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Age-related changes in human bone marrow-derived mesenchymal stem cells: Consequences for cell therapies

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Abstract

Human mesenchymal stem cells (hMSC) represent a promising cell-based therapy for a number of degenerative conditions. Understanding the effect of aging on hMSCs is crucial for autologous therapy development in older subject whom degenerative diseases typically afflict. Previous investigations into the effects of aging on hMSC have proved contradictory due to the relative narrow age ranges of subjects assessed and the exclusive reliance of in vitro assays. This study seeks to address this controversy by using a wider range of donor ages and by measuring indices of cellular aging as well as hMSC numbers ex vivo and proliferation rates.

CFU-f analysis and flow cytometry analysis using a $CD45^{low}/D7fib^{+ve}/LNGF^{+ve}$ gating strategy were employed. In addition a variety of markers of cellular aging, oxidative damage and senescence measured.

A reduction in CFU-f and $CD45^{low}/D7fib^{+ve}/LNGF^{+ve}$ cell numbers were noted in adulthood relative to childhood. Indices of aging including oxidative damage, ROS levels and p21 and p53 all increased suggesting a loss of MSC fitness with age.

These data suggest that hMSC numbers obtained by marrow aspiration decline with age. Furthermore, there is an age-related decline in overall BM MSC ''fitness'' which might lead to problems when using autologous aged MSC for cell-based therapies.

Keywords: Aging; Heat shock proteins; Human mesenchymal stem cells; Oxidative damage; Senescence

1. Introduction

The term mesenchymal stem cell (MSC) refers to a population of adult mesenchymal progenitor cells with the capability to produce progeny that differentiate into cells of various connective tissue lineages. MSC are thought to have at least five primary roles in vivo: as progenitor cells for bone formation during bone remodelling or repair (Blair et al., 2002), cartilage formation (Pittenger et al., 1999), vascular support (Hegner et al., 2005), haematopoietic support (Jang et al., 2006), and as progenitors for adipocytes (Pittenger et al., 1999).

Since MSCs appear to be endowed with a considerable regenerative capacity then it seems logical that numerical or functional deficiencies may contribute to the aging process and age-related diseases including osteoarthritis and osteoporosis. Whether the numbers of human MSC (hMSC) decline with age or not is still hotly disputed (Sethe et al., 2006) (Table 1), however MSC deficiencies, either in terms of number or cellular function, seem to be involved in musculoskeletal diseases (Bonyadi et al., 2003) and also a number of diseases remote from the musculoskeletal environment including atherosclerosis (Hegner et al., 2005) and diabetes (Kume et al., 2005). Also, with MSCs being increasingly suggested as possible autologous donors cells for cell-based therapies and tissue engineering (Safford et al., 2002), more information about age-related changes are essential for therapy development in older subjects. Initial studies in rats suggest that this may well be an issue with MSC from aged rats having a reduced capacity to generate a chondrogenic matrix in vitro (Zheng et al., 2007).

Previously reported age-related changes in mesenchymal stem cells include loss of differentiation potential, loss of

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Table 1
Age-related changes in mesenchymal stem cell numbers

CFU numbers	Age groups	Isolation	Medium	Counting technique	Reference
Decrease	Young: 0–18 years, old: 59–76 years	Ficoll gradient	DMEM 10% FCS	CFU-f count not described	Baxter et al. (2004)
Decrease	4–88 years in 10 year steps	Plastic adherence	Alpha-MEM 10% FCS	Colonies > 50 cells were counted as CFU-ALP	Nishida et al. (1999)
Decrease	13–83 years, no grouping (diseased patients)	Plastic adherence	Alpha-MEM 10% FCS	Colonies > 8 cells were counted as CFU-ALP	Muschler et al. (2001)
Decrease	8–80 years, no grouping (diseased patients)	Plastic adherence	Alpha-MEM 10% FCS	Colonies > 2 mm were counted as CFU-ALP	Majors et al. (1997)
Decrease	3–70 years no grouping (cadaver donors)	Ficoll gradient	Alpha-MEM 10% FBS	Colonies > 50 cells were counted as CFU-f	D'Ippolito et al. (1999)
No change	Young: 18–42 years, old: 66–78 years	Lymphoprep	MEM 10% FCS	No description of CFU-f definition	Justesen et al. (2002)
No change	14–48 years, no grouping	Plastic adherence	Alpha-MEM 10% FCS	Colonies were counted with an Anderman colony counter	Oreffo et al. (1998b)
No change	14–87 years, no grouping	Plastic adherence	Alpha-MEM 10% FCS	Colonies were counted with an Anderman colony counter	Oreffo et al. (1998a)
No change	Young: 22–44 years, aged: 66–74 years	Lymphoprep + stro-1 enrichment	MEM 10% FCS	Colonies were defined as 16 cells within a circular area	Stenderup et al. (2001)

proliferation potential, increases in senescent cell numbers and loss of in vivo bone formation (Sethe et al., 2006). These previous studies that have addressed the effects of aging on MSCs have provided conflicting results (Table 1).

It is equally important to investigate markers of cellular aging that might underly the above-mentioned changes in MSC number and differentiation potential. These include p53 and p21, reactive oxygen species (ROS), nitric oxide (NO), advanced glycation endproducts (AGE) and receptor for AGEs (RAGE), and lipofuscin; all of which have been found in elevated levels in aged cells and tissues and are associated with age-related degeneration (Medrano and Scrable, 2005; Rosso et al., 2006; Torella et al., 2004; Song et al., 1999; Renault et al., 2002; Stolzing et al., 2006). There is at present no term for the collective effects of these aging markers although they will lead to a generalised loss of cell functionality and will have profound physiological effects. We have therefore coined the term cell "fitness" to describe the progressive sub-lethal loss of function seen in aged cells after the accumulation of oxidative damage.

In this paper we have investigated human MSC from donors of various ages and determined their "fitness" by measuring various age and senescence markers used routinely to characterise the aging of somatic cells in relation to their differentiation capacity and functionality. We show an age-related reduction in CFU-f and $CD45^{low}/D7fib^{+ve}/LNGF^{+ve}$ cell numbers and a reduced capacity for proliferation and differentiation. In addition indices of cellular aging including oxidative damage, ROS levels and p21 and p53 all increased suggesting a progressive loss of MSC fitness with age, leading to a reduction in MSC numbers and differentiation capacity.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Sigma–Aldrich (Dorset, UK) unless stated otherwise.

