Cellular senescence in vitro and organismal ageing

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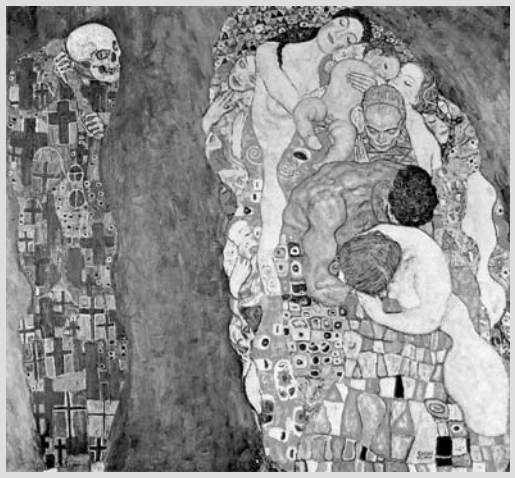

Chapter 7

Marked heterogeneity in growth characteristics of myoblast clonal cultures and myoblast mixed cultures obtained from the same individual

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Submitted for publication

Summary

Sarcopenia is a decrease in skeletal muscle mass and function while adjacent satellite cells are unable to compensate for this loss. However, myoblast cultures can be established even in the presence of sarcopenia. It is yet unknown whether satellite cells from failing muscle in older age are equally affected, as human satellite cells have been assessed using myoblast mixed cultures and not by using myoblast clonal cultures. We established a myoblast mixed culture and three myoblast clonal cultures out of the same muscle biopsy and cultured these cells for 100 days. We found marked heterogeneity between the myoblast clonal cultures that all had a significantly lower replicative capacity when compared to the mixed culture. Replicative capacity of the clonal cultures was inversely related to the β-galactosidase activity after exposure to oxidative stress. Addition of L-carnosine enhanced remaining replicative capacity in all cultures with a concomitant marginally decrease in β-galactosidase activity. It is concluded that myoblast mixed cultures *in vitro* do not reflect the marked heterogeneity between single isolated satellite cells. The consequences of the heterogeneity on muscle performance remains to be established.

Introduction

Skeletal muscle is highly plastic and responds to various stresses such as exercise and injury. Lifelong muscle function relies on the maintenance and regeneration of myofibers through activation of quiescent muscle precursor cells that after proliferation fuse to differentiated myofibers (Morgan and Partridge, 2003). These quiescent muscle precursor cells, also called satellite cells, are located between the plasma membrane and the surrounding basal lamina of the myofibers (Mauro and Dams, 1961). Exhaustion of the pool of satellite cells impairs muscle regeneration, as in certain diseases such as Duchenne muscular dystrophy (Shi and Garry, 2006).

Sarcopenia is defined as an age-related loss of muscle mass, strength and function and may result in a loss of the muscle mass of about 30-50% by the age of 80 years (Evans *et al*., 1997). This loss is generally thought to be caused by an exhaustion of the number of, and a diminished replicative capacity of satellite cells, consequent on the regeneration of muscle and accumulation of reactive oxygen species (ROS) throughout lifetime (Hepple *et al*., 2003; Fulle *et al*., 2005). In line, the fraction of satellite cells within human muscle is inversely related to the age of the donor (Renault *et al*., 2000, 2002; Sajko *et al*., 2004; Schmalbruch and Hellhammer, 1976), but others failed to find such a correlation during adulthood (Hikida *et al*., 1998; Roth *et al*., 2000). Replicative capacity of isolated satellite cells *in vitro* show a rapid decrease during childhood and adolescence but from adulthood onwards replicative capacity decreases only slightly (Decary *et al*. 1997; Renault *et al*., 2000). Remarkably however, at older age when clinical signs of sarcopenia are clearly present, satellite cells can still be cultured in myoblast mixed cultures from muscle biopsies (Renault *et al*., 2000).

It is yet unknown whether satellite cells from failing muscle in older age are equally affected, or some satellite cells are affected more than others. Myofibers are of considerable length and result from a proportional number of fused myoblasts. Each fiber thus possesses many nuclei. As satellite cells have bearing on only a distinct fragment of the myofiber and a limited number of nuclei (Allen

et al., 1999), replicative failure of individual satellite cells may have major consequences on muscle function. We questioned to what extent myoblast mixed cultures reflect the *in vivo* characteristics of single satellite cells from adult skeletal muscle. To date differences in proliferative capacity (Beauchamp *et al*., 1999; Rantanen *et al*., 1995; Rouger *et al*., 2004; Yablonka-Reuveni *et al*., 1987), differentiation (Rantanen *et al*., 1995; Zammit *et al*., 2004) and fusogenic capacity (Rouger *et al*., 2004) of individual satellite cells have only been performed in animal models. Therefore we compared the replicative capacity and oxidative stress resistance of myoblast clonal cultures with that of a myoblast mixed culture, which were established out of the same biopsy obtained from a middle aged man.

