Age-related impairment of mesenchymal progenitor cell function

Age-related impairment of mesenchymal progenitor cell function is associated with oxidative damage.

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Abstract

In most mesenchymal tissues exists a sub-compartment of multipotent progenitor cells responsible for the maintenance and repair of the tissue following trauma. With increasing age, the ability of tissues to repair themselves is diminished which may be due to reduced functional-capacity of the progenitor cells. The purpose of this study was to investigate the effect of ageing on rat mesenchymal progenitor cells. Mesenchymal progenitor cells were isolated from rats aged 3 – 56 week and viability, capacity for differentiation and cellular ageing examined. It was found that cells from the oldest group had accumulated raised levels of oxidised proteins and lipids and showed decreased levels of antioxidative enzyme activity. This was reflected by decreased CFU-f numbers, increased levels of apoptosis and reduced proliferation and potential for differentiation. These data suggest that the reduced ability to maintain mesenchymal tissue homeostasis in aged mammals is not purely due to a decline in progenitor cells numbers. There is in addition a loss of functionality due to the

accumulation of oxidative damage, which may in turn be a causative factor in a number of age-related pathologies such as arthritis, tendinosis and osteoporosis.

Key Words: Mesenchymal progenitor, oxidative stress, ageing, CFU-f

Running Header: Age-related changes of mesenchymal stem cells

Introduction

Mesenchymal stem or progenitor cells (MPC) are thought to be involved in mesenchymal tissue maintenance and repair. Exactly how this is achieved is unclear however, tissue resident mesenchymal progenitor cells appear to be replenished by marrow-derived progenitor cells via the circulation (Prockop et al., 2003). Depletion of stem cells has been suggested to contribute to degenerative diseases of a number of tissues including brain, liver, skin, bone and may also be involved in the degeneration associated with ageing (Rao and Mattson, 2001). MPC reside in the bone marrow along with haematopoietic stem cells (Bentley, 1982) and can differentiate into a variety of tissues including bone, fat, cartilage and tendon in vitro and in vivo (Deans and Moseley, 2000; Pittenger et al., 1999). In addition, differentiation into muscle, endothelial cells and neurons has been shown *in vivo* but it is not clear if this is due to fusion events, differentiation or a combination of both (Levy et al., 2003; Ying et al., 2002).

Although present in only very small numbers in the bone marrow (Barry et al., 1999; Hung et al., 2002). MPC are capable of extensive proliferation and expansion in culture (Colter et al., 2001). Undifferentiated MPC exhibit a fibroblast-like morphology and a characteristic pattern of cell-surface antigens (Deans and Moseley, 2000). These criteria, along with the ability to differentiate into multiple cell types, have been used to define a prototypic mesenchymal stem cell phenotype, which is consistent between a number of species (Devine et al., 2001; Kadiyala et al., 1997; Martin et al., 2002; Ringe et al., 2002).

The use of mesenchymal stem cells from autologous sources is very attractive for tissue engineering and also gene therapy. However, although they are capable of considerable expansion in culture, they are not immortal and cellular senescence has

been reported in *in vitro* cultures (Banfi et al., 2002; Digirolamo et al., 1999). It has been demonstrated that MPC exhibit characteristics typical of the Hayflick model of cellular senescence having a limited life span (Banfi et al., 2002; Stenderup et al., 2003) due to telomere shortening (Banfi et al., 2002) accumulation of senescent (β-galactosidase positive) cells and the impairment of differentiation (Digirolamo et al., 1999). Furthermore, cells obtained from elderly donors exhibited decreased proliferation potential and accelerated senescence compared with cells obtained from younger donors (Stenderup et al., 2003). Despite this information, little is known about how and why MPC age *in vivo*.

Free radical-derived reactive oxygen species (ROS) are constantly generated in most living tissue and can potentially damage DNA, proteins and lipids. ROS can be formed by exogenous sources and by the cells themselves, both actively and as a by-product of biological processes. The main intracellular sources of ROS are peroxisomes, lipoxygenases, nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase complex and mitochondrial respiratory chain reactions (Inoue et al., 2003). ROS are involved in the pathogenesis of several diseases including arthritis (Hitchon and El-Gabalawy, 2004), cancer (Pelicano et al., 2004), cardiovascular disease (Li and Shah, 2004) and aging (Huang and Manton, 2004; Mates and Sanchez-Jimenez, 1999) and the free radical theory of aging proposes ROS to be one of the central causes of the aging process (Harman, 1956) causing damage to all of the macromolecules of the cell. Excessive oxidative stress caused by increased ROS levels can induce the stress-related senescence pathways regulated by p53/p19 and p16RB leading either to senescence or apoptosis (Pelicci, 2004). Cellular senescence drives cells into growth arrest (Foreman and Tang, 2003) and apoptosis can kill cells avoiding an inflammatory response (Gregory and Devitt, 2004). Both are tumor-

supressive mechanisms but may also contribute to organismal aging as they would exhaust the stem/progenitor cell pools normally responsible for replenishing tissues and organs (Bell and Van Zant, 2004).

