# Exposing mesenchymal stem cells to chondroitin sulphated proteoglycans reduces their angiogenic and neuro-adhesive paracrine activity

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## Abstract

The multifactorial complexity of spinal cord injuries includes the formation of a glial scar, of which chondroitin sulphated proteoglycans (CSPG) are an integral component. Previous studies have shown CSPG to have inhibitory effects on endothelial and neuronal cell growth, highlighting the difficulty of spinal cord regeneration. Mesenchymal stem/stromal cells (MSC) are widely used as a cell therapy, and there is mounting evidence for their angiogenic and neurotrophic paracrine properties. However, in vivo studies have observed poor engraftment and survival of MSC when injected into SCI. Currently, it is not known whether increasing CSPG concentrations seen after SCI may affect MSC; therefore we have investigated the effects of CSPG exposure to MSC in vitro. CSPG-mediated inhibition of MSC adhesion was observed when MSC were cultured on substrates of increasing CSPG concentration, however MSC viability was not affected even up to five days of culture. Culture conditioned medium harvested from these cultures (primed MSC CM) was used as both culture substrata and soluble medium for EA.hy926 endothelial cells and SH-SY5Y neuronal cells. MSC CM was angiogenic, promoting endothelial cell adhesion, proliferation and tubule formation. However, exposing MSC to CSPG reduced the effects of CSPG-primed MSC CM on endothelial cell adhesion and proliferation, but did not reduce MSC-induced endothelial tubule formation. Primed MSC CM also promoted neuronal cell adhesion, which was reduced following exposure to CSPG. There were no marked differences in neurite outgrowth in MSC CM from CSPG primed MSC cultures versus control conditions, although non-primed MSC CM from the same donors was found to significantly enhance neurite outgrowth. Taken together, these studies demonstrate that MSC are resilient to CSPG exposure, but that there is a marked effect of CSPG on their paracrine regenerative activity. The findings increase our understanding of how the wound microenvironment after SCI can mitigate the beneficial effects of MSC transplantation.

## Introduction

Mesenchymal stem/stromal cells (MSC) are routinely characterised using criteria released from the International Society of Cellular Therapy (ISCT), which includes plastic adherence, a specific CD marker profile and tri-lineage differentiation potential to formosteoblasts, chondrocytes and adipocytes [1,2]. Hence, MSC havebeen isolated and cultured from bone marrow and adipose tissuefrom a variety of species, including rodents, humans and dogs [3-6]. However, more recent studies have suggested that MSC havea wider function that simply acting as stem/progenitor cells for mesenchymal tissues and that they can elicit immunomodulatoryand wound healing activity through paracrine activities on endogenous cells already present at sites of injury [7].

The potential of using MSC as a therapy for spinal cord injury (SCI) has been investigated using smaller animal models [5,6,8].These studies, coupled with *in vitro* experiments, have provided evidence that transplanted MSC stimulate SCI repair through a number of mechanisms, including MSC-mediated secretion of soluble neurotrophic and angiogenic growth factors, direct cell-cell contact and the deposition of MSC-secreted extracellular matrix components that can bridge the wound microenvironment toenhance axonal regeneration [4,7-11]. However, MSC survival has been found to decrease rapidly following their transplantation into SCI lesion sites [12-14], which limits their wound healing effects[13].

Currently, little is known as to which factors in the SCI lesion site may cause transplanted MSC numbers to decrease so quickly when used as a therapeutic. What is known is that the SCI lesion becomes an increasingly hostile microenvironment after injury wherein a combination of excitotoxicity, immune cell influx and the generation of a glial scar, which is formed by reactive astrocytes secreting chondroitin sulphated proteoglycans (CSPG), prevents tissue repair[15-17]. The glial scar, in particular, is a major block to SCI repair mechanisms because although it provides a

mechanism to limit the damage caused by SCI and hence prevent further damage to the blood-spinal cord barrier (BSCB), the CSPG present within the scar inhibits sufficient axonal regeneration [15,17].