2.2. Bone marrow processing

Bone marrow (BM) aspirates were obtained from the posterior iliac crest of 33 normal donors (aged 5–55) following written consent from the participant. In case of juveniles, an assent was sought from all children able to comprehend the nature of the project and written consent was obtained from their parents/legal guardians. The protocol was approved by Leeds Teaching Hospitals Research Ethics committee. 24 additional frozen normal BM MNCs samples (donors aged 19–39) were purchased from Biowhittaker (Wokingham, UK or from AllCell, Emeryville, USA). Following separation with Lymphoprep (Nycomed, Oslo, Norway) BM mononuclear cells (MNCs) were collected and cryo-preserved in liquid nitrogen. Comparison of three samples from fresh and frozen material was performed using the CFU-f test.

2.3. Primary MSC culture

For primary culture, 5×10^6 BM mononuclear cells (MNCs) were plated in 25 cm^2 flasks (SLS, Nottingham, UK) in DMEM/10% FCS, supplemented with antibiotics. Next day media was changed and growing cultures of adherent cells were fed twice weekly with 1/2 media changes. Initial confluent cultures were designated "passage 0" (P0) and subsequent passaging was performed as 1:2 splits in 25 cm^2 flasks. Cells were kept non-confluent at all times.

2.4. Immunophenotyping of passaged MSC

Early-passage (passages 1–5) MSC were harvested by trypsinisation and the cells were stained with antibodies against CD10 (1:10), CD13 (2 μg/ml), CD31 (1:10), CD34 (20 μg/ml), CD44 (0.5 μg/ml), CD45 (100 μg/ml), CD90 (10 μg/ml), CD105 (200 μg/ml), CD117 (20 μg/ml), CD133 (5 μg/ml), Stro-1 (1:100 cell supernatant), D7fib (10 μg/ml), MHCII (20 μg/ml) and incubated at (4 °C for 30 min followed by a staining with PE conjugated anti-mouse IgG or IgM (Stro-1) (1:100; 30 min; 4 °C). All antibodies were from Serotec (Oxford, UK) except CD34 which was from Santa Cruz (Calne, UK) and Stro-1 which was from Developmental Studies Hybridoma Bank (Iowa, USA). The cells were analysed using a Guava personal flow cytometry system (GUAVA Instruments, Hayward, USA).

2.5. Growth curve, apoptosis and cell morphology

MSC were serially subcultured under standard conditions. Briefly, the cells were cultured at 1×10^6 cells in T75 culture flasks in the above medium. When cells were 90% confluent they were trypsinised and replated into T75 culture flasks at the same starting density of 1×10^6 cells. Cultures were kept until the

cultures stopped becoming 90% confluent in 4 weeks. The number of population doublings (NPD) between subcultures was calculated according to the following equation:

$$\mathrm{NPD} = \log 10\left(\frac{N}{N_0}\right) \times 3.33$$

where N is the no. of cells at the end of growth period and N_0 the no. of cells seeded.

In addition, changes in cell size were monitored by flow cytometry in early-passage cells (passages 1–5) using forward scatter as an index of cell size. Aliquots of these cells were sub-cultivated at 20,000 cells per well in 24 well plates and grown for 3 days and apoptosis was measured in early-passage cells by flow cytometric analysis of annexin V expression. MSC were incubated with anti-annexin V-PE antibody (Abcam, 1:1000, Cambridge, UK) for 10 min at RT and then analysed in a personal flow cytometry system (Guava Instruments, Hayward, USA).

2.6. Colony-forming unit-fibroblastic assay

The colony-forming unit-fibroblast (CFU-f) assay was used for MSC enumeration in uncultured bone marrow samples. It was performed in triplicate wells of six well plates (SLS, Nottingham, UK) at a cell seeding density of $5 \times 10^4/\mathrm{cm}^2$. The media consisted of DMEM, 100 U/ml penicillin, 100 g/ml streptomycin (all from Invitrogen, Paisley, UK) and 10% pre-selected FCS (Stem Cell Technologies, Vancouver, Canada). Cultures were fed twice weekly and colonies of more than 30 cells were scored on day 14.

2.7. MSC enumeration by multiparameter flow cytometry

Uncultured BM MSCs were first positively enriched using anti-fibroblast (D7-FIB-conjugated) microbeads. $CD45^{low}D7\text{-}FIB^{+}LNGFR^{+}$ cells were next quantified in the enriched fraction using CD45-FITC (DAKO, Ely, UK) and LNGFR-PE (BD Bioscience, Oxford, UK) in combination with propidium iodide (PI). The data were collected and analysed using Becton Dickinson FACScan equipped with CellQuest software. The final frequency of $CD45^{low}D7\text{-}FIB^{+}LNGFR^{+}$ cells was calculated as their proportion in relation to total recovered cells, as previously described (Jones et al., 2006).

2.8. Effect of age on differentiation potential

2.8.1. In vitro osteogenesis

MSC were subcultured for quantitative ALP expression. 20,000 cells were seeded in 24-well plates and incubated in bone-inducing medium (containing 10^{-8} M dexamethasone and 50 μg/ml ascorbate) for 10 days. ALP quantitation was performed using a colorimetric assay using ρ-NPP as substrate (Dennis and Caplan, 1996). The cells were washed, and cell number was determined using the crystal violet assay. Specific ALP activity was quantified against a standard curve of 0–200 nM ρ-NP and expressed as nmol ρ-NP per minute per 10^4 cells.

2.8.2. In vitro chondrogenesis

For chondrogenic differentiation, up to 5 replicate cultures of 2.5×10^5 cells were cultured in Eppendorf tubes in 0.5 ml of chondrogenic media for 21 days (Mackay et al., 1998). Sulfated glycosaminoglycan (GAG) was visualized on frozen sections (5 mm thick) with 1% toluidine blue. Production of sulphated GAG was measured in an Alcian blue binding assay (Immunodiagnostic Systems, Boldon, UK) following digestion in 100 ml of papain solution (Murphy et al., 2002). Absorbance was read at 630 nm.