Materials and Methods

Muscle sample and cultures

A 464 mg muscle biopsy from the musculus vastus lateralis was obtained from a 58 year old male during elective hip replacement operation. The biopsy was washed with solution-A (10 mM glucose, 130 mM NaCl, 3 mM KCL, 1 mM $Na_2HPO_42H_2O$, 40 g per mL NaOH (reagents were obtained from Merck, Amsterdam, the Netherlands), 30 mM HEPES, 3.3 nM Red Phenol (Sigma, Zwijndrecht, the Netherlands), H_2O and connective tissue was removed. The muscle was minced and digested in a trypsine-EDTA solution (solution-A, 0.005% trypsine, 0.02% EDTA (Gibco, Breda, the Netherlands)) during one hour at 37°C. Thereafter trypsine was blocked using 10% fetal calve serum (FCS, Lot 40G4932F, Gibco). Myoblasts were washed and collected in growth medium (GM) consisting out of Ham's F-10 (Gibco), 0.50 mg per mL bovine serum albumine, 0.5 mg per mL fetuin, 0.18 mg per mL insuline (all Sigma), 1 mM creatine (Fluka, Zwijndrecht, the Netherlands), 0.39 µg per mL dexamethason, 100 µg per mL pyrovate, 50 µg per mL uridine (all Sigma), 5 µg per mL Gentamycin (Gibco), 10 ng per mL epidermal growth factor (Collaborative Research, MA, USA) and 15%

FCS. A number of 30,000 cells were obtained out of the biopsy (64 x 10^3 cells per g tissue). 8000 cells of the established cell suspension were cultured as mixed culture in a 6 well plate. First subcultivation using a split ration of 1:4 was carried out when cells covered the entire bottom of the well plate with a confluent monolayer of myoblasts.

Myoblast clonal cultures were established out of the cell suspension by manual cloning using a micropipette und a microscope in two 96 well plates, each well containing 0.1 mL GM. The three clones with the highest growth speed and juvenile morphologic phenotype were selected for further culturing and testing.

Both, the mixed culture and the three clones were frozen at early passage and simultaneously cultured as secondary cultures in GM for 100 days. Cultures were subcultured under standardized conditions using a trypsine (0.05%) EDTA solution when 95-100% cell confluence was reached using a spit ratio of 1:4. The cumulative population doubling (PD) level was calculated by tracking the increase in cell number in sequential passages and therefore the sum of all previous PDs. All three myoblast clonal cultures reached the onset of senescence which was defined as failure of the culture to reach confluence within three weeks.

Myogenic origin of all culture was tested by differentiation of the cultures and desmine staining (Dako, Enschede, the Netherlands). All cultures proved to form myotubes.

Oxidative stress and β-galactosidase staining

To assess senescence associated β-galactosidase activity, myoblasts were seeded in Permanox chamber slides (Nunc, VWR, Amsterdam, the Netherlands) and grown up to 40-50% confluence. Hereafter, cultures were exposed to hydrogen peroxide (H_2O_2, Merck) using concentrations of 1 mM and 5 mM for 30 min. After exposure, cells were washed with GM. After three days, cultures were fixed using formalin (3.7%, Merck) and stained for β-galactosidase activity, which was measured using the cytochemical assay described by Dimri *et al*. (1995). In brief, adhered myoblasts were washed two times in phosphate-buffered saline (PBS),

fixed with 3% formaldehyde in PBS for 3 min, washed two times in PBS, and then stained for 18 hours at 37°C with fresh β-galactosidase staining solution. The solution contained 1 mg per mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) in dimethylforamide (20 mg X-Gal per mL stock), 40 mM citric acid, 40 mM sodium phosphate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$, pH 6.0. Formaldehyde was purchased from Merck and all other reagents were obtained from Sigma. After staining, cells were washed twice with PBS. Cells were counterstained with Mayers–Hematoxylin staining solution (Sigma) for 5 min and washed twice in PBS. Only myoblast cultures at subconfluence (maximal 75% confluence) were analyzed. Cells were viewed by phase contrast on a Leica microscope (Leica Microsystems, Rijswijk, the Netherlands) and photographed at a 100×magnification by a digital color camera. Experiments were conducted in duplicate and for each experiment at least 500 randomly selected cells were counted. The number of positive, blue cells was divided by the total number of counted cells resulting in the percentage of β-galactosidase positive cells. Results from both experiments were averaged.