After SCI, CSPG concentrations within the glial scar increase over time, reaching peak concentrations at 2 weeks post injury [18,19]. Furthermore, studies observing decreased MSC viability *in vivo* after SCI, reported reduced viability as early as 2 weeks following transplantation [12-14]. This temporal coincidence of an increase in CSPG levels with decreased MSC survival raises the possibility that the formation of the glial scar may contribute, at least in part, to how successful MSC transplantation may prove. Nonetheless, even with the decline in MSC numbers post graft, beneficial actions of MSC transplantation have still been observed [13,14]. Furthermore, *in vitro* studies of MSC culture conditioned medium have also reported angiogenic and neurotrophic effects [4,9,10,20], strengthening theories that the reparative activities of MSC in SCI derive from the MSC secretome. Enhancing angiogenesis is especially important during the recovery stages of SCI, as blood vessels are the system of delivery for nutrients and oxygen, as well as the removal of waste products. Following ischaemia, angiogenesis can also reduce secondary damage to axons [21,22].

To our knowledge, no studies have examined whether CSPG influence MSC viability or MSC-mediated wound healing activity. Therefore, this study has used *in vitro* techniques and established cell reporter assays [4,11] to establish both the effects of CSPG on MSC viability and also on MSC-mediated angiogenic and neuro- trophic paracrine activity.

## Materials and methods

### MSC isolation and growth

Institutional ethical approval was obtained for this study (University of Chester: 060/16/CW/BS, 18th May 2016). After consent, samples of adipose tissue were surgically extracted from inguinal fat pads of dogs (n = 3 donors) undergoing MSC transplantation treatments of joint pathology. MSC were isolated via collagenase digestion and preferential plastic adhesion, as described previously [3,4], and subsequently cultured in Dulbecco's modified Eagle medium/F-12 GlutaMAX™ (DMEM/F-12), supplemented with 1% penicillin/streptomycin (P/S) and 10% foetal bovine serum (FBS) (standard culture medium) (all Gibco®, Life Technologies™, Paisley, UK) in a humidified atmosphere of 5% $CO_2$ at 37 C. Routine passaging was completed when cultures reached 80% confluences using 0.25% trypsin-EDTA (Gibco®, Life Technologies™). The culture-expanded cells were characterised for plastic adherence, immunophenotyped and assessed for tri-lineage differentiation potential, as we have reported recently [4], and hence shown to exhibit an MSC phenotype.

### Culturing MSC on CSPG and the generation of CSPG-primed MSC conditioned medium

6 well culture plates were incubated with solution of 0 μg/ml, 10 μg/ml or 100 μg/ml chondroitin sulphated proteoglycans (CSPG1; Sigma Aldrich, Dorset, UK) at 4° C overnight to allow CSPG adherence to the culture plastic. CSPG-coated wells were washed three times with Dulbecco's phosphate buffered saline (PBS, Gibco®, Life Technologies™), completely aspirated and the plate warmed for a minimum of 1 h at 37° C/5% $CO_2$ to allow the wells to completely dry. MSC were then seeded into the wells at a density of 1.8 $10^5$ cells/well in 10 ml of serum-free conditioning medium (DMEM/F12 supplemented with ITS+, NEAA and penicillin/streptomycin, all Gibco®, Life Technologies™). The plate was incubatedat 37° C/5% $CO_2$ for a total of 5 days after which time the culture-conditioned medium (MSC CM) was harvested, filtered through a 0.20 μm filter to remove any cell debris, and stored at 20° C for further experimentation. Control medium was generated by incubating the same volume of serum-free medium used to culture MSC at 37° C for 120 h and then harvesting in the same manner as each of the MSC CM. In some experiments, MSC CM was generated separately by seeding MSC into T75 flasks in DMEM/F12 medium supplemented with 10% fetal bovine serum and antibiotics, as reported previously [4].