2.9. Senescence analysis

The intracellular senescence marker p21 and p53 were measured using flow cytometry in early-passage cells (three different passage levels from three different patients; passages 1–5; young = 2.5, 9, 9; adult = 3 patients aged 18; aged = 50, 50, 55). MSC pellets were incubated with an anti-$p21^{waf1}$ (diluted 1:100; 4 °C; 30 min; Serotec, Oxford, UK) and anti-p53 antibody (diluted 1:100; 4 °C; 30 min; Serotec, Oxford, UK) after fixation in 4% paraformaldeheyde and permeabilized followed by incubation with a secondary antibody labelled with PE (Serotec, Oxford, UK) for 30 min at 4 °C. The cells were analysed in a personalised flow cytometry system (Guava Instruments, Hayward, USA).

2.10. Analysis of the stress levels in MSC

Viability was measured as the incorporation of 7-AAD using a commercial reagent from Guava International. Cells (three different passage levels of three different samples; passages 1–5; years of patient see above) were harvested, washed and re-suspended in a 1:3 dilution in the viacount reagent (Guava Instruments, Hayward, USA). Analyses were performed in a guava personal flow cytometry system. Nitrite concentration was measured using standard Griess reagent. To determine ROS levels, cells were incubated with H_2DCF-DA (5 μM) for 30 min, washed and analysed in a personal flow cytometry system (Guava Instruments, Hayward, USA). Superoxide dismutase (SOD) activity was evaluated using a commercially available kit (OxisResearch, Portland, USA) according to the manufacturer's instructions. Levels of advanced glycation endproducts (AGE) (1:100, Abcam, Cambridge, UK), carbonyls (1:100 Sigma–Aldrich, Dorset, UK) and the receptor for AGEs (RAGE) (1:100, Abcam, Cambridge, UK) were measured using flow cytometry. MSC were incubated with antibody against the HSP27 (1:10, Calbiochem, Nottingham, UK), HSP60 (1:100, Calbiochem, Nottingham, UK), HSP70 (1:100, Calbiochem, Nottingham, UK) and HSP90 (1:10, Calbiochem, Nottingham, UK) (37 °C, 30 min), followed by incubation with a PE labelled secondary antibody (1:100; 37 °C; 30 min). For all measurement cells were fixed in 4% paraformaldehyde and permeabilized. The cells were analysed in a personalised flow cytometry system (Guava Instruments, Hayward, USA).

2.11. Receptors involved in osteoblastic differentiation

MSC from passages 1–5 (three different passages of three different patients; years of patients see above) were incubated with antibody against the vitamin D receptor (VDR) (1:10, Abcam, Cambridge, UK), notch-1 receptor (Upstate, NY, USA) and the glucocorticoid receptor (GR) (1:50, Oncogene Research Products, London, UK) at 4 °C for 30 min, followed by incubation with a PE labelled secondary antibody 1:100, Abcam, Oxford, UK) at 4 °C for 30 min in fixed and permeabilized cells. The cells were analysed in a personalised flow cytometry system (Guava Instruments, Hayward, USA).

2.12. MSC stress resistance

MSC and MSC-derived osteoblast-like cells were incubated for 1 h with 0.1 mM H_2O_2 and levels of apoptotic cells were measured as described earlier. In addition "young", "adult" or "aged" MSC (see groups as described in the last section) were subcultured for quantitative ALP expression after H_2O_2 incubation. 20,000 cells were seeded in 24-well plates and incubated in bone-inducing medium (containing 10^{-8} M dexamethasone and 50 μg/ml Vitamin C) for 10 days. ALP quantitation was performed as described above.

2.13. Data analysis

Frequencies of CFU-f and CD45lowD7-FIB + LNGFR + cells between different donor groups were compared using a non-parametric Mann–Whitney test. Pearson test was used to test for age-dependency using SPSS v11 software. Comparison of all other results was performed by one-way analysis of variance (ANOVA) and multiple comparisons made using Tukey's test using the Sigmastat software and values were expressed as mean ± S.D. Data was considered significantly different when $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3. Results

MSC analysed for qualitative changes derived from donors in the age range from 7 to 18 years will be referred to as "young" MSC, for the age range 19–40 years "adult" MSC and for cells from over 40 years old donors will be named

''aged'' MSC unless otherwise stated. These groupings were chosen as they best represent the different stages of bone/body maturity or age. Up to the age of around 18 years bone still grows (Whiting et al., 2004), after this some groups have reported a steady decline of CFU-f numbers and bone thickness till the age of 40 years (D'Ippolito et al., 1999), with no further stem cell loss after 40 years.

3.1. Effect of age on stem cell number

CFU-f numbers were compared using fresh and cryo-preserved material to control for freezing effects on CFU-f numbers. No changes were found (data not shown). CFU-f numbers in bone marrow determined directly after MNC isolation were found to decline with the age of the donor (Fig. 1a). Furthermore, when the population was sorted into two age groups (0–20 years and 21–40 years of age), a significant decline in CFU-f numbers was seen in the older age group (Fig. 1b). These data correlated well with the measurements of BM MSCs by multiparameter flow cytometry. In these experiments the frequency of CD45lowD7-FIB + LNGFR + -cells was also found to show an age-related decrease which paralleled that of the CFU-f levels (Fig. 1c and d).

To confirm that these changes were not due to changes in the expression markers used to identify MSC in bone marrow, we measured the mean intensity of the cell surface markers (LNGR, D7fib and CD45) on un-cultivated MSC. No significant changes in the mean expression intensity with the age of donors were found (Fig. 2b).

3.2. Effect of age on MSC marker phenotype

To confirm the identity of the growing cultures, we analysed early-passage cells by flow cytometry for a series of markers commonly used to identify mesenchymal stem cells. We also correlated the expression of these markers with the age range of our samples in order to identify new markers which have a stable expression during aging and could potentially be used to reliably isolate populations of MSC regardless of age.

The cells were found to be positive for CD13, CD44, CD90, CD105, Stro-1 and D7-Fib regardless of age. However, for many of these markers (CD44, CD90, CD105, and Stro-1), significant age-related changes in the expression levels were found.