Several antioxidative enzyme systems exist to prevent damage caused by ROS to tissue and cells including catalase, superoxide dismutase (SOD) and glutathione peroxide (De Haan et al., 2003). SOD, mainly found in the cytosol, converts superoxide anions to hydrogen peroxide and oxygen (Michiels et al., 1994) which is in turn decomposed to water and oxygen by catalase (Makino et al., 2004). Glutathione peroxidase, localized mainly in the cell membrane, can also neutralize hydrogen peroxide and in addition can inhibit lipid peroxidation (Jung and Henke, 1996; Singh and Pathak, 1990).

Recent data regarding endothelial progenitors have shown that progenitor cells expressed significantly higher antioxidative defences compared to mature endothelial cells (Dernbach et al., 2004). Because of this we have investigated the role of oxidative stress in the accumulation of age-related damage and markers of aging in MPC.

Materials and Methods

Chemicals

All chemicals were obtained from Sigma-Aldrich (Dorset, UK; http://www.sigmaaldrich.com) unless stated otherwise.

Isolation of rat bone marrow stem cell

Female Wistar rats were purchase from Harlan (Harlan UK Limited, http://www.harlan.com) and kept on site according to home office regulations. Bone

marrow cells were obtained centrifugally from tibiae and femurae according to the method of Dobson et al., (Dobson et al., 1999) and MPC isolated by the method of Sekiya et al (Sekiya et al., 2002). In brief, 1 x 10^5/cm^2 mononuclear cells are plated out in expansion medium (DMEM-LG supplemented with 10% Serum Supreme (Cambrex Bio Science, Wokingham, UK; http://www.cambrex.com) and antibiotics). After 24 hours, non-adherent debris is removed, and adherent cells are cultured further keeping the cell density below 5 x 10^3/cm^2.

Growth curve

Rat MPC were serially subcultured under standard conditions. Briefly, the cells were cultured at 5 x 10^3 cells per cm^2 in T25 culture flasks in the above medium. When cells were 90% confluent they were trypsinised and replated into T25 culture flasks and the cultures continued until the cells stopped dividing. The number of population doublings (NPD) between subcultures was calculated according to the following equation: NPD = Log10(N/N0) x 3.33

Where: N = No. of cells in the flask at the end of growth period.

N0 = No. of cells plated in the growth vessel.

Colony-forming unit fibroblastic assay

The fibroblastic-colony forming unit (CFU-f) assay used with a modification of the technique originally described by Kuznetsov et al., (Kuznetsov et al., 1997). 2 x 10^6 mononuclear BMC were suspended in 0.5 ml media and plated on 55 cm^2 petri dishes. Cells were incubated for 14 days and then washed with PBS. The cells were then fixed in ethanol, sequentially stained for alkaline phosphatase, calcium, lipid and collagen-positive colonies and photographed as previously described (Scutt and Bertram, 1995).

Colony analysis

Colony numbers were assessed using the method of Dobson et al., (Dobson et al., 1999). Briefly, the acquired digital images were imported into Photoshop, colony irregularities smoothed out and converted into a 256 level greyscale format. The greyscale images were then imported into Bioimage "Intelligent Quantifier" and the colony number and the colony size calculated.

Morphology

Cells were seeded on a 24 well plate and grown over night. They were fixed in 4% paraformaldehyde, washed 3x with PBS and then incubated for 10 min in 0.1% Triton X 100 (in PBS). After washing the cells were incubated with 714 nM FITC-phalloidin in the dark for 20 min (RT). After washing the cells were analysed in an ImageXpress® 5000A (Axon Instruments). In addition pictures of these cells were also taken using a conventional light microscope.

Senescence associated β-galactosidase staining

Senescence associated β-galactosidase (β-gal) staining was performed as described previously (Dimri et al., 1995). Cells were fixed in 4% formaldehyde for five minutes, followed by three washes in PBS at room temperature. They were immersed in freshly prepared staining solution (0.05 mg/ml 5-bromo-4-chloro-3-indolyl-D galactoside, 40 mM citric acid/sodium phosphate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$, pH 6.0) and incubated at 37°C for 24 hours. To detect lysosomal β-gal activity the staining solution was used at pH 4.

Apoptosis measurement

Apoptosis was measured using the APOpercentage kit (Biocolor Ltd., Belfast, Ireland; http://www.biocolor.co.uk). 5 x 10^4 cells were seeded in 96 well plates and incubated for 24 h. The culture medium was replaced with Apomedium (9.5 ml culture medium containing 0.5 ml Apo dye) and incubated for 1 h. The cells were then washed twice

with PBS, dye release reagent added to the cells for 10 min and then the absorbance was measured at 550 nm in a plate reader.

Reactive Oxygen Species generation

MSC were cultured for the experiment in 96 well plates. The cells were washed with PBS and the MSC were supplemented with new medium. After 0 h and 24 h, medium samples were taken, mixed with DCF-DA (100 µM) and the amount of DCF fluorescence measured in a plate reader at 388 nm/ 456 nm. In addition MSC were cultured in the presence of DCF-DA (5µM) at 37°C for 30 min and then harvested. The cells were analysed in a GUAVA personal flow cytometry system to measure intracellular produced ROS.

