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ORIGINAL ARTICLE

The cell culture expansion of bone marrow stromal cells from humans with spinal cord injury: implications for future cell transplantation therapy

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Study design: Previous studies have shown that transplantation of bone marrow stromal cells (MSCs) in animal models of spinal cord injury (SCI) encourages functional recovery. Here, we have examined the growth in cell culture of MSCs isolated from individuals with SCI, compared with non-SCI donors.
Setting: Centre for Spinal Studies, Midland Centre for Spinal Injuries, RJAH Orthopaedic Hospital, Oswestry, UK.
Methods: Bone marrow was harvested from the iliac crest of donors with long-term SCI (>3 months, $n=9$) or from non-SCI donors ($n=7$). Mononuclear cells were plated out into tissue culture flasks and the adherent MSC population subsequently expanded in monolayer culture. MSC were passaged by trypsinization at 70% confluence and routinely seeded into new flasks at a density of 5×10^3 cells per cm^2. Expanded cell cultures were phenotypically characterized by CD-immunoprofiling and by their differentiation potential along chondrocyte, osteoblast and adipocyte lineages. The influence of cell-seeding density on the rate of cell culture expansion and degree of cell senescence was examined in separate experiments.
Results: In SCI, but not in non-SCI donors the number of adherent cells harvested at passage I was age-related. The proliferation rate (culture doubling times) between passages I and II was significantly greater in cultures from SCI donors with cervical lesions than in those with thoracic lesions. There was no significant difference, however, in either the overall cell harvests at passages I or II or in the culture doubling times between SCI and non-SCI donors. At passage II, more than 95% of cells were CD34−ve, CD45−ve and CD105 + ve, which is characteristic of human MSC cultures. Furthermore, passage II cells differentiated along all three mesenchymal lineages tested. Seeding passage I–III cells at cell densities lower than 5×10^3 cells per cm^2 significantly reduced culture doubling times and significantly increased overall cell harvests while having no effect on cell senescence.
Conclusion: MSCs from individuals with SCI can be successfully isolated and expanded in culture; this is encouraging for the future development of MSC transplantation therapies to treat SCI. Age, level of spinal injury and cell-seeding density were all found to relate to the growth kinetics of MSC cultures *in vitro*, albeit in a small sample group. Therefore, these factors should be considered if either the overall number or the timing of MSC transplantations post-injury is found to relate to functional recovery.
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Keywords: spinal cord injury; cell transplantation; bone marrow stromal cells; cell proliferation

Introduction

Bone marrow contains a population of cells that differentiate along various mesenchymal cell lineages, for example, to form chondrocytes, osteoblasts and adipocytes; these multipotent cells have been referred to as mesenchymal stem cells or simply as bone marrow-derived stromal cells (MSCs).[1] Somewhat surprisingly, MSCs have received considerable interest as possible donor cells in the development of cell transplantation therapies for spinal cord injury (SCI).[2–7] There are, however, a number of reasons for this. First and foremost, MSC transplantation has been demonstrated to enhance axonal regeneration and promote functional recovery in a variety of animal models of SCI.[2–7] These

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studies have shown that transplanted MSCs integrate into host tissue following transplantation, supporting and remyelinating axons that traverse the injury site. MSCs also synthesize a number of growth factors that may contribute to tissue sparing and axonal regeneration, including nerve growth factor and brain-derived neurotrophic factor.[8] Importantly, though, MSCs represent an attractive donor source for various cell transplantation programmes, as they can be repeatedly isolated from bone marrow with relative ease, normally expanded into large numbers *in vitro* and re-introduced into patients as autografts, which prevents the need for immune rejection drugs in the clinical setting.[9]

If donor cell number is a critical factor to the success of cell grafting for SCI treatment, as is likely to be the case, then it is important to note that the typical injury in rodent models of SCI is only a few millimetres in length,[2–7] whereas the length of lesions in humans can reach several centimetres. Recently, using an *in vitro* model, we have shown that human MSCs promote nerve growth over inhibitory molecules present at the lesion site in SCI; furthermore, the number of nerves that were subsequently able to grow over these molecules was related to the number of MSCs present.[10] Extrapolating these data to the *in vivo* situation suggests that the number of MSCs generated in cell culture could be a limiting factor to the success of MSC therapy. However, there are little, if any, data on the culture expansion of MSCs from individuals with SCI. In this study, we have examined the isolation and growth kinetics of MSCs from the bone marrow of individuals with complete long-term SCI compared with non-SCI donors undergoing treatment for low back pain. In separate experiments, we have also examined the influence of cell-seeding density on culture doubling times, the overall MSC harvest and the degree of cell senescence, as delineated by expression of senescence-associated β galactosidase.[11]