3.3. Effect of age on the proliferation potential of human MSC

To determine changes in the proliferative potential of hMSC in culture we followed the expansion of hMSC from ''young'', ''adult'' and ''aged'' donors. After 4 months, the cultures attained between 17 and 35 PD cumulative population doublings (Fig. 2a). The initial phase of growth was similar in all cultures but after about 5 weeks of culture the first

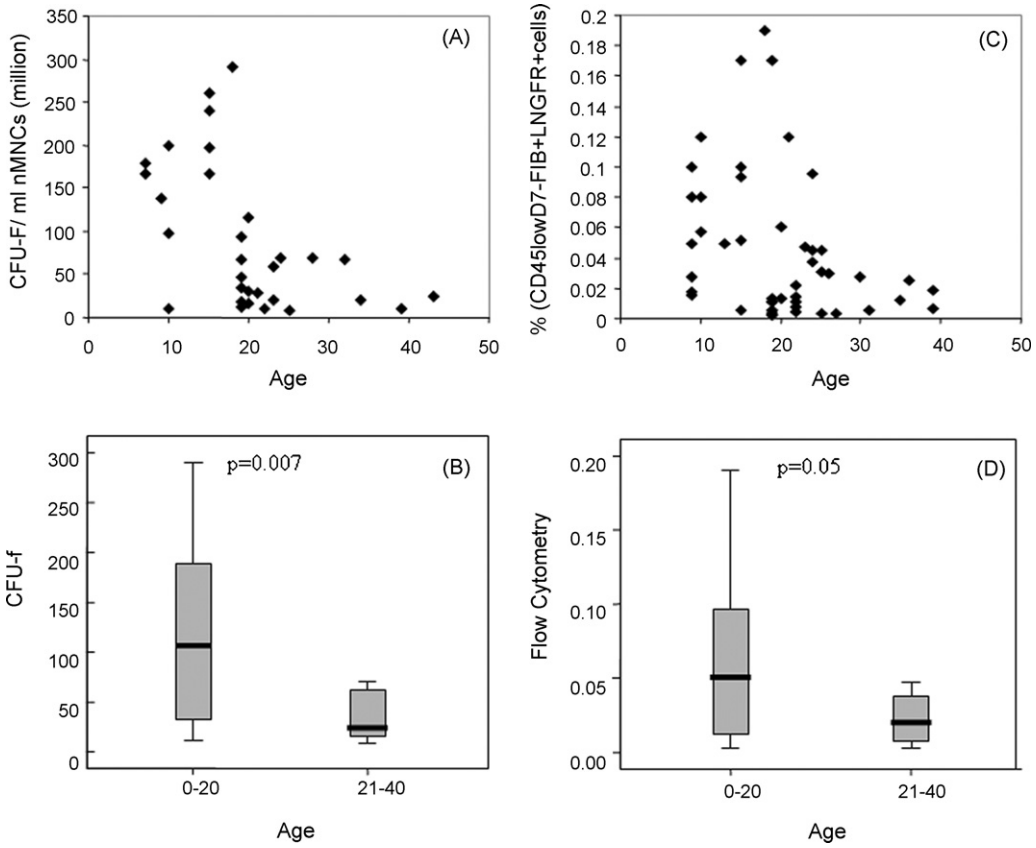

Fig. 1. CFU-f numbers in the bone marrow of healthy donors of different ages (A) and the same data grouped into two age groups (B). The frequency of MSCs identified as CD45lowD7-FIB + LNGFR+ cells from the bone marrow of healthy donors (C) and the same data grouped into two age groups (D).

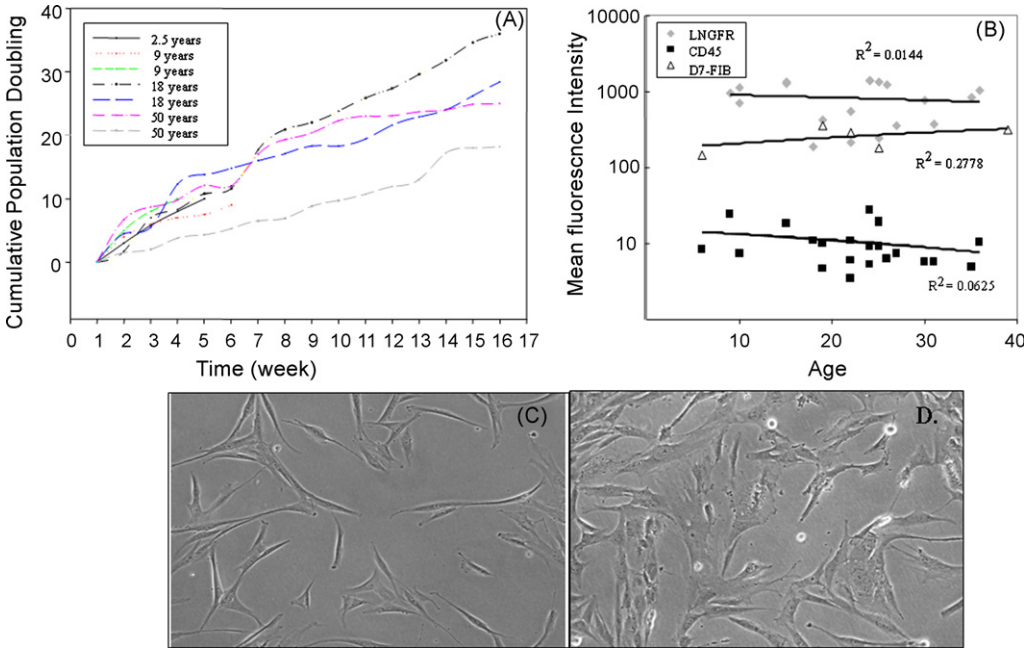

Fig. 2. Growth kinetics of hMSC of different ages. Cumulative population doublings is plotted against time in culture (A). Mean fluorescence intensity of the three used surface marker to isolate MSC from bone marrow in relation to the age of donor (B). Typical cultures of early-passage (passage 3; 18 year old donor) (C) MSC or late-passage MSC (passage 12; 18 year old donor) (D) taken with a 20× magnification using a light microscope.

age-related differences appeared. Proliferation in the "aged" MSC cultures began to decline and growth curve reached a plateau. In contrast, cultures derived from "adult" donors continued to proliferate throughout. The growth kinetics of "young" MSCs was not investigated beyond 10 PDs. During the initial passages, MSC of all ages had the typical spindle-type morphology (Digirolamo et al., 1999) whereas in the later passages, numbers of enlarged cells increased (Fig. 2C and D).

