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The Effects of Ageing on the Isolation, Proliferation and Differentiation of Mesenchymal Stem Cells

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Abstract: The reported effects of ageing on mesenchymal stem cells are variable. A number of studies have shown an age-related decline in the number and proliferation of bone marrow derived mesenchymal stem cells, however a number of other studies found no difference. A number of studies have shown an age-related change in the differentiation potential of mesenchymal stem cells, but this has not been reported in some other studies. Much of the conflicting data in the literature may be due to variations between mesenchymal stem cell donors and in the culture conditions used. It may be that the effect of age on mesenchymal stem cells includes induction of senescence that translates to a reduced cellular output upon culture and transplantation. The molecular pathways involved in the ageing process are complicated and involve the INK4a locus on chromosome 9, p21 that encodes the proteins p16INK4a. It regulates the p53 pathways that promotes senescence or apoptosis. The expression of p16INK4a in particular increases with age and regulates age-dependant senescence, and has been proposed as a biomarker of physiologic as opposed to chronologic age, especially as the expression increases with stress and the accumulation of reactive oxygen species. All of the studies looking at the effects of ageing have concentrated on chronological age as opposed to physiological age, and very few have looked at senescence markers. In this study, we provide a comprehensive review on the effects of ageing in mesenchymal stem cells in humans, mice, rats, rabbit and sheep.

Key words: Ageing, proliferation, differentiation, mesenchymal stem cells, senescence, cell culture

INTRODUCTION

The reported effects of ageing on mesenchymal stem cells are variable. A number of studies have shown an age-related decline in the number and proliferation of bone marrow derived MSCs whereas other studies found no difference. A number of studies showed no age-related change in cell surface characterisation whereas other studies did. Much of the conflicting data in the literature may be due to variations between patients and in the culture conditions used.

MATERIALS AND METHODS

Previous research was carried out and 97 papers with the terms ageing' and 'MSCs' were identified using Medline. Of these only 15 papers related to species other than humans, and only 11 papers related to MSC sources other than bone marrow. The abstracts of these 97 papers were reviewed and only 32 papers were found to actually relate to the effects of ageing on MSCs. Full length articles were obtained for these 32 papers either online or from printed sources, and a review performed. The papers were divided into the respective species they deal with and a literature review performed that looked at how ageing was assessed, and how ageing affected the cell number, proliferation and differentiation of MSCs.

RESULTS

Human: D'Ippolito *et al.* (1999) isolated mesenchymal stem cells (MSCs) from the bone marrows of vertebral bodies from 41 human donors of various ages. This was one of the first papers looking at the effects of ageing on MSCs. Following osteogenic differentiation, the number of colonies that were positive for alkaline phosphatise (AP) staining for the younger donors (aged 3-36 years) was significantly greater than in the older donors (aged 41-70 years).

A number of researchers have continued to experience problems isolating and expanding MSCs from older patients. Shamsul *et al.* (2004) aspirated bone marrows from 38 patients (aged 10-70 years). Fourteen of the 15 samples from patients above 40 years of age failed to proliferate. Sixteen of the 23 samples from patient below than 40 years old were successfully cultured. Bertram *et al.* (2005) obtained MSCs from 21 iliac crests (aged 11-76 years) and from 32 cancellous bone grafting material (aged 13-84 years). Although, age had no statistically significant effect on the ability to isolate and

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culture MSCs, the failure rate was 55.6% in donors beyond the age of 60, while it was 14.3-2.2% in donors under 60 years of age.

These results were supported by Huang *et al.* (2005) who performed experiments on four groups of bone marrow derived MSCs, group A from foetuses, group B from 0-20 years old donors, group C from 20-40 years old donors and group D from donors older than 40. The primary culture time of group B was however significantly shorter than other groups. The results also showed that cells were similar in morphology, antigenic phenotype, differentiation potential and cell cycle. Mareschi *et al.* (2006) looked at 17 bone marrow samples from young adult donors and 8 from paediatric donors. MSCs isolated from two groups showed no morphological differences but their cell growth was strictly related to the donor's age. The MSCs isolated from paediatric donors reached a cumulative population doubling almost twice as high as MSCs isolated from young adult donors after 112 days. The antigen expression in the MSCs isolated from two groups until 10th passage (77 days) and the expression of CD90, CD29, CD44, CD105, CD166 and CD106 showed no significant difference.

