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ORIGINAL ARTICLE

Viability, growth kinetics and stem cell markers of single and clustered cells in human intervertebral discs: implications for regenerative therapies

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Abstract
Purpose There is much interest in the development of a cellular therapy for the repair or regeneration of degenerate intervertebral discs (IVDs) utilising autologous cells, with some trials already underway. Clusters of cells are commonly found in degenerate IVDs and are formed via cell proliferation, possibly as a repair response. We investigated whether these clusters may be more suitable as a source of cells for biological repair than the single cells in the IVD.
Methods Discs were obtained at surgery from 95 patients and used to assess the cell viability, growth kinetics and stem or progenitor cell markers in both the single and clustered cell populations.
Results Sixty-nine percent (±15) of cells in disc tissue were viable. The clustered cell population consistently proliferated more slowly in monolayer than single cells, although this difference was only significant at P0–1 and P3–4. Both populations exhibited progenitor or notochordal cell markers [chondroitin sulphate epitopes (3B3(−), 7D4, 4C3 and 6C3), Notch-1, cytokeratin 8 and 19] via immunohistochemical examination; stem cell markers assessed with flow cytometry (CD73, 90 and 105 positivity) were similar to those seen on bone marrow-derived mesenchymal stem cells.
Conclusions These results confirm those of previous studies indicating that progenitor or stem cells reside in adult human intervertebral discs. However, although the cell clusters have arisen via proliferation, there appear to be no greater incidence of these progenitor cells within clusters compared to single cells. Rather, since they proliferate more slowly in vitro than the single cell population, it may be beneficial to avoid the use of clustered cells when sourcing autologous cells for regenerative therapies.

Keywords Intervertebral disc · Cell therapy · Clustered cells · Progenitor or stem cells · MSCs

Introduction

Degeneration of the intervertebral disc (IVD) in humans is an important phenomenon due to its association with common clinical conditions including spinal stenosis, back pain [1] and disc herniation [2]. Current treatment regimens for these conditions are limited, often being targeted to patients when degeneration is extensive and involving interventional surgery. There is a great deal of interest in biological treatments, as these offer the promise of a less invasive and potentially more permanent solution. Autologous chondrocyte implantation (ACI) has been used successfully for two decades for treating damaged articular cartilage, either caused by trauma or degeneration [3, 4]. A similar technique to ACI using autologous disc cells has been used to a limited extent for treating herniated discs

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[5], with other trials ongoing [6]. Such autologous cell therapy usually relies on sourcing cells from within the patient's IVDs.

There is a great deal of evidence in the literature that the viability of cells within human IVDs is low with up to 80 % of cells having a necrotic or apoptotic appearance, even in young individuals [7, 8]. Despite this, a feature of degenerate IVDs is the presence of clones or clusters of cells [9, 10] which have formed from increased proliferation [11]. These resemble cell clusters found in osteoarthritic cartilage [12]. In both articular cartilage and IVD, markers which are believed to represent progenitor or stem cells have been shown to be present in tissue sections [13–15], often occurring within clusters of cells. These include epitopes representing different sulphation patterns on chondroitin sulphate (CS) chains of glycosaminoglycans [14], molecules such as OCT3/4, indicating pluripotency, and Notch and its ligand, Jagged, which are involved in organ formation and morphogenesis [16, 17]. Cluster of differentiation (CD) markers of stem cells have also been reported [15].

We hypothesised that these cell clusters may have an enhanced population of stem cells in comparison to the remaining cells within the IVD matrix. Hence, using human surgical samples such as might be used as a source of autologous cells for biological therapies, we have firstly examined the viability of the cells and the presence of 'progenitor markers' in vivo, before determining the growth characteristics and stem cell immunoprofile of clustered compared to single cells in vitro.

Methods

Patient samples

Following local research ethical committee approval and informed consent, surgical samples of human IVD tissue were obtained from a total of 95 patients, aged 41 ± 10 years, having routine surgery for IVD disorders (Table 1). Bone marrow aspirates or iliac crest bone chips were also received from four patients undergoing spinal fusion procedures. Sub-groups of these surgical samples were used in the studies described here. Samples were dissected macroscopically and used to assess cell viability, snap-frozen for morphological and immunohistochemical studies or digested for cell isolation and subsequent monolayer culture.

Cellular viability

Freshly excised IVD tissue [containing both nucleus pulposus (NP) and annulus fibrosus (AF) tissue, when present], obtained from 40 patients, was incubated in 25 μm chloromethyl fluorescein diacetate (CMFDA; Life Technologies, UK) and 2 μm ethidium homodimer-1 (EH; Life Technologies) in phosphate buffered saline (PBS; Life Technologies) for 2 h at 37 °C, blotted dry and snap-frozen [18]. Cryosections were collected onto poly-L-lysine-coated slides and mounted under coverslips using Vectashield containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Ltd, UK) to stain the cell nuclei for cell count verification. Cell viability was quantified by counting green CMFDA-positive viable cells (due to cleavage of the fluorophore by active esterases in live cells) and red EH-positive dead cells (the EH entering through the damaged membrane of dead or dying cells and binding to the nuclear DNA) using a Leica DMLB fluorescent microscope. At least 200 cells were counted from a minimum of five fields of view using a 40× objective. Cryosections were also stained with haematoxylin and eosin (H&E) for assessing morphology.