3.4. Effect of age on senescence and apoptosis induction

Cells size measured flow cytometrically as forward scatter was also increased with age (Table 3) and passage (data not shown). In addition we also saw an increase in β-galactosidase positive cells during cultivation of all groups (data not shown). To further validate if there is an age-related increase in senescent cells in "aged" donors we used two markers associated with the senescent phenotype of somatic cells; the tumor-supressor protein p53 and the cell-cycle regulation protein p21. Measurements confirmed that there was an age-related increase in the number of positive cells for both markers. p53 positive cells were significantly up-regulated in "aged" MSC compared to "young" and "adult" MSC and numbers of p21 positive cells were significantly up-regulated in "aged" MSC compared to "young" MSC (Fig. 3a). In addition we found that the mean expression levels of p53 in a sub-population of cells increased with age as seen in the scatter plots (Fig. 3b).

Another important biomarker of aging is the rate of apoptosis in cultures. "Adult" MSC showed no increase in their apoptosis rate compared to "young" MSC. Only "aged" MSC showed significantly increased levels of apoptosis compared to "young" and "adult" MSC (Fig. 3c).

3.5. Effect of donor age on in vitro differentiation potential

The differentiation potential of hMSC was evaluated by culturing the cells in high-density cultures in either osteogenic, chondrogenic or adipogenic medium. Levels of specific ALP activity declined progressively with age (Table 3). "Aged" MSC displayed significantly less ALP activity compared to both "young" and "adult" MSC and "adult" MSC were reduced by 40% compared to "young" MSC. Chondrogenic differentiation also declined in "aged" MSC compared to "young" and "adult" MSC although this did not reach significance (Table 3). Adipogenic differentiation did not change significantly with age (Table 3).

3.6. Effect of age on hMSC stress levels and age markers

ROS levels in "adult" MSC were not changed compared to "young" MSC, however "aged" MSC showed significantly higher ROS level compared to both other age groups (Fig. 4a). NO did increase progressively in MSC with age with "adult" and "aged" MSC producing approximately three- and sixfold more NO than "young" MSC, respectively (Fig. 4b). SOD activity declined significantly with age with "aged" MSC producing approximately 60% of the levels of "young" MSC (Fig. 4c). Levels of oxidised proteins (carbonyls), AGEs and lipofuscin content were measured in MSC as biomarkers of aging. All three markers were seen to significantly increase in both "adult" and "aged" MSC (Table 2). In addition, the receptor for the uptake of AGEs (RAGE) was significantly increased in "adult" and "aged" MSC compared to "young" MSC (Table 3).

We found a significant age-related decrease of mean HSP levels in "aged" or "adult" MSC when compared to "young"

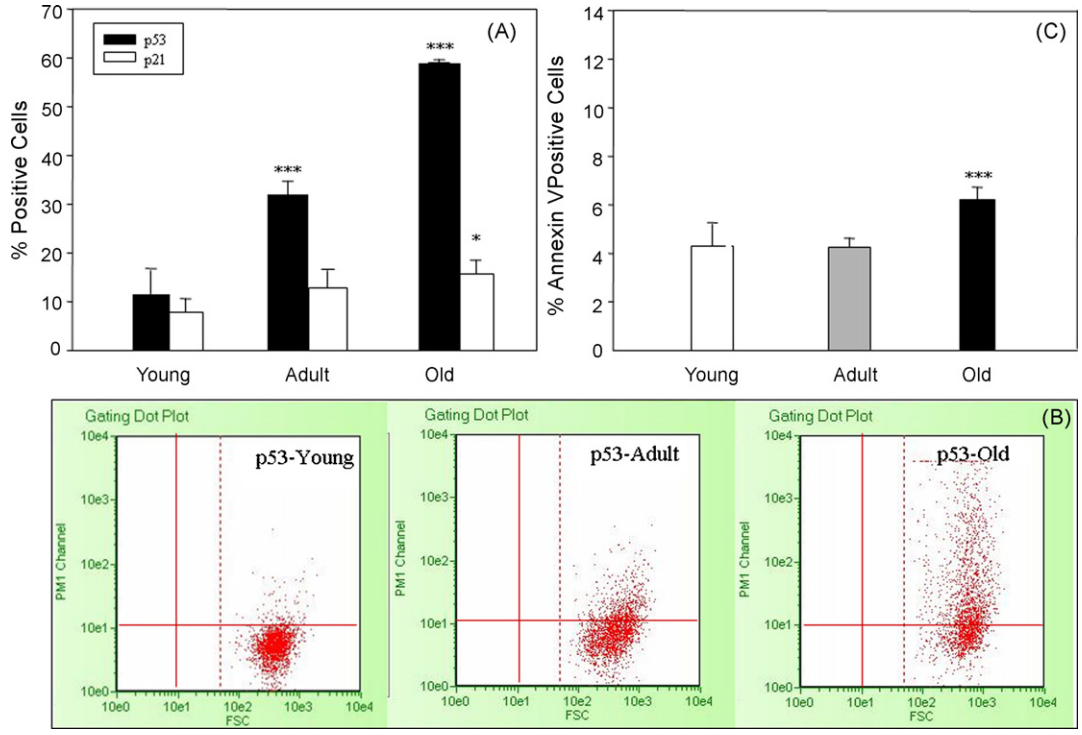

Fig. 3. Number of p21 and p53 positive MSC in early-passage MSC (passages 1–5) grouped according to the age of donors (A). Typical scatter plots of p53 levels in "young", "adult" and "aged" MSC measured by flow cytometry (B). Levels of apoptotic cells in cultures of "young", "adult" and "aged" MSC. $N = 3$ donors for each age group.