L-Carnosine supplementation

At day 30 after establishment of the cultures, these were split up in cultures fed with regular GM and GM supplemented with L-carnosine (20 mM, ≥ 90.0% pure, BioChemika, Zwijndrecht, the Netherlands). Cultures were refreshed twice a week with both media up to 100 days.

The myoblast mixed culture was exposed to oxidative stress after supplementation with L-carnosine for 14 days. β-Galactosidase activity was measured three days after exposure.

Results

After initiation of the cultures, the myoblast clonal cultures and the myoblast mixed culture did not significantly differ in growth speed being 0.26 PD per day.

As shown in Figure 1, we found a significantly lower remaining replicative capacity of all three myoblast clonal cultures that varied between 17.5 and 27.1 PDs compared to the myoblast mixed culture undergoing 43 PDs.

To test the relation between the remaining replicative capacity and resistance to oxidative stress, two myoblast clonal cultures as well as the myoblast mixed culture were exposed to hydrogen peroxide during the early replicative phase and tested for β-galactosidase activity, as marker for cellular senescence. As shown in Figure 2, after exposure to hydrogen peroxide, a significantly higher β-galactosidase activity was found in the myoblast clonal cultures compared to the myoblast mixed culture. Within the myoblast clonal cultures, the one with the lowest remaining replicative capacity expressed the highest β-galactosidase capacity after exposure to oxidative stress.

Figure 1. Heterogeneity between growth kinetics of three myoblast clonal cultures and a mixed myoblast cell culture all obtained from one muscle biopsy.

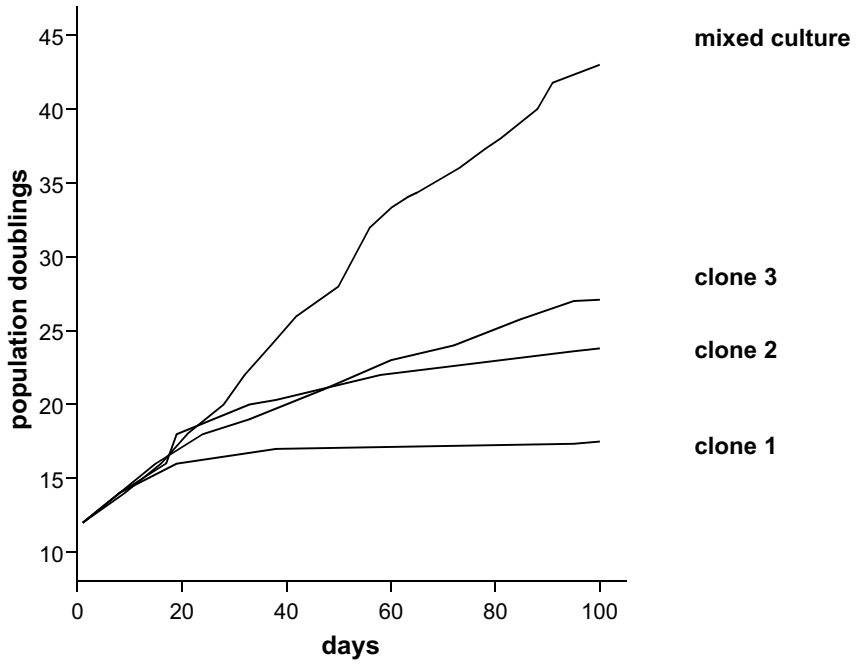
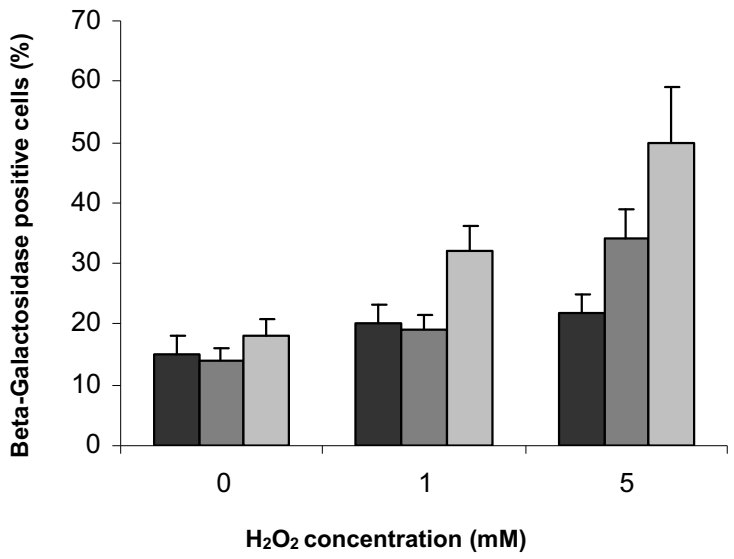