Table 1 Summary of patient demographics from whom IVDs were sourced

Age ± SD (range)	Sex	Spinal level	Pathology
41 ± 10 (14–72)	Female: 45 Male: 50	C3/4: 1 C5/6: 1 L3/4: 2 L4/5: 44 L5/S1: 47	DDD: 12 Herniation (protrusion): 32 Herniation (extrusion): 33 Herniation (sequestration): 12 Spondylolisthesis: 6

C cervical, *L* lumbar, *DDD* degenerative disc disease

Stem cell markers: immunohistochemistry

Immunohistochemistry for putative markers of progenitor cells [Notch-1 (Developmental Studies Hybridoma Bank, USA) and chondroitin sulphate (CS) epitopes, 3B3(−), 4C3, 6C3 and 7D4 [13, 19], as well as notochordal cell markers (cytokeratins 8 and 19; Acris Antibodies, Herford, Germany)] [20], was performed as previously described [21] using IVD samples (NP tissue) obtained from 37 patients. Cryosections were incubated overnight at 4 °C with the primary antibody (Table 2). For negative controls, the primary antibody was replaced with isotype-matched immunoglobulins (Dako UK Ltd, UK). After incubating with a biotinylated secondary antibody, endogenous peroxidase was blocked by incubation with 0.3 % (v/v) hydrogen peroxide (VWR International, UK) in methanol for 30 min. Labelling was amplified by incubating with an avidin biotin complex (Vectastain Elite kit, Vector Laboratories, UK) for 30 min and visualised with 0.06 % (w/v)

Table 2 Antibodies used in immunohistochemical investigations

Antibody name (clone)	Epitope	Isotype (species)	Dilution in PBS	Reference
3B3(−)	'Native' chondroitin sulphate chain	IgM (mouse)	1:500	[54, 55]
7D4	'Native' chondroitin sulphate chain	IgM (mouse)	1:50	[55]
4C3	'Native' chondroitin sulphate chain	IgM (mouse)	1:20	[55]
6C3	'Native' chondroitin sulphate chain	IgM (mouse)	1:10	[55]
Notch-1	Notch family cell membrane receptor	IgG (rat)	1:4	[19]
CK8 (C-43)	Cytokeratin 8	IgG (mouse)	1:200	[20]
CK19 (KS19.2)	Cytokeratin 19	IgG (mouse)	1:10	[42]

diaminobenzidine tetrachloride (DAB, Sigma-Aldrich, UK). The intensity of DAB staining in cellular, pericellular, territorial matrix and inter-territorial matrices within the tissue sections (Fig. 1e) was evaluated semi-quantitatively by scoring each region as 0–7, ranging from none to very strong staining; in addition, the percentage of positively stained cells was assessed, as before.

Cell culture

Samples of NP and AF tissue obtained from six patients (and NP alone from a further four patients) were finely minced and digested for 16 h at 37 °C with 0.8 mg/ml collagenase type XI (Sigma-Aldrich, UK) in DMEM/F12 (Life Technologies) before passing sequentially through cell strainers of pore sizes 70 and 40 μm (BD Biosciences, UK), forming three fractions of <40, 40–70 and >70 μm size. This digestion protocol was established as the optimum for differentially isolating single and clustered cell populations. Those cells existing within clusters following isolation could not be dissociated either with further enzymatic digestion (including with trypsin, collagenase XI and collagenase I), or with mechanical agitation (extensive vortex mixing or rapid pipetting). This suggests that the isolated single and clustered populations, following collagenase XI digestion, used routinely in this study, reflects how they would occur in vivo. Cytospins (Shandon, UK) of the freshly isolated cell populations were also generated to monitor the cell populations, via H&E staining. Cell size was determined using Nikon NIS-Elements image analysis software. For monolayer culture, cells were seeded at 5×10^3 cells/cm^3. At 80 % confluence, cells were passaged, counted [using the trypan blue (Sigma) exclusion method] and re-seeded. Population doublings (PD) were calculated as previously reported [19]. Culture was continued to passage (P) 5 and maintained as previously described [22]. Bone marrow aspirates or iliac crest bone washouts were layered over Lymphoprep (Fresenius Kabi AG, Germany) and centrifuged at 900*g* for 20 min. A 'buffy coat' layer of mononuclear cells, separated by density gradient, was harvested and seeded at 2×10^7 cells per 75 cm^2 culture flask and maintained as previously described [23].