Nitrite assay

Nitrite concentration was measured using standard Griess reagent. Briefly, 50 µl of the supernatant was incubated with an equal volume of Griess reagent. After 5 min incubation at room temperature, the absorbance was measured at 560 nm using a plate reader.

Carbonyl-ELISA

Protein carbonyls were measured according to Buss et al. (Buss et al., 1997) with modifications described by Sitte et al. (Sitte et al., 1998). Protein extracts were normalized to 3 mg protein/ml, derivatized with 2,4-dinitrophenylhydrazine (DNPH) and adsorbed to Maxisorb multiwell plates. Protein carbonyls were detected using an anti-DNPH primary antibody and an anti-rabbit-IgG peroxidase-linked secondary antibody. O-phenyldiamine was used to develop the plate, and the absorbance was determined using a multiwell plate reader using a detection wavelength of 492 nm (reference filter: 750 nm).

Thiobarbituric Acid Reactive Substances

Oxidative damage to lipid by copper-induced oxidation *in vitro* was determined by the TBARS assay. The resulting complex was measured at 535nm spectrophotometrically and the malondialdehyde concentration per mg protein was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} (Buege and Aust, 1978).

Lipofuscin content

The cellular lipofuscin content was measured as the autofluorescence intensity of unfixed cells by flow cytometry. Cell pellets were suspended in serum-free medium and analysed in a GUAVA personal cytometry system using an excitation of 488nm and an emission of 530 nm and 630 nm using at least 10.000 cells for each sample.

Measurement of proteasomal activity

Cells were washed with PBS, trypsinized and the cell pellet lysed in 200 µl lysis buffer (250 mM sucrose, 25 mM HEPES pH 7.8, 10 mM $MgCl_2$, 1mM DTT) by repeated freeze/ thawing cycles. The remaining non-lysed cells, membranes and nuclei were removed by centrifugation at 14,000×g for 30 min at 4°C. The supernatant was incubated in a buffer consisting of 225 mM Tris (pH 7.8), 7.5 mM magnesium acetate, 7.5 mM $MgCl_2$, 45 mM KCl and 1 mM DTT. The chymotrypsin like activity of the proteasome was measured using 200 µM suc-LLVY MCA (Bachem Ltd., Merseyside, UK; http://www.bachem.com). After 60min of incubation at 37°C, the fluorescence of the reaction mixture was measured using 360 nm excitation/485 nm emission and free MCA as standards.

Superoxide dismutase activity and gluthatione peroxidase activity

The activity of both enzymes was evaluated using a commercially available kit (OxisResearch, Portland, USA; http://www.oxisresearch.com) according to the manufacturers instructions.

Data analysis

Values were expressed as mean +/- standard deviation. The statistical significance of differences among experimental groups was evaluated by ANOVA with $p < 0.05$ being considered significant.

Results

Effect of age on CFU-f numbers.

When bone marrow cells are plated out at low densities individual MPC adhere and proliferate to form fibroblastic colonies. Due to the low plating density the colonies grow essentially in isolation and therefore represent the clonal expansion of a single "CFU-f" or MPC. With increasing age, the number of fibroblastic-colonies formed by rat mesenchymal progenitor cells was significantly reduced. Cultures derived from 3 week old rats gave rise to ~160 colonies per dish whereas those derived from 56 week old rats produced ~90 colonies per dish (Table 1). The proliferative potential of the MPC was estimated by calculating the mean colony size (Table 1). It was found that mean size of the colonies also decreased with age with 52 week old rat MPC producing colonies only half the size of those produced by 3 week old rat MPC.

There was also a relationship between age and MPC differentiation potential. The numbers of alkaline phosphatase positive colonies were reduced 4 fold from ~85 for MPC from 3 week old rats to ~21 for the oldest age group. In addition, the size of the ALP positive colonies was also decreased in the oldest age group by around 30%. Similarly, the number of calcium and collagen positive colonies decreased with age. Numbers of calcium positive colonies were reduced from 47.9 to 13.2 and collagen positive colonies from 39.2 to 21.3 in 3 week old rats and 56 week old rats respectively. Mean calcium and collagen-positive colony sizes also declined in the old group but the effect was smaller than that seen for ALP-positive colonies. In contrast,

no significant age-related effect was observed for Oil red O positive colony numbers or sizes.

Growth curve

The growth kinetics of MPC derived from rats of increasing age were observed from the primary passage until cells in culture ceased to replicate for at least 4 weeks. MPC from older rats showed a much slower rate of growth and achieved fewer PD before reaching senescence (Fig. 1A). The morphology of the cells changed from mostly spindle-shape fibroblastic cells to more round and flat cells indicating a senescent phenotype (Fig. 1B,C). These changes were accompanied with an accumulation of actin fibres in the senescent cells and MPC loosed the ability to reach confluence. (Fig. 1D,E).