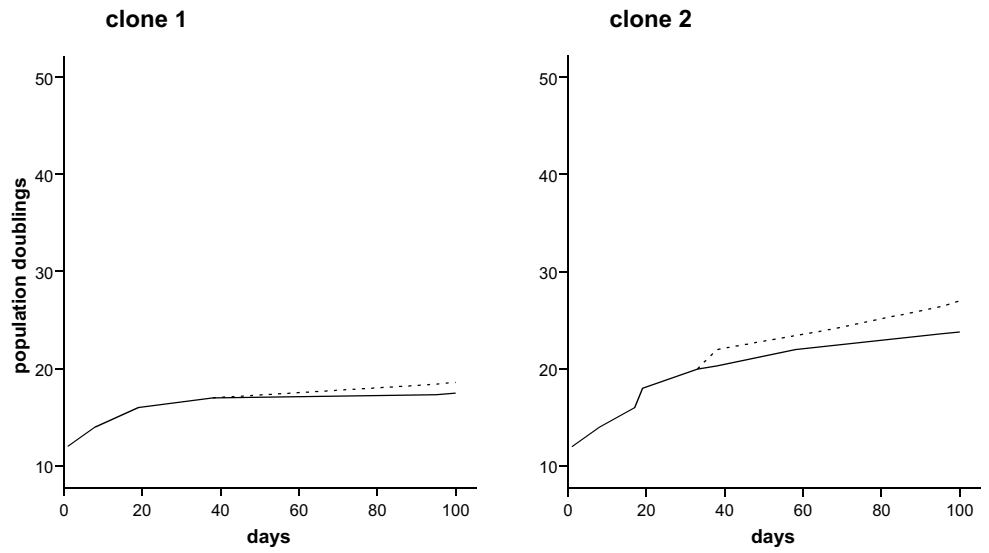
Figure 2. β-galactosidase activity (mean ± standard deviation) at three days after exposure to oxidative stress by hydrogen peroxide; myoblast mixed culture (black bars), 'clone 2' (grey bars), 'clone 1' (light bars).



To test if the replicative capacity of the cultures can be influenced by supplementation of specific nutrients, which have been suggested to influence sarcopenia, cultures were incubated with 20 mM L-carnosine upon day 30 after establishment of the cultures. Figure 3 shows that the growth speed of all cultures as well as the maximal replicative capacity increases after continuous exposure to L-carnosine. The effect was small during the first exposure period of the myoblast mixed culture when growth speed maintained at high level.

However, the β-galactosidase activity of the myoblast mixed culture during the early replicative phase did not significantly decrease after L-carnosine supplementation for a period of 14 days (16.9% vs 15.0%). When the mixed culture was incubated in the presence of L-carnosine (20 mM) prior to exposure to oxidative stress by hydrogen peroxide, a marginally decrease in β-galactosidase activity was observed (1 mM H_2O_2: 23.2% versus 19.5%; 5 mM H_2O_2: 41.1% versus 35.2%).

Figure 3. Growth characteristics of myoblast cultures supplemented with 20 mM L-carnosine upon day 30 after initiation of the culture (broken line) compared to cultures without L-carnosine supplementation (continuous line).



Discussion

We found marked heterogeneity in replicative capacity between myoblast clonal cultures when compared to the replicative capacity of a myoblast mixed culture that all originated from the same muscle biopsy. Impaired replicative potential was mirrored by a higher expression of β-galactosidase activity after exposure of the various clones to oxidative stress. Addition of L-carnosine reversed the replicative capacity by a limited number of extra PDs in all cultures studied.