MSC (Fig. 5a). To study this further, MSC were aged in vitro and divided into three groups—non-senescent (PD 1–10), pre-senescent (PD 10–20) and senescent cultures (PD over 20). The number of cells positive for HSP expression increased in pre-senescent and senescent MSC compared to non-senescent cultures (Fig. 5b). However, it was found that although the percent positive cells increased, in the same cell cultures it was found that the mean expression levels of the HSP positive cells decreased suggesting an overall reduction in their ability to respond to stress (Fig. 5c).

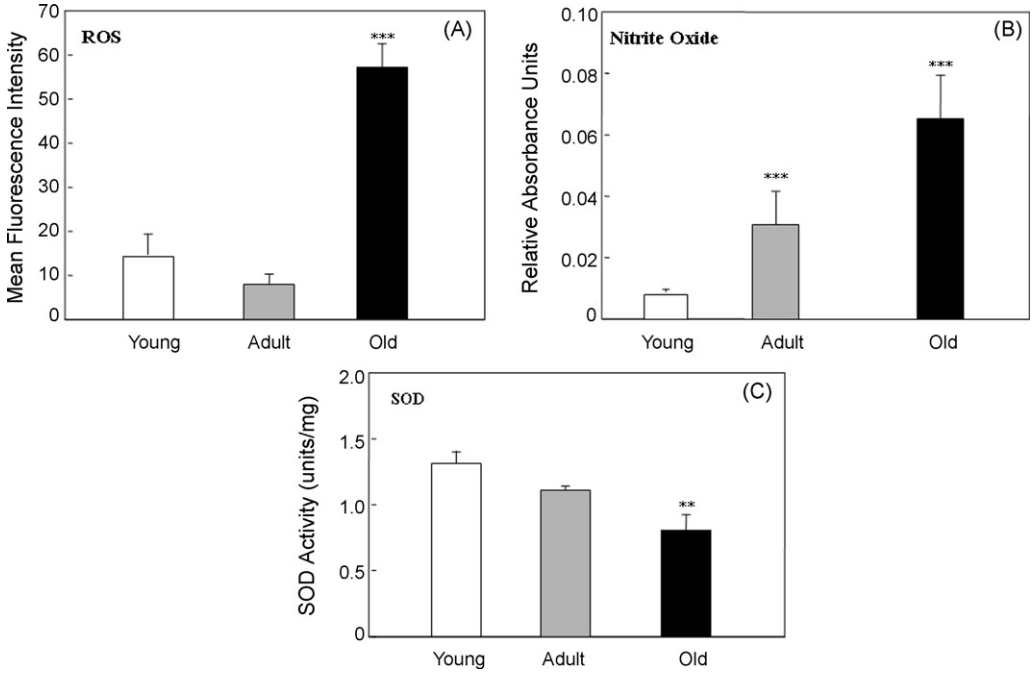

Fig. 4. Levels of reactive oxygen species (A), nitric oxide (B) and superoxide dismutase activity (C) was measured in early-passage MSC. $N = 3$ donors for each age group.

Table 2
Surface marker expression on early-passage hMSC. All values represent means ± S.D.s of samples from at least three donors

Marker	Percent Control		
	"Young" (7–18)	"Adult" (19–40)	"Aged" (>40)
CD10−	9.2 ± 0.9	8 ± 2.5	10.3 ± 3.1
CD13+	33.4 ± 9.2	40 ± 10	31.5 ± 2
CD31−	10 ± 5.6	6.2 ± 3.8	9.2 ± 4
CD34−	7.8 ± 0.1	6.5 ± 2.6	16.2 ± 2 **
CD44+	83.7 ± 7.6 **	61 ± 4	91.5 ± 2 ***
CD90+	85 ± 5.8 **	77 ± 4	50.7 ± 0.4 ***
CD105+	43 ± 0.3	29.5 ± 6 **	23 ± 0.1 **
CD133−	11.4 ± 2.8	5 ± 1.6	9.6 ± 0.6
MHC II−	11.7 ± 6.6	5 ± 1.9	2.4 ± 0.4
Stro-1+	88 ± 4.2	84 ± 6 **	57 ± 3 ***
Isotype control	7 ± 2	6.3 ± 0.9	7.4 ± 2.2

To see how these changes in the stress response system of MSC modulates their ability to cope with oxidative stress and capacity for osteoblastic differentiation after stress exposure, we measured the levels of apoptosis and alkaline phosphatase activity after treatment with 1 mM H_2O_2. "Aged" MSC showed a 10-fold increase in apoptosis after H_2O_2 treatment compared to "young" and "adult" MSC or MSC grown in osteogenic medium (Fig. 6a). The ability of these stressed cells to differentiate under osteogenic conditions was significantly decreased in all groups. Alkaline phosphatase activity after hydrogen peroxide treatment was reduced to about half the control levels in the "young" MSC, a fourth in "adult" MSC and a third in the aged MSC (Fig. 6b).

3.7. Effect of age on receptors involved in hMSC osteogenesis

As bone formation is compromised in aging we specifically investigated changes in three of the most important receptors for bone formation in aged MSC: the VDR, GR and the Notch-1 receptor. The mean fluorescent levels after staining for Notch-1 and the vitamin D receptor in MSC showed a significant decrease with age. However, levels of the GR increased significantly in "aged" MSC compared to "young" and "adult" MSC (Table 3).

4. Discussion

Since adult stem cells are a major source of cells for tissue regeneration, age-related alterations to their numbers and function may contribute to this. Widely disparate results have been reported with regards to MSC numbers and aging with some groups finding an age-related decline whereas others see no change (Table 1). This disparity probably arises from the choice of age groups, group size, gender, inclusion of pathological states and isolation and cultivation conditions (Sethe et al., 2006). In this report we used CFU-f analysis of unfractionated bone marrow and under these conditions found an age-related decline in MSC numbers. Such bioassays may not be truly representative of the biology of MSCs in vivo. To address this, BMMSCs in BM aspirates were also evaluated by flow cytometry using a previously published immunophenotype (Jones et al., 2006). A good correlation between CFU-fs and MSC frequencies obtained by the flow cytometry was found. The age-related decline in CFU-f number is therefore reflected by a decrease in MSCs measured directly ex vivo and is not solely due to changes in the functional capacity of the MSC.