During the MSC culture on CSPG-coated substrata, the cells were imaged digitally under phase contrast microscopy at 2h,

24 h and 120 h post seeding. The proportion of MSC that appeared adherent, as delineated by the presence of cell processes extending onto the culture surface, versus non-adherent (no visible cell processes and a spherical cell morphology) was determined scoring a minimum of 50 cells for each condition and each MSC tested. MSC viability was assessed by phase contrast microscopy and further by incubating MSC seeded into CSPG-coated substrata in Live/Dead staining solution, wherein calcein AM stains viable cells with green fluorescence and propidium iodide stains non-viable cells with red fluorescence, following the manufacturer's instructions (Live/Dead Cell Double Staining Kit, 04511, Sigma Ltd). The proportion of cellsthat were green (live) or red (dead) when viewed under fluorescence microscopy (490 nm) was scored and digitised images collected using a GX-CAM HiChrome-S camera.

**EA.hy926 endothelial cell and SH-SY5Y neuronal cell adhesion assays**

The effects of MSC CM on EA.hy926 endothelial cell and SH- SY5Y neuronal cell adhesion was assessed by MTS assay [23,24] as follows: briefly, 96 well tissue culture plates were coated with 50 ml of MSC CM versus control medium overnight at 4° C, followedby washes in PBS (as described above for CSPG coating), prior to seeding with EA.hy926 or SH-SY5Y cells at a density of $5 \times 10^3$ cells in 100 ml of serum-free medium per well (triplicate wells for each MSC CM). After 2 h of incubation at 37° C/5% $CO_2$, wells were washed to remove non-adherent cells and 100 ml serum-free medium plus 20 ml of MTS solution (Promega CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Southampton, UK) added to each well and the plate was incubated for a further 2 h at 37° C/5% $CO_2$. Absorbance was measured at 490nm (iMark micro-plate reader, Bio-Rad, Kidlington, UK). Cells were also imaged digitally under phase contrast microscopy.

**EA.hy926 endothelial and SH-SY5Y neuronal cell proliferation assays**

The effects of MSC CM on EA.hy926 endothelial cell and SH- SY5Y neuronal cell proliferation was assessed by culturing cells in MSC CM versus control medium and then performing MTS assays to determine the relative numbers of metabolically active cells, as described previously [4]. In brief, EA.hy926 or SH-SY5Y cells were seeded into 96 well plates at a density of $5 \times 10_3$ cells per well in 100 ml of standard serum supplemented culture medium and incubated for 24 h at 37° C/5% $CO_2$ to permit cell adherence. Then the serum supplemented culture medium was removed and wells were washed twice with 50 ml of serum-free medium prior to the addition of 100 ml of MSC CM or control medium (n = 3), and incubated at 37° C/5% $CO_2$. After 48 (endothelial cell assays) and 72 (neuronal cell assays) hours, 20 ml of MTS solution was added directly to wells and absorbance measured using a spectrophotometer, as described above. Cells were also imaged digitally under phase contrast microscopy.

**Matrigel assays for EA.hy926 endothelial tubule formation**

The effects of MSC CM versus control medium on the formation of endothelial tubule-like structures were assessed using the Matrigel assay, as described previously [4,25]. Briefly, Matrigel™ (Corning, New York, USA) was used to coat 96 well plates (100ml/well) for 30 min at 37° C/5% $CO_2$ and then seeded with EA.hy926 cells at a density of $2 \times 10^4$ cells per well in 200 ml of MSCCM or control medium and incubated at 37° C/5% $CO_2$ for 24 h. The plate was then loaded into the Cell-IQ imaging system (Chipman Technologies, Tampere, Finland) under x10 magnification and digitised images analysed for tubule formation using the Cell-IQ imaging software.

**SH-SY5Y neurite outgrowth assays**

SH-SY5Y neuronal cells were seeded into a 24 well plate at a density of $1 \times 10^4$ cells per well in 1 ml of standard serum sup- plemented culture medium and incubated for 24 h at 37° C/5% $CO_2$ to permit cell adherence. After this period, the culture medium was removed, the wells were washed with serum-free medium and then 1 ml of MSC CM or control

medium added prior to incubation at 37° C/5% $CO_2$ for 72 h. At 72 h post-treatment, digitised images were collected under phase contrast microscopy and neurite outgrowth measured using Cell-IQ imaging software, as previously described [4].

