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Telomerase protects adult rodent olfactory ensheathing glia from early senescence ☆

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ABSTRACT

Adult olfactory bulb ensheathing glia (OB-OEG) promote the repair of acute, subacute, and chronic spinal cord injuries and autologous transplantation is a feasible approach. There are interspecies differences between adult rodent and primate OB-OEG related to their longevity in culture. Whereas primate OB-OEG exhibit a relatively long life span, under the same culture conditions rodent OB-OEG divide just three to four times, are sensitive to oxidative stress and become senescent after the third week *in vitro*. Telomerase is a "physiological key regulator" of the life span of normal somatic cells and also has extratelomeric functions such as increased resistance to oxidative stress. To elucidate whether telomerase has a role in the senescence of rodent OB-OEG, we have introduced the catalytic subunit of telomerase mTERT into cultures of these cells by retroviral infection. Native and modified adult rat OB-OEG behaved as telomerase-competent cells as they divided while expressing mTERT but entered senescence once the gene switched off. After ectopic expression of mTERT, OB-OEG resumed division at a nonsenescent rate, expressed p75 and other OEG markers, and exhibited the morphology of nonsenescent OB-OEG. The nonsenescent period of mTERT-OEG lasted 9 weeks and then ectopic mTERT switched off and cells entered senescence again. Our results suggest a role of telomerase in early senescence of adult rodent OB-OEG cultures and a protection from oxidative damage. This article is part of a Special Issue entitled: Understanding olfactory ensheathing glia and their prospect for nervous system repair.

Introduction

Olfactory bulb ensheathing glia (OB-OEG) are unique cells capable of supporting axonal elongation within the adult mammalian CNS throughout life (Graziadei and Graziadei, 1979; Doucette et al., 1983; Raisman, 1985). Transplants of these cells were first used to foster the regeneration of injured sensory axons into the spinal cords of adult rats (Ramon-Cueto and Nieto-Sampedro, 1994). Then, subsequent studies demonstrated that implants of adult OB-OEG promote functional recovery and regeneration of damaged axons after spinal cord injuries (SCI) of various types and severities (reviewed in Franssen et al., 2007), including complete transection (Imaizumi et al., 2000; Kubasak et al., 2008; Ramon-Cueto et al., 2000) and contusion (Plant et al., 2003). In most of these studies, OB-OEG were grafted immediately after injury (reviewed in Franssen et al., 2007), but recently, the repair efficacy of adult OB-OEG transplants after chronic SCI, and also, the feasibility of an autologous therapy with these cells have been demonstrated (Munoz-Quiles et al., 2009; Rubio et al., 2008). Under normal culture conditions, adult native nonhuman primate (Guest et al., 2008; Rubio et al., 2008) and human OB-OEG (Barnett et al., 2000; Lim et al., 2010) divide for more than 2.5 months and up to 34 passages, yielding enough cells for either auto- or allografting and for storage. Successful results in experimental animals with SCI and the availability of large numbers of adult native OB-OEG are strengthening the idea that transplantation of these cells might constitute a future treatment for this pathology in humans.

Most of the experimental studies involving OB-OEG transplantation for neural repair have been carried out in rodents. However, in contrast to adult OB-OEG from other species, including humans (Barnett et al., 2000; Krudewig et al., 2006; Lim et al., 2010; Techangamsuwan et al., 2008), these cells from adult rodents become presenescent immediately after plating, senescent after the third week in culture and are sensitive to the oxidative stress of the hyperoxic environment of normal incubators (Rubio et al., 2008). This species-related differential growth and *in vitro* behaviour exhibited by adult OB-OEG also occur in other cell types (Parrinello et al., 2003; Shimizu et al., 2003; Steinert et al., 2002). The number of times a cell divides *in vitro* before senescence is variable depending on the cell type, species, and organism of origin (Hayflick, 1965; Martin et al., 1970; Rohme, 1981) and sensitivity to the artificial *ex vivo* environment (Parrinello et al., 2003; Sherr and DePinho, 2000). Telomerase is considered a "physiological key regulator" of the life span of normal somatic cells (Kilian et al., 1997; Kim et al., 1994; Meyerson et al., 1997; Nakamura et al., 1997; Nakayama et al., 1998).