Materials and methods

Bone marrow stromal cell culture

Following local research ethical committee approval and informed consent, bone marrow aspirates were harvested from the iliac crest of individuals with a complete long-term Frankel grade A SCI ($n=9$; 3+ months post-injury) or from non-SCI-patients undergoing spinal fusion in the treatment of low back pain ($n=7$) (Table 1). Mononuclear cells isolated by density gradient centrifugation (Lymphoprep, Fresenius Kabi Norge, AS) were plated out in Dulbecco's Modified Eagle's Medium (DMEM)/F12, supplemented with 10% fetal bovine serum, penicillin and streptomycin (DMEM/10% fetal bovine serum medium; Invitrogen Life Technologies, Paisley, UK) at a seeding density of 20×10^6 cells per flask (Falcon 250 ml Polystyrene Tissue Culture Flask, BD Biosciences, Cowley, Oxford, UK). After 24 h, non-adherent cells were removed and the adherent cell population was cultured in monolayer in DMEM/10% fetal bovine serum medium. Cells were routinely passaged at 70% confluence by trypsinization (0.05% Trypsin-EDTA, Invitrogen) and re-seeded at 5×10^3 cells per cm^2. In separate experiments, cells were seeded at 5×10^2, 1×10^3 and 5×10^3 cells per cm^2 at passages I–III.

Cell characterization

Flow cytometry was used to assess the immunoreactivity of cells expanded in monolayer culture, targeting a CD-immunoprofile that is characteristic of MSC populations.[12] Briefly, this was performed as follows: after trypsinization, cells were resuspended in phosphate-buffered saline (Sigma-Aldrich, Poole, UK) containing 2% bovine serum albumin (Sigma-Aldrich) and 10% normal human Ig (Grifols, Cambridge, UK) for 60 min to block nonspecific binding.

Table 1 Bone marrow donor demographics

Sex	*Age (years)*	*Neurological level of SCI*	*Months since SCI*
SCI donors			
Female	23	C1	6.5
Male	39	C4/5	272
Male	38	C5	7
Female	66	C6	61
Male	42	T4	72
Male	44	T6	217
Male	51	T9	5
Male	47	T9	82
Male	41	T11	14
Sex	*Age*	*Neurological level of fusion*	*Other conditions/symptoms*
Non-SCI donors			
Female	43	L4–S1	Spondylolisthesis and L5 nerve root compression
Female	64	L4–S1	None
Female	61	L4–S1	Osteoarthritis and spondylolisthesis
Male	52	L5/S1	None
Female	32	L5/S1	Spondylolisthesis, narrowing of L5 nerve roots
Male	40	L5/S1	Right-sided leg pain
Female	38	L5/S1	Pain in both legs

Abbreviation: SCI, spinal cord injury.
All SCI donors were complete Frankel grade A and none of these individuals required anaesthesia before bone marrow was harvested. Non-SCI donors were spinal fusion patients receiving treatment for low back pain caused by disc degeneration. In this group, the bone marrow donor site was neurologically intact and harvested under general anaesthetic.

Cells were then incubated with fluorescently tagged antibodies specific for CD34, CD45 (both phycoerythrin-conjugated) and CD105 (fluorescein-conjugated) (Immunotools, Friesoythe, Germany) for a further 30 min. Cells were also incubated with isotype-matched IgG (Sigma-Aldrich) as a negative control. Immunoreactivity was determined using a FACScan flow cytometer and analysed using Cell Quest software (BD Biosciences).