Senescence

Cultured IVD cells from eight of the patient samples were assessed for senescence associated-β-galactosidase (SA-β-Gal) at the later passages, as previously described [24]. Senescent cells were determined as those containing positively stained lysosomes at pH 6, in comparison to pH 4 where all cell lysosomes should be positive. Percentage cell positivity was calculated as before, counting at least 200 cells.

Stem cell markers: flow cytometry

Flow cytometry was performed to determine the presence of CD markers, described as part of the minimal criteria for defining mesenchymal stem cells (MSCs) by the International Society for Cellular Therapy (ISCT) [25] [i.e. positive for CD73, CD90 and CD105 (BD Biosciences) and negative for CD14, CD19, CD31, CD34, CD45 and HLA-DR (Immunotools, Germany)]. Both clustered and single cell populations cultured to varying passages between 1 and 5 were examined and compared to bone marrow-derived MSCs, as previously reported [26]. Cell populations were isolated from NP tissue obtained from five patients and bone marrow MSCs from three of these patients for this analysis. Immunoreactivity was assessed using the BD FACScan flow cytometer and analysed with Cell Quest software (BD Biosciences), where staining was expressed as the percentage of the cells stained positively in comparison to the isotype-matched immunoglobulin negative control.

Statistical analysis

Data was analysed using Microsoft Excel and Analyse-It (version 2.21; Analyse-It Software Ltd, UK) add-on software. Where data were normally distributed, differences were calculated using a Student's *t* test or one-way