These data should be evaluated in the context of known age-related changes in bone metabolism. Indices of bone formation in post-pubescent males are maximal in late teens after which they decrease until the age of 50 and then remain stable (Szulc et al., 2007). In women the situation differs in that both bone formation and resorption are increased after the menopause leading to a net reduction in BMD (Szulc et al., 2007). This would suggest that (i) if younger subjects are not included in such studies, age-related changes will be greatly reduced and the value of the data compromised and (ii) combining data from male and female subjects will tend to minimise age-related effects. In this study, we have used samples from a wide age range (5–55 years) and the CFU-f and FACS data presented correlate well with published age-related changes in indices of bone formation (Szulc et al., 2007). This trend is borne out by examination of groupings used in other investigations; studies where proportionally large numbers of younger subjects (∼20 years of age or less) tend to show a significant reduction in CFU-f number with age (D'Ippolito et al., 1999; Muschler et al., 2001; Nishida et al., 1999) whereas those with relatively

Table 3
Age-related parameters in early-passage BM hMSC from different donor groups

Age of donor	"Young" (7–18)	"Adult" (19–40)	"Aged" (>40)
Viability	93%	95%	92.3%
Cell size (forward scatter/arbitary units)	276 ± 34	306 ± 61	376 ± 55 *
ALP activity	1.45 ± 0.1	0.83 ± 0.05 **	0.54 ± 0.04 ***
%Oil Red O positive cells	66% ± 12%	86% ± 11%	59% ± 9.5%
GAG concentration (μg/pellet)	3.2 ± 3 ($n = 6$)	7.4 ± 4.9 ($n = 3$)	1.6 ± 1.9 ($n = 4$)
Carbonyls (mean fluorescence intensity)	11 ± 1.7	29 ± 3 *	40 ± 11 **
Lipofuscin (mean fluorescence intensity)	1.9 ± 0.4	2.2 ± 0.5	16.4 ± 0.8 ***
RAGE (mean fluorescence intensity)	3.6 ± 1.4	26 ± 0.4 ***	41.2 ± 3 ***
AGE (% positive cells)	7 ± 2	34.5 ± 13	65 ± 18 **
Vitamin D receptor (mean fluorescence intensity)	17.9 ± 3	11.4 ± 3 *	8.2 ± 1.1 **
Glucocorticoid receptor (mean fluorescence intensity)	30 ± 14.6	25 ± 2.9	71 ± 1.5 **
Notch-1 receptor (mean fluorescence intensity)	37.7 ± 7.2	38 ± 2	10 ± 4 **

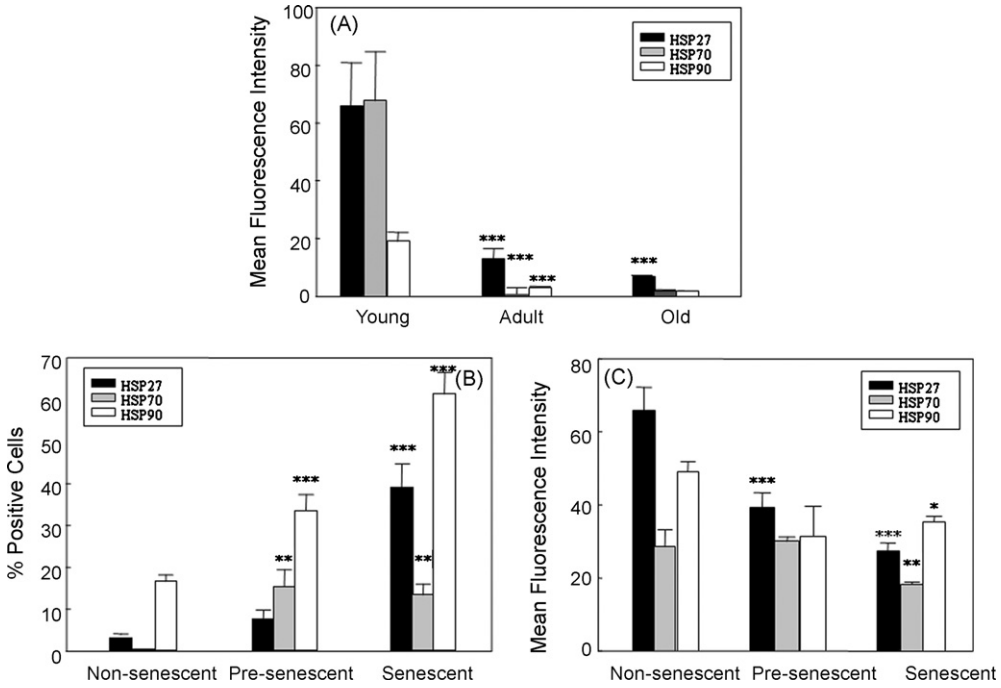

Fig. 5. Level of HSP27, HSP70 and HSP90 expression was measured in ''young'', ''adult'' and ''aged'' MSC using flow cytometry (A). In addition percent positive cells (B) and mean fluorescence levels (C) of HSP staining were measured in non-senescent, pre-senescent and senescent cultures of ''young'' MSC. $N = 3$ donors for each age group.

few younger subjects showed no such reduction (Oreffo et al., 1998b; Stenderup et al., 2001).

BMMSC are somatic cells and can achieve a maximal 30–40 population doublings in vitro (Banfi et al., 2000; Baxter et al., 2004). Because of this, a decline in replicative lifespan with donor age would be expected and has been found elsewhere as well as in this study (Baxter et al., 2004). During matrix formation in vitro, MSCs follow a defined temporal sequence of proliferation, matrix development and mineralisation (Stein and Lian, 1993). An age-related reduction in proliferative capacity should therefore be accompanied by a reduction in differentiation potential. A loss of MSC osteogenic potential with age has been demonstrated previously (Roura et al., 2006) however, as with MSC number and proliferative capacity, there is some disagreement surrounding this (Oreffo et al., 1998b). Despite this, the majority of reports describe an overall decline in osteogenic potential with donor age regardless of species (Sethe et al., 2006). In contrast, age-related changes in in vitro chondrogenic differentiation have not been well investigated. Cartilage shows an age-related decline in its repair capacity (Im et al., 2006) and one report has shown a reduction in chondrogenesis in MSC from osteoarthritis patients but failed to demonstrate a direct age-related decline in the chondrogenic potential in MSC from normal donors (Murphy et al., 2002).