The differentiation potential of passage II cells along mesenchymal cell lineages was assessed using the established protocols.[1,13,14] This entailed establishing cell cultures as follows: chondrogenic differentiation.[14] pellet cultures in DMEM supplemented with ITS-X (insulin, transferrin and selenous acid; Invitrogen), ascorbate 2-phosphate (Sigma-Aldrich), dexamethasone (Sigma-Aldrich) and transforming growth factor-β1 (PeproTech Ltd., London, UK); osteoblastic differentiation[13]- monolayer cultures in DMEM/10% FCS, supplemented with ascorbate 2-phosphate, dexamethasone and β-glycerophosphate (all Sigma-Aldrich); adipogenic differentiation[1]—DMEM/10% FCS supplemented with ITS-X, dexamethasone, 3-isobutyl-1-methylxanthine and indomethacin (all Sigma-Aldrich). After 3–4 weeks in culture, the differentiation status of cultures was examined by type II collagen immunolocalization for chondrocyte differentiation, alkaline phosphatase activity for osteoblastic differentiation and oil red-O visualization of lipid accumulation for adipocyte differentiation.

MSC harvest and growth kinetics

The MSC index was defined as the number of cells harvested at passage I divided by the number of mononucleated cells originally isolated and plated out into culture flasks. This value is indicative both of the proportion of MSCs present in the bone marrow sample and/ or their capacity to expand in monolayer culture. The time taken for MSC cultures to double in cell number was calculated using the following formula: culture doubling time (DT) $= (t_2 - t_1) \times \ln(n_2)/\ln(n_2/n_1)$, where $t =$ consecutive time points and $n =$ the respective cell numbers at these time points. MSC DT was determined from passage I through to passage III where indicated.

Senescence-associated β-galactosidase activity

β-galactosidase is a lysosomal enzyme ubiquitously expressed by all cells; the enzyme has an optimal activity at pH 4.0, but it is synthesized at greater levels in senescent cells such that activity can also be detected at pH 6.0.[9] Hence, senescence-associated β-galactosidase (SA-β gal) activity was determined at pH 6.0 in experiments in which cells were seeded at different cell densities at passages I–III, as an indication of cell senescence. Briefly, this was performed as follows: cells were fixed in 10% paraformaldehyde (in phosphate-buffered saline), then immersed for 24 h at 37 °C in freshly prepared 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (1 mg ml^{-1} 40 mM citric acid/sodium phosphate pH 6.0, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$; Sigma-Aldrich) and titrated to pH 6.0. Lysosomal (non-senescent) β-galactosidase activity was detected in parallel cultures using the same solution adjusted to pH 4.0 as a positive control. These cells were subsequently washed in ice-cold phosphate-buffered saline and the proportion of SA-β gal-positive cells present were determined by scoring a minimum of 200 cells over five random fields.

Statistical analysis

The relationship between the MSC index and donor age was evaluated using the Spearman ranked correlation coefficient, *rs*. The Mann–Whitney *U*-test was used to examine differences both between SCI and non-SCI donors, and between SCI donors with cervical versus thoracic lesions, in the MSC index and MSC culture DT. A two-way analysis of variance (ANOVA) was used to evaluate relationships between cell seeding density and MSC culture DT or overall MSC harvest.

Results

During the culture expansion of MSC from bone marrow isolates, stromal cells outgrew any fully differentiated and non-proliferating cells, for example, monocytes or osteoclasts (which occasionally had also adhered to the culture plates). The number of cells harvested at passage I as a proportion of the bone marrow-derived mononucleated cells originally seeded into the culture flasks was therefore termed the MSC index. No significant differences were seen either in the MSC index between SCI and non-SCI donors (Figure 1a) or in the time taken for these cultures to come to first passage, which was 2–3 weeks in both cases. Within the subgroup of SCI donors, the MSC index did not relate significantly to either the neurological level of SCI or to the period between injury onset and when the bone marrow sample was collected (Spearman rank, $P = 0.1447$ and