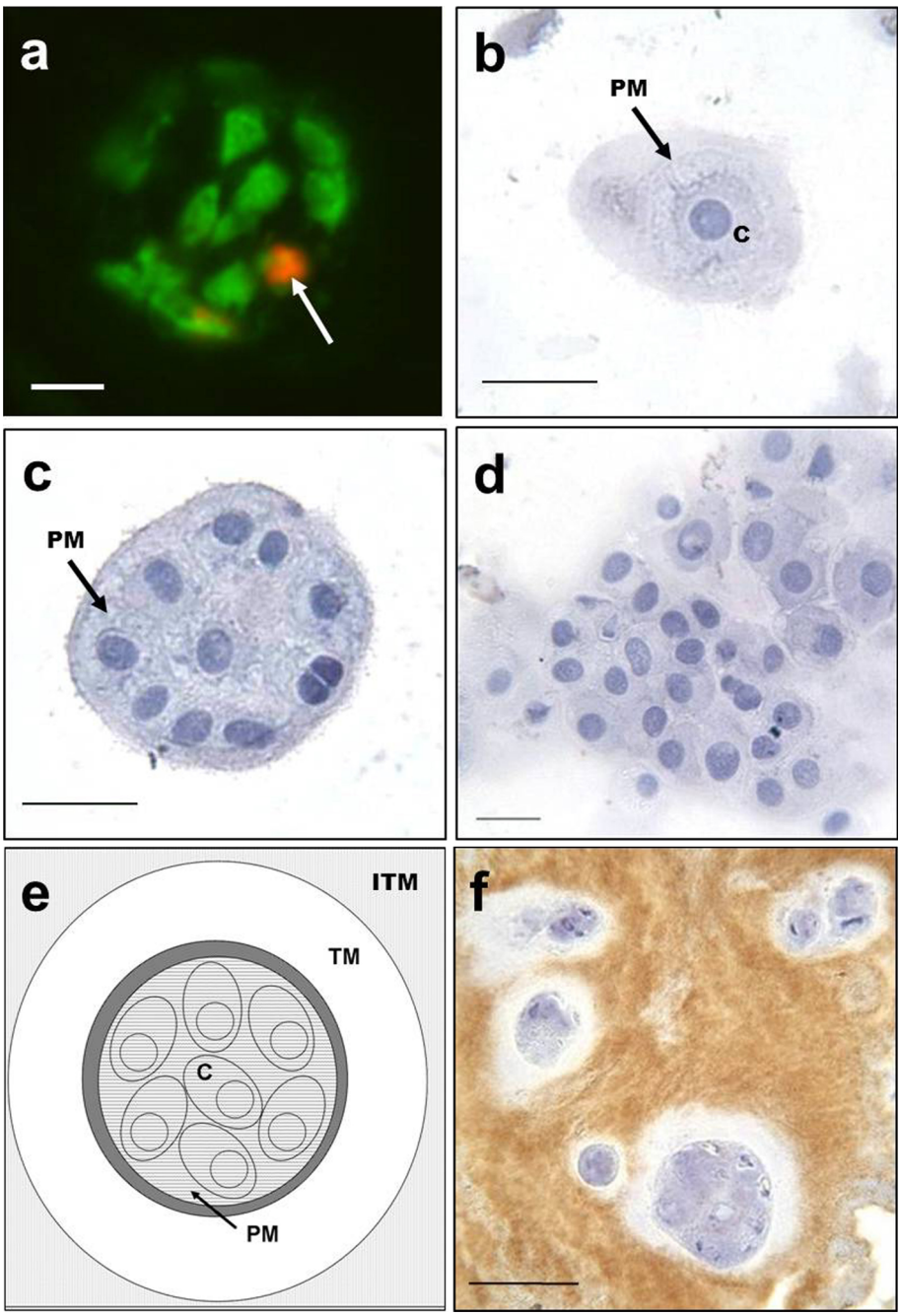

Fig. 1 Intervertebral disc cells in tissue sections and following isolation. **a** Clustered and single cell populations in a cryosection of IVD (42 year old, L4/5 IVD with protrusion) contained both live cells (*green fluorochrome*) and dead cells (*red fluorochrome*, *white arrow*). Sequential straining of enzyme-digested IVD tissue resulted in the isolation of three cell fractions: single cells <40 μm in size (**b**), small clusters 40–70 μm in size (**c**) and large clusters >70 μm in size (**d**). The cells (*C*), together with the pericellular matrix (*PM*, as seen in **b**, **c**) constitute a chondron. **e** Schematic demonstrating the different regions of IVD cryosections scored for immunohistochemical staining intensity: cellular (*C*), pericellular matrix (*PM*), territorial matrix (*TM*) and inter-territorial matrix (*ITM*); **f** Staining intensity often varied within the cryosections, particularly around the cell clusters (cryosection of 47 year old L4/5 IVD with spondylolisthesis). Cells are stained with Mayer's haematoxylin and *scale bars* 20 μm

ANOVA with Bonferroni post hoc test. Non-normally distributed data was assessed using a Mann–Whitney test or Kruskal–Wallis. Significance was taken as $p < 0.05$.

Results

Cell viability and patient demographics

Cell viability in IVD tissue was determined as 68.9 ± 14.6 % (range 30.3–100 %). Whilst EH-bound cell nuclei were found in both single and clustered cells throughout the tissue sections (Fig. 1a), they were most frequent at the tissue periphery. More clustered cells were viable than single cells in the excised samples (73.5 ± 24.4 vs. 65.9 ± 16.9 %, respectively; Mann–Whitney test, $p = 0.001$), but patient age or gender had no effect on this property or any of the following studies. Analysis of H&E stained sections showed that 50 ± 20.6 % (range 5–95 %) of cells were within clusters and there were between 3 and 30 cells in each cluster (mean 6 ± 4.5). Both clustered and single cells could often be seen to sit within a clear region