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Its catalytic subunit TERT adds telomeric repeats onto the ends of chromosomes, and cell growth is arrested when telomeric DNA cannot be maintained above the critical length (reviewed in Cong and Shay, 2008; Cong et al., 2002; Poole et al., 2001). However, there are interspecies differences in TERT regulation. Whereas most adult human somatic cells do not express TERT, rodent cells depend on telomerase for division and the absence of this enzyme leads to senescent growth arrest (reviewed in Cong et al., 2002; Ducrest et al., 2002; Forsyth et al., 2002). Telomerase has additional extratelomeric physiological functions such as increased resistance to stress, regulation of apoptosis, and cell survival (Cong and Shay, 2008; Santos et al., 2004). Under hyperoxic conditions, telomerase is translocated from the nucleus to the mitochondria, and here it diminishes superoxide production and cellular ROS levels protecting cells from oxidative damage (Ahmed et al., 2008; Cong and Shay, 2008; Santos et al., 2004). Ectopic expression of TERT extends the life span of a number of human cell types (Jiang et al., 1999; Morales et al., 1999; Murasawa et al., 2002; Shi et al., 2002; Simonsen et al., 2002; Yang et al., 1999) and also confers increased resistance against oxidative stress and OH-induced apoptosis and protects mtDNA from damage (Armstrong et al., 2005; Murasawa et al., 2002; Ren et al., 2001). Same as most adult human somatic cells, canine, and human adult OB-OEG do not express TERT and thus divide *in vitro* by a telomerase-independent mechanism (Lim et al., 2010; Techangamsuwan et al., 2008, 2009). However, the vast majority of studies using adult OB-OEG for neural repair involve rodent cells and nothing is known about telomerase competency in these cells. To elucidate whether telomerase is involved in their presenescent state in culture and their early entrance into senescence, we have analysed the levels of TERT mRNA in nonsenescent and senescent adult rat OB-OEG because this is a direct indicator of the transcriptional state of the telomerase gene. In addition, we have introduced TERT into adult rodent OB-OEG to determine whether these cells overcome senescence and can be protected from oxidative damage. Our study may help to better extrapolate experimental findings in rodents to other species.

Materials and methods

The experimental procedures adhered to the recommendations of the European Union and the US Department of Health for the care and use of laboratory animals and were approved by the ethics committee of our institution.

Culture of nonsenescent olfactory bulb ensheathing glia (OB-OEG)

Cultures were set from adult (2–2.5 months old) Wistar Hannover rats (Harlan Laboratories, Barcelona, Spain). For each culture, we used the olfactory nerve and glomerular layers of eight olfactory bulbs (OB) as previously described (Ramon-Cueto and Nieto-Sampedro, 1992; Rubio et al., 2008). A week later, OB-OEG were selected by immunoaffinity using an antibody against p75 (nonsenescent OEG are p75 positive), as described previously (Ramon-Cueto et al., 1998; Rubio et al., 2008). Purified p75-OB-OEG were seeded onto poly-L-lysine-coated (10 μg/ml; Sigma; average molecular weight, 30,000) 25-cm^2 culture flasks and grown in 1:1 DMEM/Ham's F-12 medium (Gibco-Invitrogen, Madrid, Spain) supplemented with 10% heat-inactivated fetal bovine serum (D/F-10S). Culture medium was replaced every 2 days.