A number of researchers found no difference in isolation and proliferation with ageing. Stenderup *et al.* (2001) looked at 38 normal volunteers (23 volunteers aged 22-44 years, and 15 volunteers aged 66-74 years), and 13 patients with osteoporosis (aged 58-83 years). MSCs derived from the iliac crest were identified by magnetic activated cell sorting (MACS) using STRO1 antibody. They found no significant difference in colony-forming efficiency, average colony size, cell density per colony or percentage of AP positive colonies between groups. Mineralized matrix formation as assessed by alizarin red staining was also similar between the groups.

No differences were also found by Suva *et al.* (2004) who looked at MSCs from the femoral heads of 16 patients (aged 27-81 years) undergoing hip arthroplasty. Culture doubling time was not correlated with patient age. Fourteen clonogenic cell lines isolated from 5 patients (aged 27-78 years) showed no age-related changes in their frequency, doubling time, and maximal amplification. Scharstuhl *et al.* (2007) looked at MSCs isolated from the bone marrow of 98 patients at the time of total hip replacement. They too found no correlation of age with the number of mononuclear cells, MSC yield, or cell size was found. Proliferative capacity and cellular spectrum of the harvested cells were independent of age. From all tested donors, MSCs could be differentiated into the chondrogenic lineage.

A number of studies have also sought to explore the expression of senescence related markers. Zhou *et al.* (2008) also looked at the effects of age in bone marrow derived MSCs (aged 17-90 years). They tested the effect of age on senescence-associated beta-galactosidase, proliferation, apoptosis, p53 pathway genes and osteoblast differentiation in confluent monolayers by AP activity and osteoblast gene expression analysis. There were significantly more human bone MSCs positive for senescence-associated beta-galactosidase in samples from older than younger subjects. Doubling time of MSCs was positively correlated with age. With age, significantly more cells were apoptotic. There were also age-related increases in expression of p53 and its pathway genes, p21 and BAX. Consistent with above experiments, there was a significant age-related decrease in generation of osteoblasts. Stolzing *et al.* (2008) used a wider range of donor ages and measured indices of cellular ageing as well as MSC numbers ex vivo and proliferation rates. A reduction in colonies 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.

No differences in telomere length and differentiation were noted by some groups. Roura *et al.* (2006) compared the differentiation capacity of human CD105 positive MSCs obtained from the bone marrow of young and elderly donors. Cells were obtained from 10 young (aged 24+/-6.4 years) and 9 elderly (aged 77+/-8.4 years) donors. Cells obtained from young and elderly donors showed no significant differences in relative telomere length and lipofuscin accumulation. There was also no significant difference in cell pluripotentiality as analysed by adipogenic and osteogenic induction media. Siddappa *et al.* (2007) isolated and characterized bone marrow derived MSCs from 19 donors (aged 27-85 years) and also showed that dexamethasone-induced alkaline phosphatise expression showed no correlation with age.

On the other hand some groups did notice a change in differentiation potential. Jiang *et al.* (2008) performed quantitative RT-PCR to examine mRNA expression of the major factors defining MSC lineage, cbfa1 for osteoblasts, ppar-gamma for adipocytes, sox9 for chondrocytes, and rankl for osteoclasts, in 80 healthy subjects and patients (14-79 years old). RANKL and PPAR-Gamma levels exhibited a clear positive correlation with age in female patients, but not in males, with a slight age-related decline in CBFa1 transcripts. DRAK1 (an apoptosis related gene) expression showed an age-associated ascending trend.

There are only 2 studies in literature looking at the effects of ageing on MSCs from tissues other than bone marrow. Schipper *et al.* (2008) compared MSCs harvested from subcutaneous adipose depots in 12 female patients classified into 3 age ranges (25-30, 40-45, and 55-60 years old). They noted differences in cell proliferation related to age. Younger patients had increased PPAR-gamma-2 expression, whereas the older patients have a variable expression. Additionally, they also noted age-related changes in function. Khan *et al.* (2009) looked at the effects of ageing in later life on the isolation, expansion, cell surface characterisation and osteogenic potential of fat pad derived MSCs and found no difference.

Mouse: Bergman *et al.* (1996) showed that cultures from older mice (aged 24 months) yielded an average of 41% fewer osteogenic precursor cell colonies per given number of bone marrow derived MSC than younger animals (aged 4 months).

It also showed that the basal proliferative rate in cultures from older animals, as measured by 3H-thymidine uptake, was more than three times that observed in cultures from young animals. In bone marrow derived MSC cultures grown in osteogenic media, colonies in both age groups became AP positive at the same rate and produced abundant type I collagen.