Senescence and Apoptosis

The decrease in CFU-f numbers seen in aged animals may be the result of an increase in levels of apoptosis or senescence. Freshly isolated MPC cultivated for 2 days in 24 well plates showed essentially no signs of β-gal activity at pH 6 in any of the tested age groups (Table 1, Fig. 2A,B). As a positive control, staining was also performed at pH 4 to display lysosomal β-gal activity (results not shown) and positive staining was found in MPC from all age groups. Once in culture, MPC displayed increasing senescence with passage number regardless of the age of the donor animal (Table 1, Fig. 2C,D). Levels of apoptosis were also measured in MPC from young (3 week) and old (56 week) animals. In contrast to the levels of senescence, the percentage of apoptotic MPC was increased in the oldest group by about four fold (Fig. 1E).

Reactive oxygen species and nitric oxide generation

Because the decrease in CFU-f numbers in aged rats may be affected by changes in oxidative status, we examined the effect of age on ROS and NO levels. It was found

that MPC-associated extracellular ROS formation did not change with age in female rats (Fig. 3A), whereas NO production was slightly but not significantly increased (Fig. 3B). In contrast levels of intracellular ROS formation was significantly increased by approximately 20% in the 56 week old animals compared to the 3 week old animals (Fig. 3C).

Oxidative damage to proteins and lipids

Protein carbonyl formation was increased more than four fold ($P < 0.01$) in MPC from 56 week old rats compared to 3 week old rats. Carbonyl content also increased in brain and kidney tissue isolated from the same rats and was highest in the kidney samples (Fig. 4A). TBARS levels increased around three fold in MPC from 56 week old rats compared to MPC from 3 week old rats. A gradual increase in TBARS levels was also found in brain and kidney tissues (Fig. 4B). Similarly, levels of lipofuscin in MPC also increased with age (Fig. 5A).

Antioxidative enzyme activities

As MPC showed signs of age-related damage accumulation (oxidised proteins and lipids), we studied the activity of several antioxidative defence enzymes. SOD activity and glutathione peroxidase activity decreased steadily with age in MPC with the activity of both enzymes being reduced by about 50% in cells from 56 week old rats compared to those from 7 and 12 week old rats (Fig. 6A,B). Interestingly the SOD activity found in MPC is much higher than in either brain or kidney homogenates. Glutathione peroxidase activity in the youngest group was found to be nearly as high as that in kidney homogenates but by 56 week the activity had reduced to around 20% of that found in kidney. In contrast, 20S proteasome activity showed no correlation with the age of the rat from which the MPC were isolated (Fig 5B). Due to the poor

recovery of protein from 3 and 4 weeks old rat MPC, it was not possible to perform these assays on cells derived from these animals.

Discussion

The capacity of organs to repair themselves declines with age and this may be due to a loss or failure of adult stem cells. It has also been suggested that these same adult stem cells may be used to engineer tissue constructs to replace damaged tissues or organs. MPC hold great promise for regenerative medicine (Barry and Murphy, 2004) as there are few ethical concerns regarding the use of these cells and, few concerns about possible immunological rejections as they do not appear to elicit an immune response (Aggarwal and Pittenger, 2005). However, there may be other problems with the use of adult stem cells; in particular the age of the donor may have an impact on their vitality. Tissue stem cells or progenitor cells are exposed to various toxins and detrimental events during their lifetime in the same way as differentiated cells and the resultant accumulation of damage may be one of the causes of aging (Kirkwood and Franceschi, 1992). A better understanding of age-related changes in stem cells will help to explain the degenerative changes observed in organs during aging. Experiments with embryonic, adult neural stem cells and haematopoietic stem cells have shown that they express a certain subset of genes involved in oxidative stress resistance, detoxification and DNA repair (Ivanova et al., 2002; Ramalho-Santos et al., 2002) to increase the resistance of these cells to stress and aging. In contrast, the extent to which aging affects mesenchymal stem cells is relatively unknown.

Fibroblastic colony formation

CFU-f numbers have been shown to reflect changes in bone anabolic activity seen after age and ovariectomy (Scutt et al., 1996), unloading (Tanaka et al., 2004) and the

administration of bone anabolic drugs (Erben et al., 1998; Nishida et al., 1994; Weinreb et al., 1999). The CFU-f assay has also been shown to be more sensitive than other culture systems in terms of the response to bone active agents such as PGE_2 (Still and Scutt, 2001) or PTH (Davies and Chambers, 2004). Analysis of CFU-f cultures also reveals that considerable variability occurs between colonies indicating significant heterogeneity within the MPC population (Dobson). In this study an age-related reduction in the number of CFU-f in the animals was found. Similar results have also been found senescence accelerated mice (SAM) compared to normal mice (Tsuboi et al., 1991) and in normal aged mice (C57BL) (Brockbank et al., 1983). An age-related reduction in CFU-f in rats has also been reported (D'Ippolito et al., 1999; Majors et al., 1997; Nishida et al., 1999; Scutt et al., 1996), however, there are also contradictory data where no changes in CFU-f numbers with age were found (Justesen et al., 2002; Oreffo et al., 1998; Stenderup et al., 2003) and in one case an increase has been reported (Martinez et al., 1999). A variety of factors may explain these discrepancies between studies including differences in isolation methods, counting methods, growth media, species or strain of animals as well as the age groups which were compared. The majority of studies suggest that CFU-f numbers are reduced with age however, the effect is probably multifactorial and requires further systematic study.