The most efficient culture medium to induce the proliferation of adult rat OB-OEG contains 10% serum, heregulin, FGF, and 2–4 μM forskolin; and the latter to potentiate the effects of the mitogens (Yan et al., 2001). Accordingly, 2 days after plating, we added to the culture medium 2 μM forskolin (Sigma) and 20 μg/ml bovine pituitary extract (Biomedical Technologies, Inc.). Pituitary extract was used as a source of FGF (Hadden et al., 1989; Kent and Bomser, 2003) and heregulin (De Mello et al., 2007). We decided to add pituitary extract because it also contains other mitogenic factors for adult rat OB-OEG such as PDGF and IGF-1 (Yan et al., 2001) and provides comparable results for OEG proliferation (De Mello et al., 2007). In addition, pituitary extract provides remarkable protection again oxidative stress (Kent and Bomser, 2003).

Transfection of packaging cells and retroviral production

The retroviral construct pBabe-mTERT linked to a puromycin resistance gene was kindly provided by Dr María Blasco from Centro Nacional de Investigaciones Oncológicas (Martin-Rivera et al., 1998). Phoenix packaging cells were transfected with this construct to produce virus as follows. We used the Phoenix Amphotropic packaging cells, which are based on the 293T cell line (a human embryonic kidney line transformed with adenovirus E1a and carrying a temperature sensitive T antigen coselected with neomycin). These cells were also provided by Dr. Maria Blasco (Sachsinger et al., 2001). Phoenix cells were seeded in Petri dishes at a density of 5×10^6 per 10 cm^2 and grown in DMEM + 10% FBS (D-10S). One day after platting, cultures were approximately 70% confluent. For transfection, we mixed 900 μl of a solution containing 20 μg DNA construct in H_2O with 100 μl of $CaCl_2$ 2.5 M, and while bubbling, we added 1 ml of Hank's balanced salt solution (HBSS, double concentrated; GIBCO). The resulting solution was added dropwise to the plates containing the Phoenix cells. After incubating at 37 °C for no more than 20 hours, we replaced the solution and added DMEM containing 1 μM of dexamethasone and 1 mM Na-butyrate, and dishes were placed in an incubator at 32 °C. After 48 h, virus-containing supernatants were collected, and dishes were incubated with 10 ml of fresh medium (the same as before) at 37 °C for another 2 days before collecting the second supernatant. Supernatants from all dishes were filtered (0.45-μm pore) and stored at −80 °C for future infections. Before use, these supernatants were centrifuged at 20,000 rpm for 5 hours, and the pellets were resuspended in fresh D/F-10S medium in 25-fold less volume than the initial one (10 ml/plate) to concentrate the virus 25×.

Retroviral infection of adult rat OB-OEG

Sixteen rats were used in this study. We used both bulbs of four rats for each primary culture to have enough cells from the same culture for all the experimental variables (five). Thus, we set 20 flasks containing cells from four different primary cultures.

Retroviral infections require that host cells divide, and thus, infections were carried out before OEG senescence (before the third week *in vitro*) (Rubio et al., 2008). All cultures used in the study contained nonsenescent OB-OEG and no other contaminant cells (all cells were p75, GFAP, S100, and O4 positive) (Rubio et al., 2008). After 2 weeks *in vitro* (1 week after immunopurification), OB-OEG were detached from the flasks, and cells from each culture plated into five 60-mm Petri dishes at a density of 300,000 cells per dish. Three dishes of each culture were infected with pBabe-mTert, pBabe-LacZ, and pBabe alone, respectively. PBabe-LacZ-infected cultures were used to monitor transduction efficiency. This was between 30% and 50%. In addition, Pbabe and Pbabe-LacZ cultures were used to evaluate any possible effect of a nontransducing (the former) and a transducing (the latter) retrovirus on OB-OEG phenotypic properties. We wanted to make sure that any change observed in Pbabe-mTERT OEG was due to the expression of mTERT and not to the vector, the infective process, or the expression of an exogenous gene. The remaining two Petri dishes per experiment were subjected to all the infection procedure except for the incubation with the virus and one was treated with puromycin while the other was not. The former was used to determine the moment when transduced cells were selected after puromycin treatment of the cultures. The latter was used to control