### Statistical analysis

At least 3 independent experiments were performed, i.e. using MSC and MSC CM generated from 3 separate tissue donors. IBM SPSS software was used for statistical analyses. Data generated from n = 3 MSC donors was pooled and tested for normal distribution using the Shapiro-Wilk test and then relationships between parameters and differences between groups were tested for significance using either a one way analysis of variance (ANOVA) with Tukeys post-hoc or T-tests for normally distributed data or a Kruskal-Wallis test with Mann Whitney U tests post-hoc for non-normally distributed data. All data have been shown as mean-s±standard deviations (SD). Significance was accepted below the 5% level.

## Results

### CSPG substrata inhibited MSC adhesion, but did not affect MSC viability

Using CSPG to coat wells as a culture substratum inhibited MSC adhesion in a concentration-dependent manner (Fig. 1). Hence, at 2 h post-seeding, the MSC morphology, i.e., a more stromal appearance with cytoplasmic extensions onto the culture surface and the extent of cell spreading indicated a greater adhesion in control wells compared with reduced adhesion as the coating concentration of CSPG increased. This evident inhibition of MSC adhesion by CSPG coating was also seen at 24 h post-cell seeding, but by 120 h post-seeding there was no apparent difference in the morphology of MSC on all culture substrata. Image analyses of digitised images collected throughout time course demonstrated that both proportions of adherent versus non-adherent MSC and the extent of MSC spreading were significantly inhibited by CSPG coated in such a transient manner (Fig. 1B). However, MSC appeared phase bright at all time, indicating viable cultures, and Live/Dead staining at 24 and 120 h post-seeding demonstrated that culturing MSC on increasing CSPG concentrations had little to no effect on their viability (Fig. 1A: inset). The lowest MSC viability recorded was 98 ± 1% viable cells at 24 h post-seeding and 96 ± 3% viable cells at 120 h post-seeding.

### Culturing MSC on CSPG-coated substrata reduced the pro-adhesive and proliferative effects of primed MSC CM on EA.hy926 endothelial cells with little effect on the formation of endothelial tubule-like structures

MSC CM generated from MSC cultured on 0 μg/ml and 10 μg/ml CSPG-coated substrata significantly increased EA.hy926 endothelial cell adhesion after 2 h of culture compared to control medium. However, there was a marked reduction in this pro-adhesive effect from MSC CM generated from MSC cultures on 100 μg/ml CSPG-coated substrata (Fig. 2). Similarly, MSC CM from MSC cultured on 0 μg/ml and 10 μg/ml CSPG-coated substrata increased EA.hy926 endothelial cell proliferation at 48 h of treatment compared to control medium, which also was not seen using MSC CM from 100 μg/ml CSPG-coated substrata (Fig. 3). Conversely, the effects of SC CM in enhancing the formation of tubule-like structures by EA.hy926 cells on Matrigel were not diminished by prior priming of the MSC CM through CSPG exposure (Fig. 4). There was a significant increase in EA.hy926 endothelial tubule formation when the endothelial cells were cultured in MSC CM harvested from 0 μg/ml, 10 μg/ml and 100 μg/ml CSPG coated wells (all $p < 0.05$; Mann-Whitney *U* test) compared to control medium.

### Culturing MSC on CSPG-coated substrata reduced the pro-adhesive effects of primed MSC CM on SH-SY5Y neuronal

**cells with little effect on SH-SY5Y neurite outgrowth**

MSC CM generated from MSC cultured on 0 μg/ml, 10 μg/ml and 100 μg/ml CSPG-coated substrata significantly increased SH-SY5Y neuronal cell proliferation after 72 h of culture compared to control medium. However, there was a marked reduction in this pro-adhesive effect from MSC CM generated from MSC cultures on 100 μg/ml CSPG-coated substrata (Fig. 5). Conversely, none of the CSPG primed MSC CM, including the conditioned medium generated from MSC cultures that had been seeded onto PBS-only coated wells (0 μg/ml primed MSC CM) enhanced SH-SY5Y neurite outgrowth compared to control medium (Fig. 6). Wepreviously observed that MSC CM harvested from MSC culturesseeded in T75 flasks in serum-supplemented medium to initially permit cell adhesion then washed and cultured in serum-free medium enhanced SH-SY5Y neurite outgrowth [4]. Therefore, in separate experiments using MSC from the same donors that have been used in the current study we generated MSC CM using the technique previously reported. Similar to our previous results, we found that this MSC CM was neurotrophic, significantly enhancing SY-SY5Y neurite outgrowth compared to control medium (Fig. 6C).