Proliferative Capacity

The age-related reduction in the mean size of the colonies derived from old rats seen in this study perhaps reflects the functional competence of the aged stem cells and has also been seen in mice (Globerson, 1997). Colony size is a measure of the proliferative capacity of individual CFU-f (commonly thought to be closely related to MPC) *ex vivo* and is therefore an indicator of qualitative changes in the CFU-f. This is

in contrast to colony numbers, which simply reflects total CFU-f numbers regardless of their proliferative capacity. This is also subtly different to *in vitro* growth curves in that the cells are assessed directly *ex vivo* whereas in growth curves the cells are pre-selected for rapid proliferation. Despite this, a loss of proliferative potential with age during *in vitro* expansion was seen in this study has also been found in human (Baxter et al., 2004) and murine (Wolf and Arora, 1982) MSC. This loss of proliferative potential due to age has also been found in skin stem cells (Martin et al., 1998), haematopoietic stem cells (Vaziri et al., 1994), muscle stem cells (Conboy et al., 2003) and myocardial stem cells (Torella et al., 2004), suggesting that stem cell aging may be a general phenomenon.

Differentiation of MPC

There was an age-related decrease in the number and size of colonies that stained positive for collagen, calcium or alkaline phosphatase. This is consistent with previous investigations showing a reduced capacity for differentiation in aged animals (Bergman et al., 1996; Meunier et al., 1971; Nakahara et al., 1991). It has however, been shown that osteoblastic differentiation requires an initial phase of rapid proliferation and that inhibition of this prevents differentiation (Stein et al., 1990). Therefore this apparent decrease in differentiation may not necessarily be intrinsic to the cells themselves but may be a result of their decreased proliferative capacity.

Senescence of MPC

In this study no age-related increase in the numbers of senescent MPC were found. This is in contrast to the findings of other groups who found signs of senescence in MPC from humans aged between 19-49 years. However, in these studies the MPC had undergone at least 15 populations doublings before they displayed signs of senescence (Bruder et al., 1998; Digirolamo et al., 1999). Cellular senescence is a

complex phenotype that entails changes in both function and replicative capacity. Unlike apoptosis, which eliminates damaged cells from tissues, senescent cells remain alive despite changes in morphology, metabolism, and derangement of differentiated functions (Itahana et al., 2001). A weakness of the results of DiGirolamo et al and Bruder et al, is that they only measured senescence in MPC by testing the loss of differentiation potential or by morphology after expansion in culture and did not assess senescence in freshly isolated MSC without *in vitro* replication. As we only found very low levels of β-gal staining in MPC from both young and old rats we must concluded that MPC isolated from rats of all ages *in vivo* are largely non-senescent. The increased levels of senescent MPC seen after *in vitro* expansion may be an artefact due to the higher stresses experienced by the cells during *in vitro* cultivation (Rubin, 1997).

Age-related Oxidative stress and damage accumulation in MPC

It has been suggested that the expression of high levels of antioxidative enzymes may be a characteristic of stem cells that is lost with differentiation (Dernbach et al., 2004). For example, it has been shown that endothelial progenitor cells express significantly higher levels of catalase, SOD and glutathione peroxidase mRNA than mature endothelial cells (He et al., 2004). Consistent with this, MPC from young animals expressed high levels of SOD and glutathione peroxidase, considerably higher than in brain or kidney, but this activity was progressively reduced with age. Accompanying this, levels of intracellular ROS (but not extracellular ROS or NO) were increased leading to the accumulation of oxidised proteins, oxidised lipids and lipofuscin with age. This may be due in part to declining antioxidative defence enzyme activities with no increase in 20S proteasome activity to cope with the increasing levels of oxidised proteins. It is known from other cell types that lipofuscin content increases as soon as

the growth rate in a cell culture declines (Sitte et al., 2001). This is consistent with the suggestion that *in vivo* aged MPC have a lower rate of proliferation than younger MPC and therefore begin to accumulate lipofuscin.

It is known that ROS and NO generation increases in other tissues, such as bone marrow, with age and this may play a contributory role in damage accumulation by MPC. Consistent with this, the differentiation of haematopoietic progenitors *in vitro* is inhibited by the addition of ROS producing macrophages (Hoffman et al., 1993). Furthermore, an age-related increase in NO production has been found in rat bone marrow cells and mouse bone marrow-derived macrophages after challenge with LPS (Chen et al., 1996; Ota et al., 2003). It has been hypothesized that oxidative stress increases the number of single strand breaks in the telomeres causing the cells to exit the cell cycle or enter apoptosis. Consistent with this, an age-related increase in apoptosis was also observed in this study.