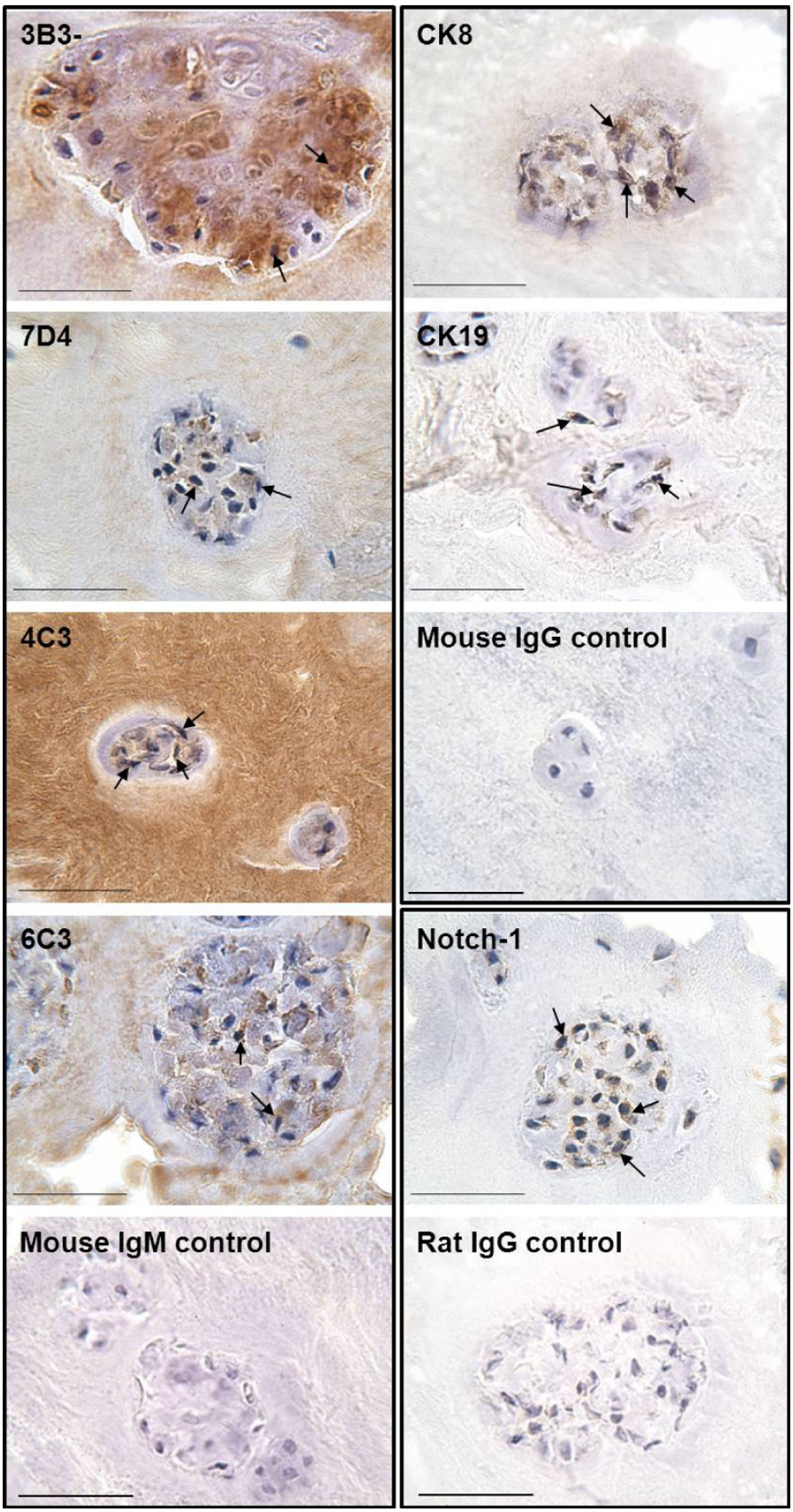

Fig. 2 Immunohistochemical staining of surgical IVD samples, for chondroitin sulphate epitopes [3B3(−), 7D4, 4C3 and 6C3], Notch-1 and cytokeratins 8 and 19. Staining patterns varied throughout the inter-territorial matrices and also in and around cell clusters and single cells (*black arrows*). Frequently paler intensity staining was seen around the cell clusters (e.g. 4C3). Isotype-matched negative controls are also shown. Cell nuclei are counterstained with Mayer's haematoxylin and *scale bars* 50 μm

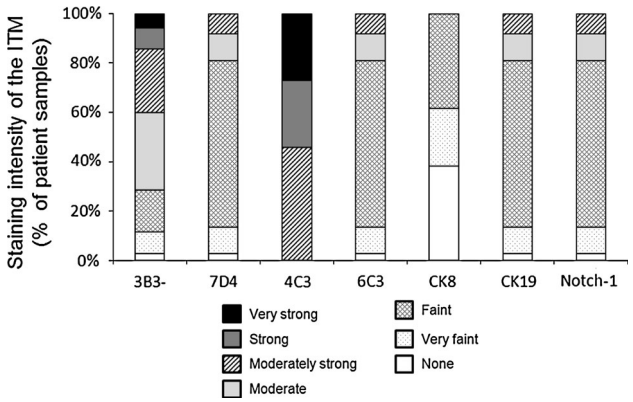

Fig. 3 Intensity and frequency of immunohistochemical staining of the inter-territorial matrix (*ITM*) for all antibodies used. Staining intensity was scored from none to very strong. The IVD tissue sections stained with the CS epitope, 4C3, all showed at least moderately strong staining in the ITM, whereas the other epitopes resulted in mostly faint ITM staining

Table 3 Positive cellular immunohistochemical staining for clustered and single cells

Epitope	Percentage of immunopositive cells Mean ± SD (range)	
	Clustered cells	Single cells
3B3(−)	32 ± 13.1 (8.8–56.7)	31.4 ± 12.6 (1.9–57.7)
7D4	19.1 ± 18.3 (0–81.3)	18.9 ± 11.4 (0–40)
4C3	35.9 ± 18.4 (5.2–71.9)	38.7 ± 17.4 (3.6–86.2)
6C3	31.2 ± 18.2 (0–66.8)	31.5 ± 11.7 (8.2–55.6)
CK8	20 ± 4.2 (0–78.8)	15.4 ± 18.5 (0–62.1)
CK19	19.1 ± 21.2 (0–72.3)	15.3 ± 16.2 (0–63)
Notch-1	10.6 ± 19.8 (0–80.1)	10.6 ± 19.5 (0–95.8)

No significant difference between clustered and single cell positivity was seen for any of the epitopes examined, when tested with the Student's *t* test or Mann–Whitney *U* test, as appropriate

of pericellular matrix or capsule, forming a chondron (Fig. 1b, c).

Stem cell markers: immunohistochemistry

Immunostaining for the putative stem cell markers within the IVD tissue varied between patient samples, in both location and intensity of staining and between epitopes investigated (Fig. 2). Staining was generally widespread, but no individual regions or populations had a consistent staining pattern. The territorial matrices of cell clusters were often fainter in intensity than the surrounding inter-territorial matrices, suggesting a difference in matrix composition. With the exception of 3B3(−) and 4C3 which showed strong staining, the inter-territorial matrices showed little to no staining across the patient samples (Fig. 3). There was no significant difference between the percentage of positively stained cells in the clustered and single cell populations (Table 3). Within cell clusters, staining for the CS epitopes was often variable and both positive and negative cells could be seen within any one cluster. However, the staining seen for the Notch-1 and CK epitopes was less varied, with cells within a particular cluster generally being entirely positive or entirely negative (Fig. 2).