## Discussion

Following SCI, the combination of inflammatory cytokines, widespread cell death and immune cell influx causes surviving local astrocytes to become reactive. These reactive cells secrete CSPG, which, along with debris from dying cells and myelin sheath degradation, form the glial scar [16]. Although perhaps the primary action of the glial scar is to aid in restoration of the BSCB, so limiting further CNS damage, the CSPG present is inhibitory to new nerve growth [15,17]. Hence, the presence of CSPG in the damaged spinal cord is a major consideration to strategies that target axonal regeneration and spinal cord repair.

Transplanting MSC directly into SCI lesion sites in animal models increases functional recovery, which is thought to be mediated by the secretion of factors by the transplanted MSC [9,20]. However, the viability of MSC decreases dramatically within weeks of transplantation, limiting their wound healing capacity [12-14].The causes of MSC death following transplant could be due to the severe microenvironment within the injured area of the spinal cord [12-14]. As described above, the accumulation of a CSPG-rich matrix in the SCI lesion is one aspect of this microenvironment. Therefore, this study has examined whether exposing MSC to CSPG has any effect on their growth and viability, as well as their capacity to stimulate angiogenesis and nerve growth, both of which are integral to SCI repair processes. We used these concentrations ofCSPG specifically because previous research has shown that EA.hy926 endothelial cell migration [26] and SH-SY5Y neurite outgrowth [27] were not inhibited at 10 μg/ml CSPG, but were inhibited at 100 μg/ml CSPG. Increased CSPG is thought to an important role in inhibiting CNS repair [18,19] and although the exact CSPG concentrations seen *in vivo* after SCI are not well documented, the range that we have examined includes those levels of CSPG that have little/no effect on endothelial cells and nerve growth and those that are inhibitory, at least *in vitro*. Hence, the range was intended to mimic those levels that may be seen in the CNS lesions into which MSC have been transplanted.

Our data shows that MSC adhesion was transiently inhibited by CSPG-coated substrata, as has been reported previously for a variety of other cell types including neurons and endothelial cells [9,10,28-30]. However, the MSC became adherent and more spread on the CSPG-coated substrata with time, which may indicate eitherthat CSPG was being lost from the substrata, possibly due to MSC-mediated degradation [31-33], or that the MSC were secreting other adhesive ECM, e.g. collagens, laminin or fibronectin [20], which masked the CSPG. Further experimentation is required to determine how MSC adhesion to the CSPG substrata increased withtime. However, exposure to CSPG did not significantly affect MSC viability at any time point during this study. Hence, our results do not support the hypothesis that increased levels of CSPG seen in the glial scar play a direct role in reducing the numbers of viable MSC after their transplantation, suggesting that other microenvironmental factors may be responsible for this cell loss.

In contrast, exposure of the MSC to CSPG reduced the extent to which the MSC secretome, in the form of MSC CM, promoted some aspects of angiogenesis. Similar to previous reports [11], MSC CM generated from MSC cultures that had not been exposed to CSPG enhanced EA.hy926 endothelial cell adhesion (when used as a MSC CM-coated culture