Shi *et al.* (2005) looked at adipose tissue-derived MSCs harvested from juvenile (aged 6 days) and adult (aged 60 days) mice. Greater attachment, proliferation, and proliferating cell nuclear antigen production were seen in juvenile as compared with adult adipose-derived mesenchymal cells. The juvenile cells underwent significantly greater adipogenic differentiation, determined by Oil Red O staining, than did adult cells. Early osteogenic differentiation was determined with AP staining, and terminal differentiation with von Kossa staining and determination of extracellular matrix calcium content. Interestingly, the adult cells were capable of robust early and terminal osteogenic differentiation, with levels of all three osteogenic assays being similar to those seen in juvenile cells.

More recently Kretlow *et al.* (2008) have shown that cells from younger donors adhere to tissue culture polystyrene better and proliferate in greater number than those from older animals. Chondrogenic and osteogenic potential decreased with age for each group, and adipogenic differentiation decreased only in cells from the oldest donors.

Rat: Scutt *et al.* (1996) found that in rats with increasing age the numbers of colonies from the bone marrow are drastically reduced. Stolzing and Scutt (2006) looked at MSCs isolated from rats aged 3, 7, 12 and 56 weeks. Cells from the oldest group accumulated raised levels of oxidized proteins and lipids and showed decreased levels of antioxidative enzyme activity. This was reflected in decreased colony numbers, increased levels of apoptosis and reduced proliferation and potential for differentiation. Tokalov *et al.* (2007a) performed a study to investigate the age-associated variations of bone marrow derived MSCs. The amount of bone marrow tissue obtainable from femurs and tibiae increased with age and reached a maximum in 8- to 12-week-old rats. At the same time, the proportional number of mononuclear cells containing MSCs decreased. As a result, after 2 weeks of culture, the maximum yield of MSC number was registered from the youngest age group (4 weeks).

Zheng *et al.* (2007) measured the responses of rat bone marrow derived MSCs to chondrogenic induction *In vitro*. Cells from immature rats (1 week old), young adult rats (12 weeks old) and old adult rats (1 year old) were analyzed for cartilage extracellular matrix (ECM) production. Histological analysis showed strong cartilage ECM formation by MSCs from 1-week-old rats, but not by MSCs from 12-week-old or 1-year-old rats. Real-time polymerase chain reaction revealed age-related declines in messenger RNA encoding type II collagen, aggrecan, and link protein. Microarray analysis indicated significant age-related differences in the expression of genes that influence cartilage ECM formation. Zhang *et al.* (2008) looked at rat bone marrow derived MSCs in *In vitro* culture and differentiation into cardiomyocytes. The tissue content of MSCs in bone marrow decreased with increased age of animal and MSCs from old donor rats exhibited less myogenic cells than those from the young rats after exposure to 5-azacytidine.

The fall in number of MSCs may not be accompanied by a fall in differentiation potential. Yue *et al.* (2005) compared the number of MSCs in bone marrow in juvenile, adult and old male rats (aged 1, 9 and 24 months). To assay the influence of ageing on osteogenic differentiation ability, MSCs from the three age groups were transduced with the BMP2 gene. Following gene transduction, expression of osteogenic proteins e.g., alkaline phosphatase, type Ialpha 1 collagen, osteopontin, and bone sialoprotein, as well as ectopic bone formation in athymic mice were compared. Results showed that the number of MSCs in bone marrow decreased with age, but no significant differences between the three age groups were found with regard to capability of BMP2 gene-modified MSCs to differentiate osteogenically. Tokalov *et al.* (2007b) investigated age associated changes in variations of bone marrow cell composition, phenotype and differentiation capacities of MSCs. Age related changes in bone marrow cell composition, proportions of separated MSC and yield of MSC in 2 weeks of *In vitro* culture were found. At the same time, neither change in phenotype nor in osteogenic and adipogenic differentiation capacities of MSC was registered.

Some groups have found a better differentiation potential in higher age groups. Cei *et al.* (2006) compared the capacity of bone marrow derived MSCs harvested from young and adult rats to proliferate, migrate, and differentiate into the osteogenic lineage following exposure to platelet-released supernatant (PRS) or (BMP-6). MSCs were isolated from 12 young rats (aged 6 weeks) and 12 adult rats (aged 9 months). Proliferation was assessed by 3[H] thymidine incorporation, migration was evaluated with the Boyden chamber assay, and osteogenic differentiation was deduced from AP activity. Cells from both age groups showed similar mitogenic activity and chemotactic motility when exposed to PRS. Adult bone marrow stromal cells had higher alkaline phosphatase activities at baseline and upon incubation with BMP-6 than cells from young animals. There was no difference between the two groups in the slope of the AP activity curve following stimulation with BMP-6.