Myofiber regeneration during life depends on the proliferation and fusion of adjacent satellite cells. The number of PDs of myoblasts *in vitro* may thus represent the remaining replicative potential of satellite cells dependent on the replicative history *in vivo*. Despite stressors as oxidative radicals may affect all satellite cells equally, replicative histories of satellite cells may well be different as damage may occur locally and randomly. The marked heterogeneity in remaining replicative capacity between the various clones that are reported here are in line with this notion. Our results in man concur with earlier observations in turkeys showing that satellite cells are composed of a heterogeneous population that reflect their replicative capacity as measured by colony sizes (Rouger *et al*., 2004).

Cells out of a biopsy form a mixture of clones with varying growth potential because of difference in their *in vivo* history (Bayreuther *et al*., 1988). Satellite cells with certain cellular characteristics, namely a high growth speed, which have also been associated with characteristics in differentiation (Rantanen *et al*., 1995; Rouger *et al*., 2004), and a highly remaining replicative capacity will dominate the culture during the phase of steady proliferation. The commitment theory of cellular ageing predicts that an uncommitted cell population doubles in size until the first cells die, thereafter cells grow at a reduced but steady rate until the last clone has reached the senescent state (Holliday *et al*., 1977). Therefore, it is not surprising that the replicative capacity of the mixed culture, dominated by the clones with the highest replicative capacity and growth speed, was superior to that of the individual clones.

Oxidative damage is generally assumed to negatively influence replicative capacity, an effect that has predominantly been studied in human fibroblast cultures (Chen *et al*., *1995*; von Zglinicki *et al*., 1995). In rat and human skeletal muscle, a decreased anti-oxidative capacity is present in an age dependent manner (Hepple *et al*., 2003; Fulle *et al*., 2005), which suggests a clear role in the progression of sarcopenia and its significant contribution of the regenerative potential of muscle fibers (Fulle *et al*., 2004). Renault *et al*. (2002) showed a dramatic decrease of viable myoblasts after treatment with hydrogen peroxide, whereas the replicative capacity of the remaining cells, forming the mixed culture, was only slightly decreased and differentiation not affected. Our experiments focused on the heterogeneity between myoblast clones and myoblast mixed cultures. In line with the superior replicative potential of the myoblast culture, the mixed culture was far more resistant to oxidative stress as measured by the induction of β-galactosidase activity. As we also found expression of β-galactosidase activity after exposure to oxidative stress to be proportional to the remaining replicative capacity of the cultures, these findings suggest that satellite cells have accumulated different loads of oxidative damage and / or replicative history.

L-carnosine, an endogenous cytoplasmatic dipeptide (beta-alanyl-L-histidine) is predominantly present in skeletal muscle / neuronal cells (Kohen *et al*., 1988) and possesses several biological functions, including pH buffering (Harris *et al*., 1990; Boldyrev *et al*., 1993), anti-oxidation (Boldyrev *et al*., 1993), membrane stabilization, sensitizing the contractile apparatus to Ca^{2+} and potentiating Ca^{2+} release (Lamont and Miller, 1992). The production decreases in an age-dependent manner. Here, for the first time we showed that L-carnosine continuously supplemented to the culture medium, increases the growth speed as well as the replicative potential of human myoblasts, which underlines the anti-oxidant free radical scavenging property of L-carnosine. Extended *in vitro* lifespan in the presence of L-carnosine has also been reported in fibroblasts cultures (Shao *et al*., 2004; McFarland and Holliday, 1999) and in T-helper cells (Hyland *et al*., 2000).

The cellular rejuvenation induced by L-carnosine has been related to a reduction in telomere damage and shortening rate (Shao *et al*., 2004). In fibroblasts cultures at the end of their replicative lifespan β-galactosidase activity was lower in addition of L-carnosine (Shao *et al*., 2004); however, in our preliminary set of experiments β-galactosidase activity in the mixed myoblast culture was only marginally reduced after exposure to oxidative stress.

It is concluded that myoblast mixed cultures *in vitro* do not reflect the marked heterogeneity in replicative capacity between single isolated satellite cells and may overestimate the remaining replicative capacity and with that the remaining fiber regenerative capacity *in vivo*. There is a need for focus on the consequences of the heterogeneity of single satellite cells on muscle performance in aged individuals suffering from sarcopenia.

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