The increased levels of apoptotic MPC in aged animals may have an impact on the relationship between aging, stem cells and cancer. Mathematical models of a theoretical stem cell niche have suggested that stem cells dying by apoptosis rather then going into senescence in aged animals may lead to a higher possibility of developing cancer (Lynch, 2004). It is suggested that as stem cells die by apoptosis they are replaced by increasing the rate of symmetrical stem cell self-renewal thus increasing the likelihood of their transformation into cancer cells.

Conclusion

MPC display a significantly decreased functional capacity and vitality in aged animals and these data suggest that these changes are the result of age-related damage accumulation due to a loss of SOD and glutathione peroxidase activity. ROS production by neighbouring cells may also play a role in this process but this needs

further investigation. The decreased functionality of the MPC will have repercussions on physiological tissue maintenance and the repair of tissue damage and may therefore be a factor in organ aging.

Additionally the changes in apoptosis in aged stem cells may explain the increased cancer risk in older organisms. These findings may have profound implications for the use of adult tissue stem cells in cellular therapy and tissue engineering as the target population for these forms of therapy will typically be the elderly. These data demonstrate that MPC progressively lose their capacity to proliferate and differentiate with age which will in turn compromise engraftment success and the lifetime of the tissue engineered construct.

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Legend

Figure 1) Growth kinetics and morphology of rat MPC in culture.

a) MPC isolated from rats aged 3, 4, 7 and 56 weeks were cultured under standard conditions for up to 4 months and the cumulative number of population doublings (PD) were calculated.

Cell morphology of MPC form 3 week old rat early passage (b) and late passage (c). Actin staining in early passage MPC cultures (d) and late passage MPC (e).

Figure 2) Senescence and apoptosis of MPC.

MPC were stained with x-Gal for the determination of senescence level in early (a) and late (c) passage cultures of cells isolated from 3 week old rat and 56 week old rats (b,d). e) Levels of apoptotic cells were determined in 1st passage cultures of MPC from 3

Figure 3) Peroxide and nitrite oxide production of MPC.

Levels of peroxides were determined in 1st passage cultures of MPC from 3 week and 56 week old rats using DCF-DA (5 μM). Fluorescence intensity of DCF in the MPC supernatant was then determined after 0h and 24h using a fluorescence plate reader (a). NO release by MPC of rat of different ages. 1st passage cultures of MPC from 3 week and 56 week old rats were cultured for 24 h and aliquots of the supernatant were taken out and incubated with the same amount of Griess reagent to determine NO levels (b). In addition MPC were loaded with DCF-DA to measure intracellular Levels of ROS (c).

Figure 4) Oxidative damage accumulation in MPC.

Protein were extracted from MPC derived from rats of different ages and from kidney and brain tissue homogenates of the same rats. Carbonyl content in these proteins were determined using a ELISA (a). Oxidised lipids and aldehydes were measured in MPC, kidney and brain homogenates from rats of different age using the TBAR assay. Protein extract were analysed for TBARS and expressed as MDA equivalents (b).

Figure 5) Lipofuscin and proteasome activity of MPC.

Total protein was isolated from MPC, kidney and brain homogenates and the chymotrypsin-like activity of the 20S proteasome was measured using a fluoropeptide (a). 1st passage cultures of MPC from rats of different ages were analysed in a GUAVA personal flow cytometer for there lipofuscin content. The wavelengths at 530 nm and 630 nm were measure as equivalent to the intracellular lipofusine content.

Figure 6) Antioxidative defence enzyme activities in MPC.

Total protein was isolated from MPC, kidney and brain homogenates of rats of different ages. The activity of superoxide dismutase (a) and glutathione peroxide activity (b) was determined using commercial kits.

Table 1. The effect of age on colony number colony size and MPC senescence.

Whole bone marrow cells were isolated from rats of increasing age and cultured in the fibroblastic-colony forming unit assay. The number and size of the colonies was determined by image analysis as described in the methods. MPC were isolated from rats of different ages and the percentage of senescent cells after 0 and 15 passages determined by staining for β-gal.

Figures

Table 1:

AGE OF RAT	3 week	4 week	7 week	12 week	56 week
Colony Number					
Total	160±8.6	123±4.7	124±4.3	121±6.2	92±5.2
Calcium	47.9±2.8	43.5±2.2	33.8±3.1	21.8±5.2	13.2±2.5
Collagen	39.2±5.2	31.2±5.3	30.4±6.6	29.2±5.8	21.3±3.8
Alkaline Phosphatase	85±1.5	72.3±4.2	56.4±2.8	34.9±3.7	21.1±2.8
Oil Red O	12.1±3	11.5±1.7	13.6±2.9	12.5±1.7	12.4±1.5
Mean size of colonies (mm^2)					
Total	34.2±2.5	33.7±1.7	32.2±2.9	23.8±3.1	17.9±1.9
Calcium	20.1±2.4	19.3±1.1	15.3±2	9.6±0.9	8.8±0.8
Collagen	15.9±2.3	15.8±1	13.4±2.1	12.9±0.8	10.5±1.2
Alkaline Phosphatase	15.8±3.3	14.5±2.6	12.5±1.7	11.1±0.6	10.8±2.4
Oil Red O	24.6±3	25.3±2.8	23.9±4.1	23.7±2.4	23.3±2.8
% β-gal positive colonies					
0 passage	5.6±0.4	6.5±0.3	4.6±0.5	6.1±0.6	4.3±0.2
15 passage	22.4±1.6	21.8±1.57	19.7±2.1	18.6±0.8	22.6±1.3