There is one study looking at the effects of ageing in rat MSCs not from the bone marrow. Tarnowski *et al.* (2007) isolated, cultured, and characterized MSCs from livers of young and old rats and tested their multipotential for differentiation. The MSCs in liver sections were identified by the presence of markers, respectively for primary stem cells Thy-1 and CD34, for differentiation to early cholangiocytes and for differentiation to

hepatocytes. Ki67 was detected as the cell proliferation marker. The results revealed age-dependent changes in the number of recovered primary MSCs. In both age groups they observed cells changing under differentiating conditions to liver cell lineages, such as cholangiocytes and hepatocytes, as well as to non-liver cells such as adipocytes, astrocytes, neuroblasts and osteoblasts.

Rabbit: Huibregtse *et al.* (2000) obtained bone marrow aspirations from the iliac crest of rabbits, aged 4 months or 1, 2, or 3 years and assayed *In vitro* and *in vivo* chondro-osteogenic potential. MSCs were isolated and expanded in culture, and were placed subcutaneously in mice for 3 and 6 weeks. On histological examination, the cubes were scored for the presence of bone and cartilage, and average values for age groups were compared. At aspiration, the samples exhibited an overall reduction in cell concentration with increasing age and at the first harvest time, they showed a decrease in colony-forming efficiency and cube score with increasing age.

Similar age-related changes in chondrogenesis were noted in MSCs derived from the periosteum. O'Driscoll *et al.* (2001) looked at 736 periosteal explants from the proximal medial tibiae of 82 rabbits, aged 2 weeks to 2 years, and cultured them in agarose suspension conditions conductive for chondrogenesis. The samples were analyzed using histomorphometry, collagen typing, wet weight measurement, 35S-sulfate uptake. Chondrogenesis declined significantly with age. In parallel with this decrease in chondrogenic potential similar decreases were measured in 3H-thymidine uptake, 35S-sulfate uptake, as well as the thickness and the total number of cells in the cambium layer of the periosteum. Autoradiography with 3H-thymidine and PCNA immunostaining confirmed the measured decrease in proliferative activity in the cambium layer where the chondrocyte precursors reside, although the percentage of proliferating cells did not change significantly with age.

No differences were noted when MSCs were used to repair tendon defects. Dressler *et al.* (2005) looked at MSCs isolated from 1-year old rabbits, culture expanded, and cryogenically preserved. After 3 years, MSCs were re-harvested from the 4-year old rabbits and expanded. The MSCs were mixed with type I collagen gel and the resulting constructs implanted in bilateral full-length central third tendon defects. Intra-animal (paired) comparisons between repair tissue treated with 1-year old MSCs and repair tissue treated with 4-year old MSCs resulted in no significant differences in material properties including maximum stress, modulus and strain energy density. There was no significant difference in structural properties including maximum force, stiffness and strain energy.

Zhang *et al.* (2008) looked at rabbit adipose tissue derived MSCs in *In vitro* culture and differentiation into cardiomyocytes. The age of animal had no significant influence on the tissue content, proliferation and differentiation rate of MSCs.

Sheep: Rhodes *et al.* (2004) looked at bone marrow from the iliac crest of 28 individual sheep (aged 4 months to 8 years) and MSCs were isolated. There was no statistically significant correlation between the age of the sheep and MSC proliferative potential or estimated initial MSC number.

DISCUSSION

Much of the conflicting data in the literature on the effects of ageing in mesenchymal stem cells may be due to variations between mesenchymal stem cell donors and in the culture conditions used. In future studies, the effect of such variations can be reduced by examinations of the effect of age on the phenotype, proliferation and differentiation potential of patient-matched samples during identical culture conditions.

It may be that the effect of age on MSCs includes induction of senescence (Zhou *et al.*, 2008). Cellular senescence within MSC populations presumably translates to a reduced cellular output upon culture and transplantation. Senescence may be present in de-novo isolated cells or may become apparent upon culture and preparation for transplantation (Shibata *et al.*, 2007). The INK4a/ARF locus on chromosome 9, p21 encodes two proteins p16INK4a and p14ARF. p16INK4a is an inhibitor of the cyclin-dependant kinases, CDK4 and CDK6, which promote proliferation (Kim and Sharpless, 2006). The p14ARF protein regulates p53 pathways that promote senescence or apoptosis. The expression of p16INK4a in particular increases with age and regulates age-dependant senescence (Zindy *et al.*, 1997) and has been proposed as a biomarker of physiologic as opposed to chronologic age (Krishnamurthy *et al.*, 2004) especially as the expression increases with stress and the accumulation of reactive oxygen species (Sharpless and DePinho, 2007). All of the studies looking at the effects of ageing have concentrated on chronological age as opposed to physiological age and very few have looked at senescence markers, and this may account for the variations seen in studies so far.

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