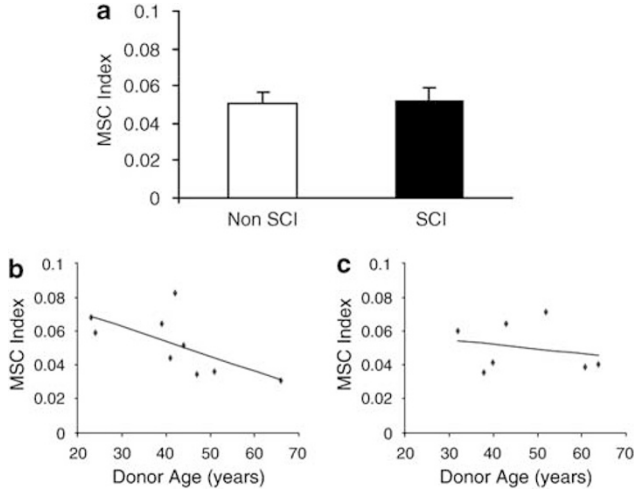

Figure 1 The number of bone marrow stromal cells (MSCs) isolated and expanded to passage I from the bone marrow of spinal cord injury (SCI) and non-SCI donors (MSC index). (**a**) No significant difference was seen between SCI (black bar) and non-SCI donors (open bar) in the MSC index (means ± s.e.m.). (**b**) The MSC Index and donor age was inversely related in SCI donors (*rs* −0.77, Spearman rank, $P = 0.0159$). (**c**) No relationship was observed between MSC index and donor age in non-SCI donors (*rs* −0.04, Spearman rank, $P = 0.9394$).

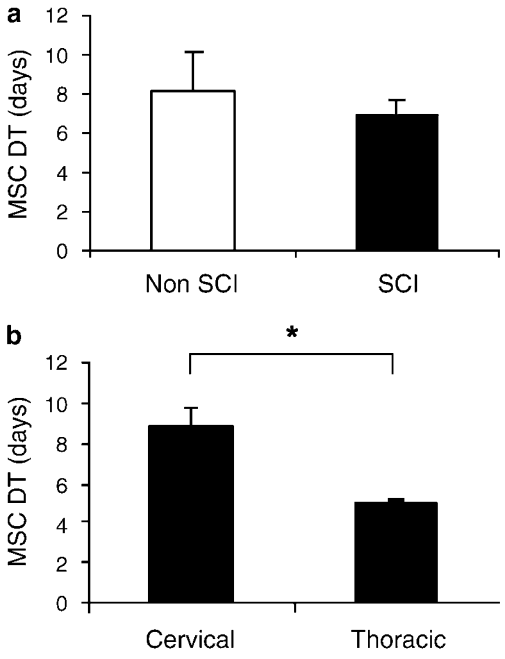

Figure 2 The doubling times (DT) of spinal cord injury (SCI) or non-SCI donor marrow stromal cells (MSCs) between passages I and II. (**a**) No significant difference was seen between SCI (black bar) and non-SCI donors (open bar) in MSC DT (means ± s.e.m.). (**b**) MSC DT from donors with cervical lesions was significantly greater than MSC isolated from individuals with thoracic injuries (means ± s.e.m., cervical $n=4$, thoracic $n=4$, $^*P=0.0286$, Mann–Whitney *U*-test).

$P=0.2894$, respectively). However, the MSC index was inversely related to donor age in SCI donors (Figure 1b, $rs=-0.77$, Spearman rank, $P=0.0159$), which was not the case for non-SCI donors (Figure 1c, $rs=-0.04$, Spearman rank, $P=0.9394$). When the SCI and non-SCI groups were pooled, there was no significant difference between the MSC index in females versus males (Mann–Whitney *U*-test, $P=0.3148$: $n=7$ females, $n=9$ males).

From passage I through to passage II, MSC from SCI and non-SCI donors proliferated at similar rates, with culture doubling times of approximately 1 week (Figure 2a). In contrast, the DT of MSC cultures from donors with thoracic SCI was significantly lower than in those with cervical lesions (Figure 2b, Mann–Whitney *U*-test, $P=0.0286$). No significant relationships were seen between MSC culture DT and donor age either in the whole sample group or in the SCI or non-SCI subgroups, or with the time of injury onset in the SCI subgroup (data not shown). Furthermore, there was no significant difference between MSC culture DT in females versus males when data from both non-SCI and SCI groups were pooled (Mann–Whitney *U*-test, $P=0.0848$). By passage II, sufficient cell numbers were generated to characterize cultures by flow cytometry and differentiation studies. Hence, we found that greater than 95% of passage II cells were CD34−ve, CD45−ve and CD105+ve (Figure 3a); this matches previously published MSC immunoprofiles.[12] Passage II cells also differentiated along all three mesenchymal cell lineages tested, as delineated by collagen type II immunolocalization, alkaline phosphatase activity and lipid accumulation following culture in chondrocytic, osteoblastic and adipocytic differentiation conditions, respectively (Figures 3b–d).