substratum) and EA.hy926 endothelial cell proliferation (when used as a soluble culture medium) compared to control medium. However, priming the MSC through CSPG exposure reduced this adhesive and proliferative effect in a CSPG concentration-dependent manner. Using Matrigel assays [4,25], MSC CM was also found to enhance EA.hy926 endothelial tubuleformation compared to control medium; however, this proangiogenic effect was not significantly changed by CSPG priming.Therefore, from this data it can be concluded: (i) MSC secreted proangiogenic factors; (ii) MSC exposure to CSPG reduced their pro-adhesive and proliferative effects on endothelial cells. This suggests that the presence of CSPG altered the MSC phenotype, at least interms of angiogenic activity, and raises the important question as to how this change may have arisen it if wasn't associated with decreased MSC viability. Previous research has suggested that as MSC differentiate to form chondrocytes, they have reduced angiogenic activity [34]. Furthermore, changes in MSC morphology to a more spherical cell shape compared to a flattened, fibroblastic shape, is associated with increased chondrogenesis [35]. Hence, one possibility may be that the transient changes seen in MSC morphology when their adhesion to the CSPG-coated substrata was inhibited also may have reduced their secretion of angiogenic factors, e.g. decreased vascular endothelial growth factor (VEGF) expression, or increased their secretion of anti-angiogenic factors, e.g. CSPG [11]. In addition, if CSPG are released from the culture substrata as a result of MSC culture, then the fact that CSPG itself inhibits endothelial cell adhesion and migration [26,36] suggests that this may also play a role. Further research is required to address these questions, which may have direct relevance to the impact that transplanted MSC have in the SCI milieu.

MSC CM was found to promote SH-SY5Y neuronal adhesion when used as an MSC CM-coated culture substratum, an effect thatwas diminished by MSC culture on high concentrations of CSPG. This suggests that the MSC CM contained molecules that are pro-adhesive to neuronal and endothelial cells (as discussed above, e.g. collagen, laminin and fibronectin), which we have previouslyreported is present in the MSC secretome [11]. The presence of ECM components that support neuronal and endothelial cell adhesioncan be related to increased nerve growth [37] as well as angiogenesis [38]. However, and contrary to our previous research [4],the MSC CM generated in this study had no effect on SH-SY5Y neurite outgrowth. To examine whether the method of MSC CM generation used in the current study could have accounted for this difference in outcome from our previous research, we also made MSC CM using our previously published methods [4] and found that this harvested conditioned medium was neurotrophic. Further research is required, therefore, to better understand this difference in outcome and fully establish the effects of exposing MSC to CSPG on their neurotrophic activity. The MSC CM generated in the current protocol was harvested after a 5 day culture period, whereas our previous protocol was harvested after only a 3 day culture period. This extra time in culture may have caused a shift in the levels of secretome components. Like all cells, MSC require growth factors and nutrients to survive [39] and to synthesise proteins [40]. Hence, the MSC used in this study may have started to reduce their secretion of trophic factors and re-uptake products that had been secreted over the first 3 days of culture to counter decreasing growth factor/nutrient levels. This could have reduced the level of some secretome components within the MSC CM, possibly even differentially, e.g. MSC possess the low affinity nerve growth factor receptor CD271 [41] hence there may have been potentially increased NGF uptake with time, which in terms would mean less neurotrophic activity within the MSC CM. The results from this study suggest that there may be more complex interactions between MSC, MSC-secreted factors, CSPG exposure and time inculture, and this warrants further investigation.

Finally, it is noteworthy that these findings have relevance to other CNS disorders, not just SCI, where stem cell transplantation has shown great potential. These include the use of different potentially therapeutic cells, including MSC, to combat stroke and traumatic brain injury [42-46]. The survival of the transplanted cells and the effects of CSPG on their wound healing activity may well play an important role in these scenarios.

## Conclusions

In conclusion, exposing MSC to CSPG prior to harvesting culture conditioned medium, possibly with a view to using MSC CM therapeutically in SCI, has shown a variety of interesting effects thatneed further investigation. CSPG priming of MSC CM reduced its trophic effects on endothelial cell adhesion and proliferation, and moderately inhibited some

aspects of neurogenesis (i.e. neuronal cell adhesion), but not neurite outgrowth. This reduction in trophic activity may influence important aspects of MSC-mediated SCI repair by limiting new blood vessel formation and nerve growth. Proteomic study of the MSC CM generated in these conditions in future research may give further insight as to how CSPG interactions in the SCI microenvironment influence repair processes. This knowledge could help the design of more effective ways of culturing MSC to enhance the MSC-mediated wound healing activity.

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