Clustered and single cell culture

When grown in monolayer culture, the cells from within the clusters migrated out and formed a confluent monolayer by the end of P0, which appeared indistinguishable from the cultures of isolated single cell populations. As both the >70 μm and the 40–70 μm fractions contained clustered cells at isolation (as seen in the cytospins; Fig. 1b–d) and there was consistently no difference seen between the growth kinetics, the results of these two groups were combined. Therefore, analyses presented here refer to the <40 μm population (single cells) and the >40 μm population. At the point of initial isolation, the cells existing within clusters had a significantly smaller diameter than the single cells (14.7 ± 3.9 vs. 19.7 ± 7.1 μm diameter, respectively; Mann–Whitney test, $p < 0.001$). The most notable PDs were seen at P0–1, where the clustered cells underwent significantly more PDs than the single cells (3.3 and 1.1 PDs, respectively; Mann–Whitney test, $p = 0.0002$; Fig. 4a). Between passages 1 and 5, PDs were fairly consistent, regardless of cell population or passage number. For each passage, clustered cells consistently took a longer time to reach 80 % confluence than single cells. This difference was significant at P0–1 and P3–4 (Mann–Whitney test, $p = 0.005$; Fig. 4b). Time taken to reach confluence decreased with increasing passage number of the clustered cell population (Pearson's correlation; $r = -0.89$, $p = 0.045$), but not for single cells. Lysosomal β-galactosidase staining within the cell cytoplasm (at pH 4) was seen in 97.7 ± 3.4 % of cells. At pH 6, SA-β-Gal staining was seen in both clustered and single cell populations. At passage 4, 15.4 ± 14.9 and 19.8 ± 7.8 % of clustered and single cells stained, respectively, whilst at passage 5, the number of cells staining increased to 20.4 ± 11.2 and 23.9 ± 11.4 of clustered and single cells, respectively. Although the percentage of SA-β-Gal positive cells was greater in the single than clustered cell population, this difference was not significant.

Stem cell markers: flow cytometry

Clustered and single IVD cell populations showed similar results for the cell surface markers suggested to indicate

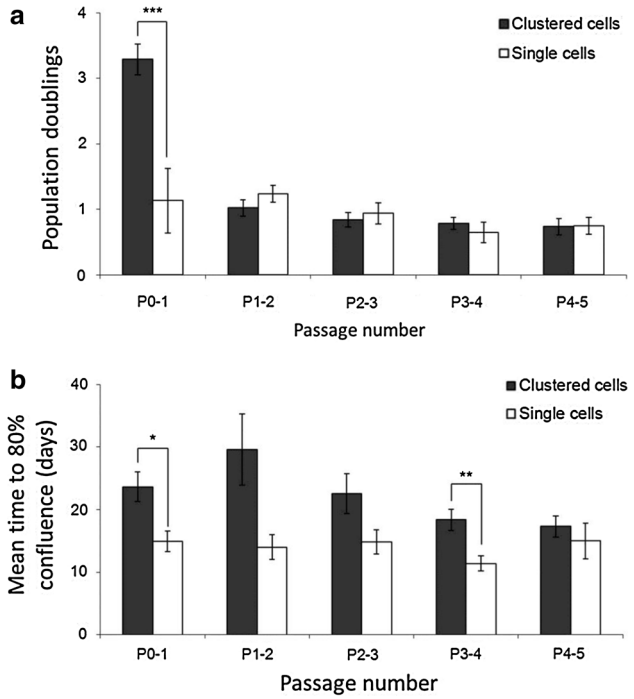

Fig. 4 Growth kinetics of clustered and single cell populations in monolayer culture. **a** Freshly isolated cell clusters underwent a significantly greater number of population doublings than the single cells (Mann–Whitney test, $p = 0.005$). After the first passage, however, there was no difference in the number of population doublings for single or clustered cell populations at subsequent passages. **b** Cells isolated from clusters consistently took longer to reach confluence than cells existing singly; at P0–1 and P3–4, this difference was significant ($p = 0.02$ and 0.007, respectively; $n = 44$ cell populations). Significant differences are indicated as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$

stem cells by the ISCT [25] (Fig. 5). However, the populations did not always fulfil the ≥95 and ≤2 % positivity or negativity, respectively, as specified but were generally >80 and <10 %, respectively, apart from CD14. The IVD cells showed unexpectedly high percentages of cells that were positive for CD14 (up to 66.5 %). There was no correlation between the passage number of the IVD cells and the percentage of expression of any of the cell surface markers analysed. Like the IVD cells, cultured MSCs fulfilled the general pattern of expression, except for cells demonstrating higher CD14 than expected (up to 50.9 % of the population).