Figure 1

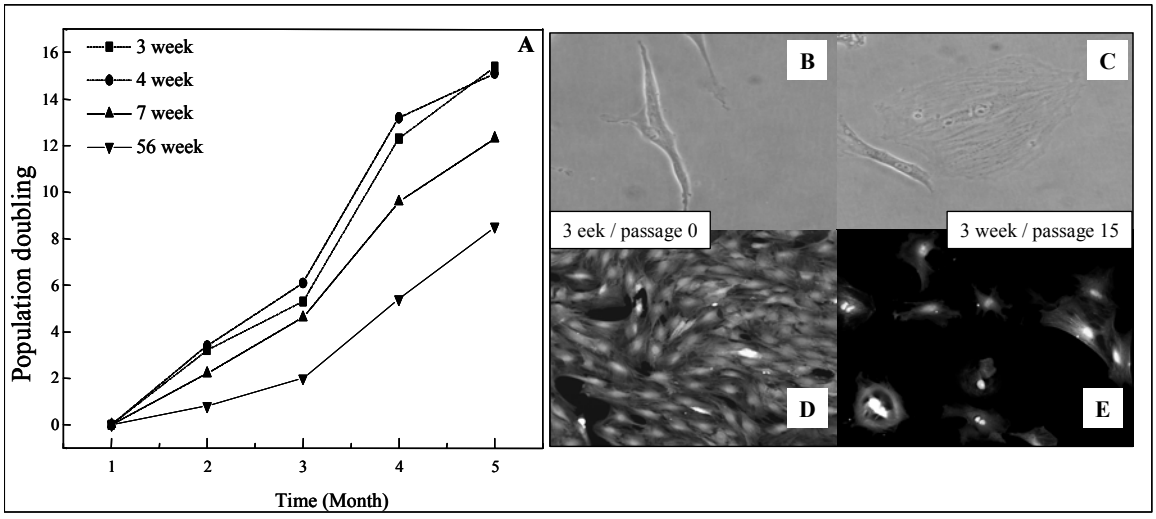

Figure 2

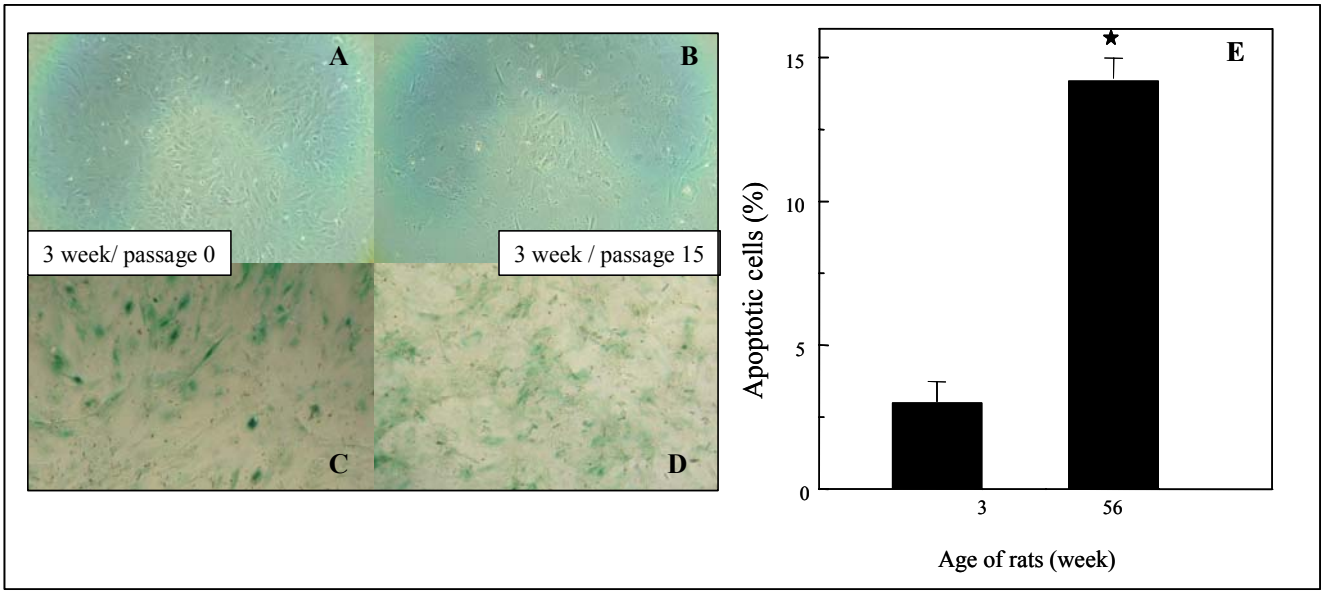

