networks in a tubule formation assay which was inhibited by an anti-endosialin antibody (Bagley et al. 2009).

In contrast to the majority of studies using co-injection of MSCs and cancer cells for tumour establishment, Qiao et al reported suppression of tumours by MSCs in a hepatoma model (Qiao and others 2008). When SCID mice were injected with H4702 cells in the presence of an equal number of MSCs, the latent time for tumour formation was prolonged and tumour size smaller. Further analysis revealed that cancer cell proliferation was decreased, and apoptosis increased, in parallel with downregulation of expression of Bcl-2, c-Myc, PCDNA (proliferating cell nuclear antigen) and survivin (Qiao et al. 2008).

There are considerable variations in study design in the reports highlighted here. It is unlikely that models using an excess of MSCs:tumour cells recapitulate the in vivo situation. However, the evidence provides vital information on interactions between tumour cells and MSCs, and supports a role for MSCs in the tumour microenvironment, potentially as precursors for pericytes and/or carcinoma associated fibroblasts.

Effect of MSCs on pre-established tumours

To investigate the potential of MSCs as gene delivery vehicles for therapeutic agents, it is necessary to understand their role following engraftment at the site of pre-established tumours. The majority of studies using MSCs engineered for delivery of a therapeutic agent have resulted in significant anti-tumour effects. In the same studies, control animals were injected with unmodified MSCs and their effect on tumour growth and progression analysed. Although not the primary focus of the research, the vast majority have shown that following systemic injection of unmodified MSCs into tumour bearing animals, there was no change in tumour volume compared to untreated animals.

In a rat glioma model, MSCs were administered either via intracranial co-injection with glioma cells, or injection 3D following tumour inoculation. Although the most marked results were seen following administration of MSCs secreting IL-2, nonetheless a significant inhibition of tumour growth and increased animal survival was also observed in animals that received unmodified MSCs (Nakamura et al. 2004). A number of studies following this have shown no pro-tumour effect of MSCs in a

range of tumour types (Stoff-Khalili et al. 2007; Kim et al. 2008; Mishra et al. 2008; Bexell et al. 2009; Sasportas et al. 2009; Dembinski et al. 2010; Gao et al. 2010).

Unmodified MSCs resulted in a significant reduction of growth of Kaposi's Sarcoma (KS) through inhibition of activation of Akt protein kinase. IV Injection of MSCs coincident with KS tumour implantation resulted in >50% decrease in tumour size (Khakoo et al. 2006). In animals with a pre-established tumour, infusion of MSCs also resulted in significant inhibition of tumour growth. The authors concluded that MSCs possess intrinsic antineoplastic properties and may be particularly useful for treating malignancies characterised by dysregulated Akt (Khakoo et al. 2006).

In a model of orthotopic pancreatic cancer, administration of unmodified MSCs caused significant tumour repression, which was enhanced further when the MSCs were engineered to express IFNB (Kidd et al.). In contrast, in another model of orthotopic, syngeneic pancreatic tumours, IV administration of MSCs weekly for three weeks resulted in increased tumour growth (Zischek et al. 2009).

In a model of cervical cancer, injection of MSCs was associated with greater tumour size compared to tumour cells alone, up to 45 days after injection. However, at 60 days, the difference disappeared in both SC and IV injected mice (Grisendi et al. 2010). This again highlights the importance of the extent of tumour monitoring and may explain discrepancies between the outcome observed in some studies.

In a murine model of orthotopic pancreatic tumours, interaction of MSCs with the endothelium of blood vessels was observed. MSCs supported tumour angiogenesis in vivo, with CD31(+) vessel density shown to be increased following transfer of MSC compared with siVEGF-MSC. This study demonstrated the migration of MSCs toward tumour vessels and further supports a role for these cells in angiogenesis (Beckermann et al. 2008).

Future Considerations

The ability of MSCs to specifically home to the sites of tumours and their metastases, while escaping host immune surveillance holds tremendous promise for tumour targeted delivery of therapeutic agents. However, a more complete understanding of MSC biology and fate following infusion is required to support translation to the clinical setting. Since ex vivo expansion of MSCs will be required prior to infusion, extensive characterisation of the cells prior to use will be necessary. The reports reviewed herein support differences in MSC stability dependent upon species and

source of isolation. It appears that human MSCs are stable in culture for at least 1-2 months, and that transformation in this scenario would be a very rare occurrence. However, it is clear that stringent screening of MSC phenotype and genotype, including karyotyping, will be an absolute requirement prior to administration to patients. Recent research has shed new light on interactions between MSCs and tumour cells through cell-cell contact, and secretion of paracrine factors in the primary tumour microenvironment. A consensus appears to be developing that MSCs have the capacity to differentiate into cells with a similar pheonotype to CAFs or pericytes, which are known to support tumour progression and angiogenesis. Their immunosuppressive qualities may also facilitate tumour cell evasion of the immune system. Considerable further research is required to determine the true prevalence of MSCs or their derivatives in primary tumours from patients. Current approaches employ a variety of methods for MSC isolation and expansion, and there are also considerable variations in the tumour models used. The site of tumour injection (orthotopic, subcutaneous), time of MSC administration (day of tumour inoculation versus following tumour establishment), proportion of each cell type, route of MSC administration (intratumoral, intraperitoneal, intravenous), length of follow-up before animal sacrifice, and tumour parameters measured will all effect the study outcome.

As increased knowledge of MSC biology emerges, isolation and culture will be further optimised, and variations between studies reduced. This is a very exciting, and relatively new area of research. Elucidation of any potential negative effect of MSCs in the tumour setting will support development of protocols to minimize these effects while taking full advantage of the remarkable tumour homing capacity of these cells.

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