In separate experiments, we compared the routine cell-seeding density of 5×10^3 cells per cm^2 at passage I to densities of 1×10^3 and 5×10^2 cells per cm^2. Seeding cells at these lower densities between passages I and III not only resulted in the harvest of greatly increased cell numbers at passage III (ANOVA, $P=0.0075$), but they were also associated with lower MSC culture DT, which was significant between passages II and III (ANOVA, $P=0.0232$) (Figure 4). The mean relative increase in MSC numbers between passage II and III was 71, 3430 and 131 633% for 5×10^3, 1×10^3 and 5×10^2 cells per cm^2, respectively. None of the cultures generated at these various cell-seeding densities demonstrated increased levels of cell senescence, as depicted by expression of SA-β gal, which was negative in all cases.

Discussion

It is clear from animal studies that transplantation of bone MSCs promotes functional recovery after SCI.[2–7] However, there is also evidence to suggest that SCI in humans may influence the activity of other bone marrow cells. SCI is known to depress natural and adaptive immunity and *in vitro* studies have demonstrated not only decreased lymphocyte function[15,16] but also reduced long-term colony formation of haemopoietic stem cells.[15] A clear question, therefore, is whether these cells can be isolated in humans with SCI and how they behave in culture. There is little published data characterizing MSC in humans with SCI, although one recent report stated that bone marrow harvests from SCI donors gave rise to 'fibroblast-like mesenchymal cells' in just 75% of cases.[17]

Using standard protocols for MSC isolation, we found no significant differences in the expansion of stromal cells from the bone marrow of SCI or non-SCI donors successfully growing cells with MSC characteristics through to passage II and beyond in all cases. Furthermore, no differences were seen in the proliferative rate of the MSC generated from either group, based on their culture DT after first passage. MSC population doublings in non-SCI humans have previously been found to relate to age, where cultures established from paediatric donors proliferated to generate nearly twice as many cells as those derived from young adults within the same time span.[18] In addition, differences in the number of mononuclear cells present in bone marrow biopsies taken from SCI donors with cervical versus thoracic injuries have been reported, where donors with cervical lesions had larger cell counts per ml of marrow.[17] We found that the number of MSCs generated at passage I as a proportion of mononucleated cells originally isolated (that is, the MSC index) was inversely related to age in SCI donors but not non-SCI donors. We observed no differences in the MSC index or culture DT of SCI donors in comparison with non-SCI donors. In contrast, MSCs from individuals with cervical SCI lesions proliferated more slowly than in those with thoracic lesions. Given the small sample sizes involved