Figure 3

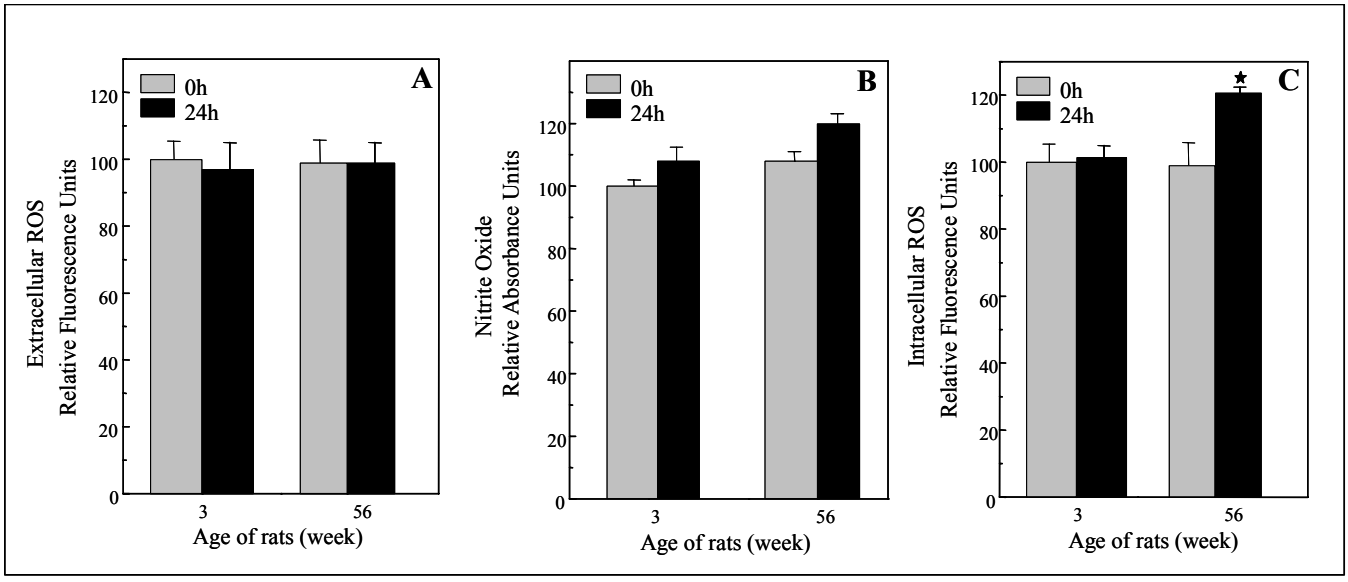

Figure 4

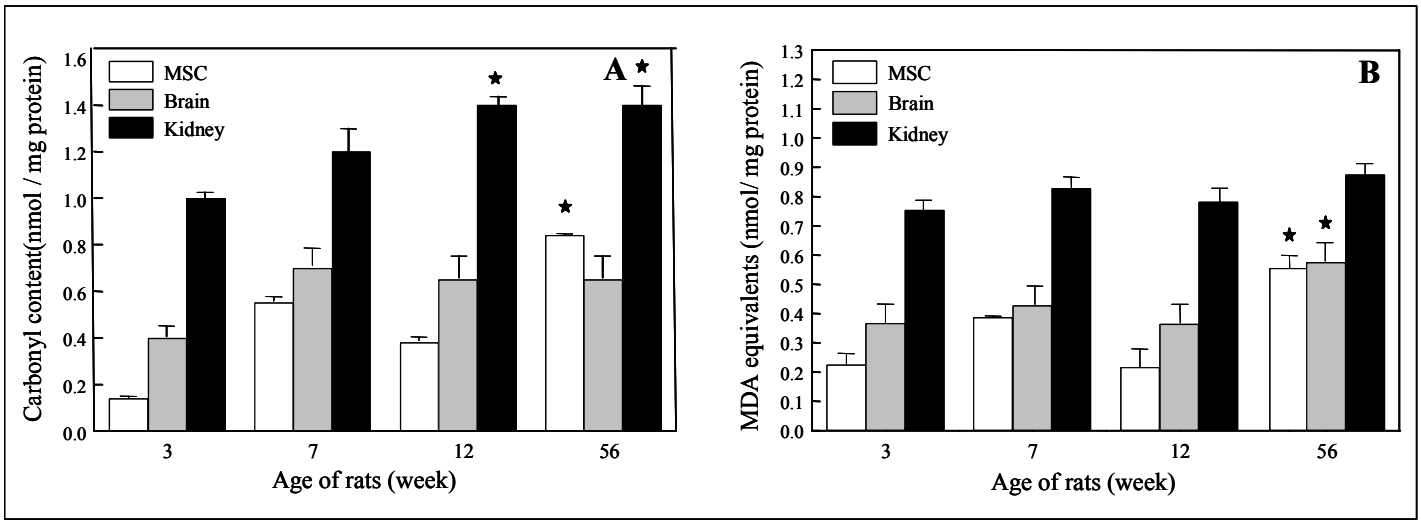

Figure 5