Little is known about the stress resistance and accumulation of oxidative damage of hMSC in relation to donor age despite being of direct relevance to their clinical application. We have shown that MSC from older patients have reduced SOD activity combined with increased levels of NO and ROS and oxidised and glycated proteins. Oxidative damage accumulates in MSC in vitro (Ebert et al., 2006), induces MSC apoptosis and senescence (Byun et al., 2005; Finkel and Holbrook, 2000) and

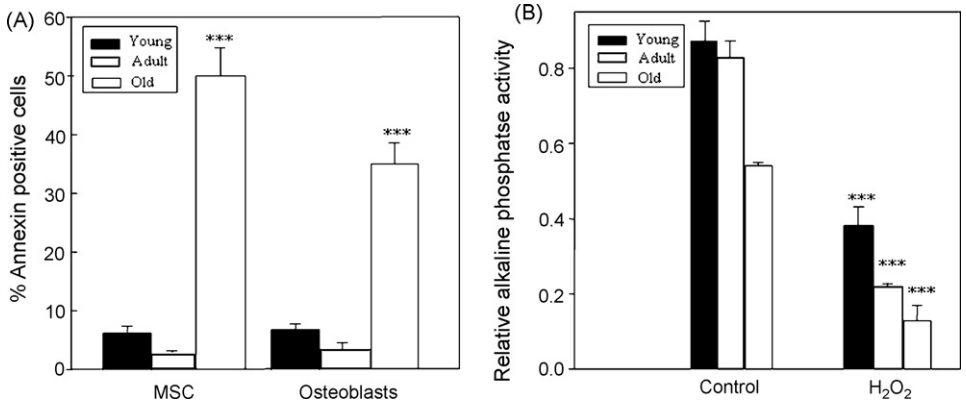

Fig. 6. Level of apoptotic cells in MSC and MSC-derived osteoblast cultures were measured after a 0.1 mM H_2O_2 exposure for 1 h (A). Alkaline phosphatase activity was measured in control and H_2O_2 stressed ''young'', ''adult'' and ''aged'' MSC (B). $N = 3$ donors for each age group.

inhibits osteoblastic differentiation (Mody et al., 2001) but no age-related studies have been performed. At the organ/tissue level, an age-related link between oxidative stress and bone mineral density has been demonstrated (Basu et al., 2001; Liu et al., 2004). Similarly, levels of plasma antioxidants are decreased in osteoporotic aged women (Maggio et al., 2003) and dietary antioxidants have a beneficial effect on bone mineral density, strengthening the link between oxidative damage of MSC and their differentiation capacity (Morton et al., 2001). We show here that artificial induction of oxidative stress induces apoptosis and inhibits MSC differentiation, especially in aged hMSC.

There have been few if any investigations into the expression hMSC cell-cycle regulator and senescence markers with relation to in vivo or in vitro aging (Byun et al., 2005). P53 levels increase with age mirroring cumulative levels of stress (Atadja et al., 1995; Campisi, 2005) and inhibition of p53 results in an extension of fibroblast lifespan (Hara et al., 1991) and osteoblastic differentiation (Wang et al., 2006). Here we show that both p53 and p21 are up-regulated in ''aged'' MSC which is consistent with their pro-apoptotic activities (Campisi, 2005).

HSP are known markers of stress resistance in cells (Mehlen et al., 1995) and play a central role in stem cell differentiation and self-renewal (Mauney et al., 2004). Moderate heat shock can induce osteogenic differentiation in rat MSC (Shui and Scutt, 2001). It has also been reported that heat stress can induce HSP90, HSP70 and HSP27 in osteoblasts (Nover, 1991) and that induction of HSPs is linked to alkaline phosphatase activity (Shui and Scutt, 2001). In this study we have shown that with aging, the number of HSP positive MSC increases, but the responsiveness of this system decreased impairing the protective mechanisms of the cells. Similar observations have been made in heavily expanded hMSC (Mauney et al., 2004) suggesting that aging leads to an impaired HSP/stress response and a reduced differentiation/proliferation capacity.

The VDR is a major regulator of calcium homeostasis and bone metabolism (Walters et al., 1992) and the GR is necessary for MSC differentiation (Sher et al., 2004, p. 87). Both receptors were down regulated with age in MSC and similar behaviour has been shown for the VDR in bones (Martinez et al., 2001). The notch receptor is a transmembrane protein involved in stem cell self-renewal but its role in osteogenic differentiation is not fully understood and diverse results have been reported. In a mesechymal progenitor cell line (Kusa) the activation of Notch-1 lead to a decrease in the osteogenic potential (Shindo et al., 2003) whereas in MC3T3-E1 cells, Notch-1 activation stimulated proliferation and osteogenic differentiation (Tezuka et al., 2002). In this study we found an age-related decline of Notch-1 receptor levels in ex vivo MSC, which would fit well with an inhibition of Notch-1 leading to a decrease in bone formation, and would explain why with age MSC lose some of their responsiveness to bone-inducing agents. Levels of all three receptors involved in bone formation were changed during aging which might seriously affect their usefulness either in tissue engineering or cell therapy.

Little attention has been devoted to the stability of so called ''MSC markers'' commonly used to isolate, enrich and characterise MSCs in vivo and in vitro. This information is however of vital importance for the analysis of stem cell numbers in relation to donor age when MSC are isolated using one or several of such ''MSC markers''. The three markers used in this study to isolate MSC (LNGR, CD45 and D7-Fib) were stable in their expression during aging verifying the age-related decline in BMMSC numbers in unselected and enriched MSC fractions. Aging does however, seem to change the expression of several other markers by MSC e.g., Stro-1 and CD105, which could potentially lead to inconsistencies when comparing reports of changes in MSC numbers from independent groups (Oreffo et al., 1998b; Roura et al., 2006). These markers cannot therefore be relied upon to identify MSC in aging studies and that further studies on aging and stem cell marker stability are required.

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