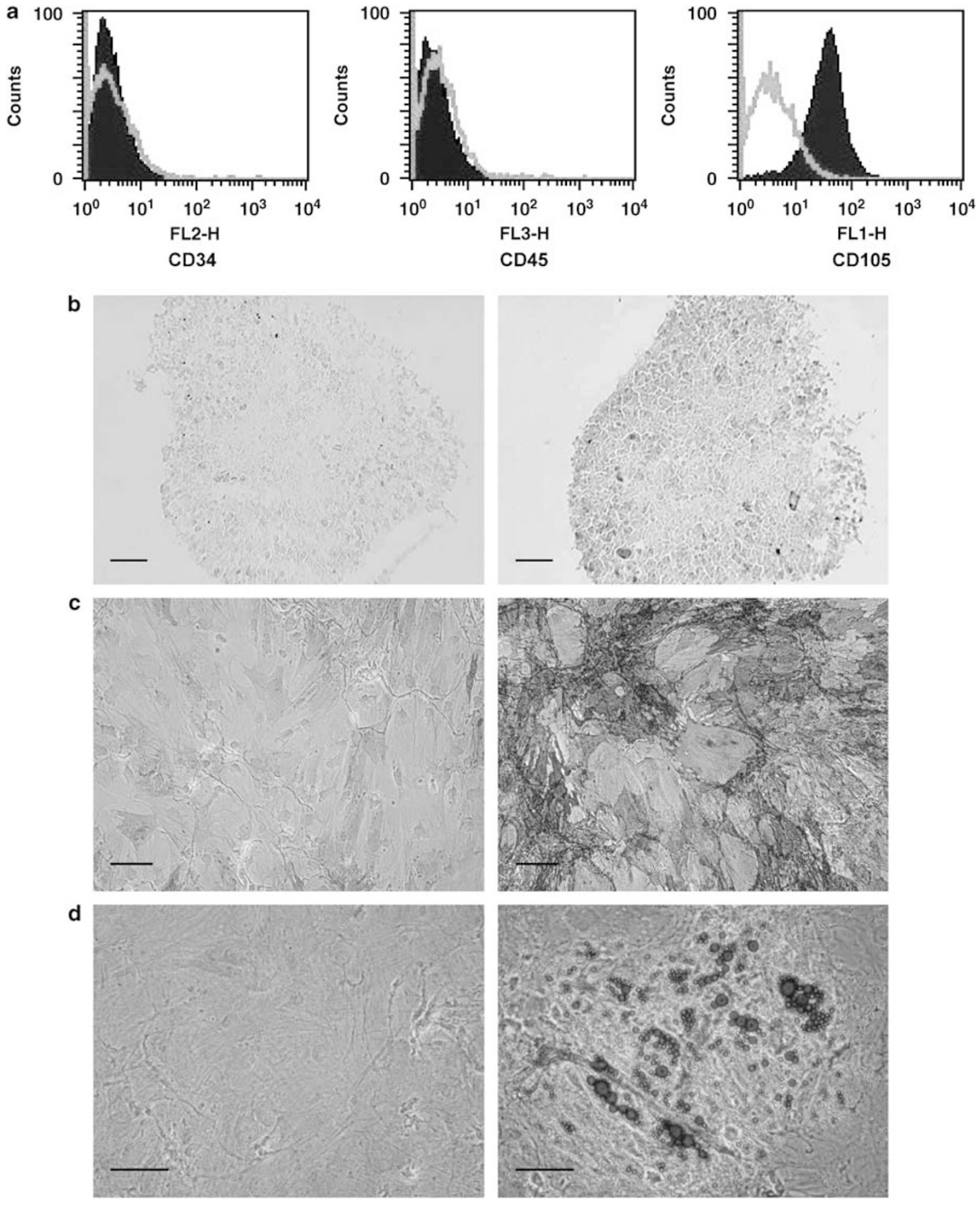

Figure 3 Phenotypic characterization of marrow stromal cells (MSCs). (**a**) Fluorescence-activated cell sorting analysis for CD-immunolabelling of MSC isolated from iliac crest biopsies (passage II cells). More than 95% of cells in all cultures tested were CD34−ve, CD45−ve and CD105+ve at passage II and thereafter. The black histogram denotes immunopositivity (fluorescence intensity) for each indicated marker, whereas the grey-lined histogram denotes immunolabelling with an isotype-matched control antibody. (**b**) Immunolocalization of collagen type II (right panel) in sections of MSC pellet cultures stimulated in chondrogenic medium. Left panel = isotype control antibody. (**c**) Alkaline phosphatase activity was markedly increased in monolayer MSC cultures treated with osteogenic stimuli (right panel) compared with MSC cultured in standard culture medium (left panel). (**d**) The presence of lipid vesicles was increased in MSC treated with adipogenic stimuli (revealed with oil red-O, right panel), compared with MSC cultured in standard culture medium (left panel). All calibration bars = 100 μm.

and the fact that two out of four of the cervical samples were female, whereas no females were included in the thoracic group, this difference may relate in some way to sex. However, no significant differences were seen in either the MSC index or MSC culture DT in female versus male donors when the SCI and non-SCI groups were pooled. These