Discussion

It has long been known that IVD cells can exist within the matrix of the degenerate human NP either singly or as groups or clusters of cells [27]. However, to our knowledge, this study is the first time that they have been isolated and studied in vitro as two separate populations. Indeed, in some reports, the clustered cell population has been discarded and therefore entirely excluded from study [28–30].

We have shown that the clustered and single cell populations were remarkably similar, both in vitro (apart from the period initially following isolation) and in vivo. The more PDs and longer time taken to reach confluence during the first passage of clustered cells, in comparison to single cells could be due to the time taken for the cells to migrate out of the clusters. Not only may the cells have to overcome contact inhibition of neighbouring cells within the cluster but also have to break down the pericellular capsule if present (Fig. 1c).

Senescent (SA-β-Gal positive) cells were seen equally in both clustered and single cell populations in vitro. This is contradictory to the previous findings of cells within sections of human IVD, i.e. in vivo [10], where the cell clusters contained significantly more senescent cells than those existing singly. This may be due to the isolation and cell culture processes in the present study resulting in the selection of a proliferative cell population with the clustered and single cell populations becoming more similar following time in monolayer culture and therefore showing no difference in SA-β-Gal positivity. A further explanation would be if the senescent disc cells within the clusters isolated from the disc tissue behave as senescent osteoblasts have been reported to, being less adherent in culture with a lower attachment frequency [31].

The environment of a stem cell niche acts to keep stem cells in a quiescent state until required for tissue regeneration or repair [32]. The presence of a stem cell niche in the IVD has been identified by the occurrence of a panel of markers including Stro-1, Jagged-1 and Notch-1 [33–35]. Notch-1 is transiently expressed by cells and is involved in both induction and inhibition of proliferation as well as cell fate in stem cell niches [11, 16]. It is also found in a region of known progenitor cells in articular cartilage [13]. Positive Notch-1 immunofluorescence staining in IVD cells, reported by Brisby et al. [15], in a small sub-population of the IVD cells, is in agreement with our findings in this study, suggesting a sub-population enriched in stem cells in both clustered and single cells.

Recently, a strong co-localisation of Notch-1 with the CS epitope, 7D4, has been reported in this IVD stem cell niche region [36]. 7D4, together with the other CS epitopes used in this study [3B3(−), 4C3 and 6C3], have been reported in both IVD tissue [14, 37] and developing, injured or repairing articular cartilage [38–40]. The presence of these epitopes in the degenerate IVD samples may be a repair response recapitulating developmental pathways, as is seen in repairing cartilage [39].

Our findings of positive staining for the cytokeratins, CK8 and CK19, which are commonly found in notochordal cells [41] [20, 42], within all or no cells of clusters are