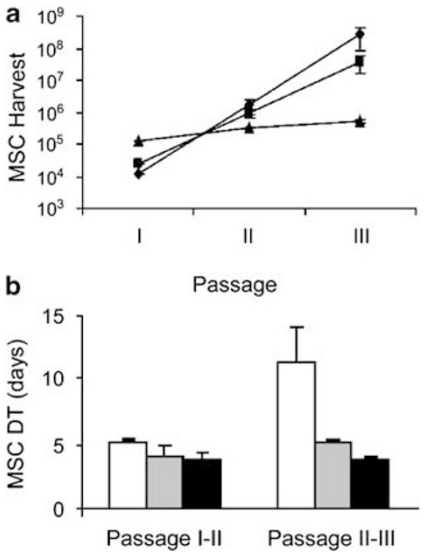

Figure 4 Lower seeding densities reduced culture periods and maximized marrow stromal cell (MSC) numbers. (**a**) MSC harvest between passages I and III at each seeding density (triangular points = 5×10^3 per cm^2, square points = 1×10^3 per cm^2 and diamond points = 5×10^2 per cm^2 means ± s.e.m.). There was a significant relationship between the seeding density of MSCs and the cell yield at passage III, where lower seeding densities produced significantly larger MSC cell yields (ANOVA, $P = 0.0075$). (**b**) MSC DT between passages I and III (open bars = 5×10^3 per cm^2, grey bars = 1×10^3 per cm^2 and black bars = 5×10^2 per cm^2 means ± s.e.m.). There was a significant relationship between the seeding density of MSCs and the culture DT between passages II and III where lower densities significantly reduced MSC DT (ANOVA, $P = 0.0232$).

findings may indicate differences in the behaviour of MSCs from SCI donors, as the MSC index is a combined indication of cell isolation, cell adhesion and cell proliferation. However, larger donor sample groups (of increased donor age range) and further study are required to ascertain (i) whether the influence of age on MSC proliferation is more pronounced in individuals with SCI compared with non-SCI donors, or (ii) why MSCs from SCI donors with cervical lesions appear to proliferate more slowly than those from donors with thoracic lesions. One possible explanation for the differences in proliferation rate is that although more mononucleated cells are present in the bone marrow of SCI donors with cervical lesions,[15] these may contain proportionally fewer MSC. If this were the case, then fewer MSCs will have been initially seeded into the culture flasks and these will have undergone increased rounds of proliferation to reach passage I, resulting in a reduced proliferation rate thereafter compared to cells undergoing fewer rounds of division before first passage. We report no significant relationships in the MSC index or culture DT and the period between injury onset and when the bone marrow sample was collected in SCI donors (5–272 months). This donor population excludes patients in the acute phase of SCI, which may well influence the behaviour of transplanted MSC in a manner that differs from the subacute or chronic setting. MSC transplantation has been demonstrated to be beneficial in acute/subacute and chronic models of SCI, and therefore these are all clinical targets.[2–7] However, following local ethical guidelines, bone marrow aspirates were harvested only from individuals with a complete long-term Frankel grade A SCI, a minimum of 3 months post-injury.

The number of cells available for transplantation may well be limiting to the success of cell transplantation therapies for SCI, especially when scaling up in lesion size from animal models to humans. Using an *in vitro* model, and supporting this assumption, we have recently reported that cell number relates directly to the capacity of MSCs to diminish the nerve-inhibitory effects of molecules found in the glial scar after SCI.[10] Here, we show that seeding MSCs at lower densities from passages I to III significantly reduced the normal culture DT and greatly increased overall MSC yield. In addition, we found that this proliferative response did not lead to increased cell senescence, as delineated by SA-β gal activity, which is generally deleterious to cell function. It is unclear how MSC seeding density relates to proliferative rate. Cell contact inhibition induces growth arrest in C3H10T1/2 cells at confluence, which is a murine fibroblast cell line with similar phenotypical characteristics to MSCs;[19] this growth arrest of C3H10T1/2 is reversed by reseeding the cells at lower density.[20] Hence, establishing MSC cultures at lower cell seeding densities may similarly reduce the effects of contact inhibition, maintaining a greater proportion of cells in cycle. If there is a window of opportunity in the acute phase of SCI when MSC grafting is therapeutic, then generating sufficient MSCs from autologous bone marrow over relatively short time periods is potentially of great importance.

In summary, we have shown that MSCs can be successfully isolated from bone marrow biopsies of donors with SCI and subsequently expanded in culture. In parallel studies, we have also demonstrated that MSCs from SCI donors promote nerve growth.[10] Together, these findings support and validate previous animal studies suggesting that MSC transplantation may provide therapeutic benefits to humans with SCI.

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