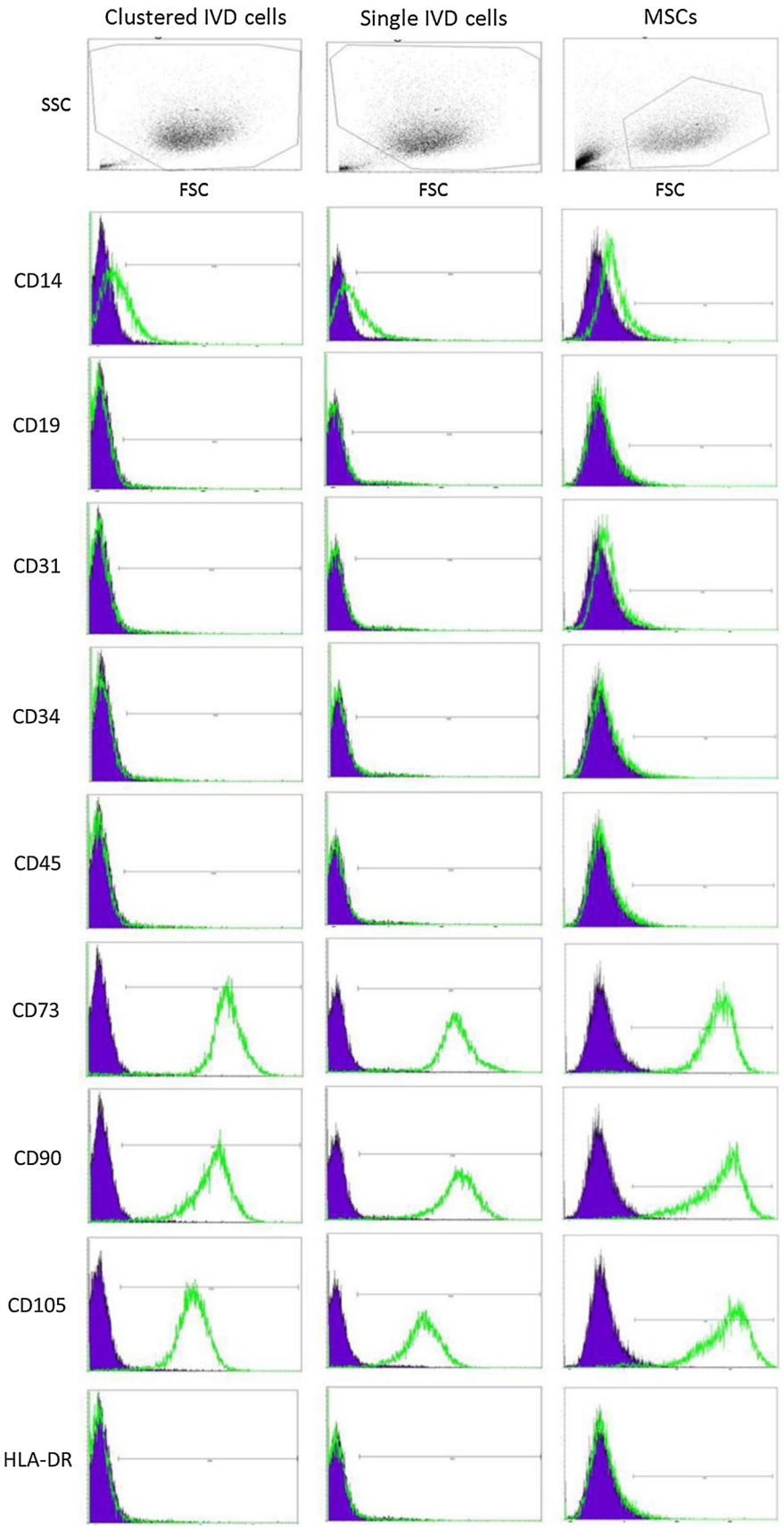

Fig. 5 Representative flow cytometry histograms of clustered and single IVD cell populations and bone marrow MSCs using the profile to define an MSC as stated by the ISCT (i.e. positive for CD73, 90 and 105 but negative for the others shown). The *dot plot* represents forward scatter (*FSC*) vs. side scatter (*SSC*), whilst the *purple histograms* represent the isotype controls

similar to those described by Sun et al. [43] who found all cells within clusters were uniformly either positive or negative. These authors suggest the positive clusters may be autophagosomes formed from notochordal cells. We found positive cytokeratin staining in samples from a wider age range than reported by Weiler et al. [42], who found no positive staining for CK8 and CK19 in surgical samples from patients older than 47 years. However, since microfilaments such as cytokeratins have been reported in normal chondrocytes [44], perhaps they are not unique markers for notochordal cells.

As well as immunohistochemistry, flow cytometry was used to assess the presence of stem cell markers. Due to the cell number required for flow cytometry, this study was only possible on the cultured populations of single and clustered cells rather than freshly isolated cells. The culturing process possibly resulted in an altered marker expression from that seen in vivo. Nonetheless, both clustered and single cell populations, as well as bone marrow-derived MSCs largely exhibited the pattern of expression specified by the ISCT [25]. However, both the IVD cells and the MSCs showed a greater expression of CD14 than expected. The presence of CD14, generally thought of as a monocyte or macrophage marker [45], may be indicative of an inflammatory environment due to its role as a co-receptor for lipopolysaccharide as an immune response (reviewed by [46]). The positive expression of CD14 by the cells may also be indicative of the expression of an epitope that cross-reacts with the CD14 antibody, as reported by Pilz et al. [47] who stated that MSCs express a similar epitope.

Following conflicting reports on the viability of cells within the degenerate IVD [7, 48], we have found that the majority of cells within surgical samples are viable, a promising finding when considering this tissue as a source of cells for regenerative therapies. As described in a previous study [49], we noted the presence of dead cells, particularly at the tissue periphery in these surgical samples. This is most likely due to cellular trauma inflicted during the surgical excision, which has been shown to cause cell death in articular cartilage [50].

Whilst this study is aimed at assessing the suitability of autologous IVD cell as a source for cellular therapy, there are still many obstacles that need to be overcome before a treatment of disc degeneration can be facilitated with cells (reviewed in [51]). For example, it is still not known whether the regeneration of the degenerate IVD would result in the reduction of pain, nor whether the conditions within the degenerate IVD would physically and nutritionally support a population of cells. Until these problems are addressed more fully, cellular therapy will remain a specialist treatment.

In conclusion, both the clustered and single cell populations examined in human IVD showed good viability in vivo and contain a similar proportion of cells with stem cell or progenitor cell properties. These cells may resemble those of resident stem cell niches found in other tissues, such as the satellite stem cells in adult skeletal muscle (making up 2–7 % of muscle cells), which remain quiescent in health but proliferate following injury to induce regeneration (reviewed by [52]). As clustered disc cells proliferate more slowly in vitro, show increased senescence and stress proteins in vivo [10, 53] and appear to have no greater enrichment of progenitor or stem cells over single disc cells, they appear to offer little or no advantage or benefit for biological therapies than single cells isolated from the disc.

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Conflict of interest None.

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