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Expression of senescence-associated β-galactosidase (SA-β-Gal) by human skin fibroblasts, effect of advanced glycation end-products and fucose or rhamnose-rich polysaccharides

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ABSTRACT

Expression by cells of the SA-β-Gal was shown to be a reliable indicator of the switch mechanism used by cells to enter the senescent phenotype. We used this method in order to explore the variation of SA-β-Gal-positive cells with passage number and time spent in culture. Both parameters produced an increase of SA-β-Gal-positive cells. The addition of a Maillard-product (advanced glycation end-product = AGE) to the fibroblast cultures also increased SA-β-Gal expression. Fucose- and rhamnose-rich oligo- and polysaccharides (FROPs and RROPs, respectively) provided a significant protection against this AGE-induced increase of SA-β-Gal-positive cells. It is speculated that these processes might well play an important role in skin aging.

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1. Introduction

Cell-aging and its modulation are one of the most intensely pursued research areas in experimental gerontology. Since the first publications by Hayflick and Moorhead on the limited proliferative capacity of normal, non transformed cells, this experimental approach became one of the most investigated models of in vitro aging (see for review, Hayflick, 1977; Macieira-Coelho, 1988). Although reproduced in a number of laboratories (Martin et al., 1974; Goldstein and Harley, 1979; Azzarone et al., 1984) this approach to understand cell-aging stirred up serious criticism also (Rubin, 1997). More recently experiments with cells from non-agerians showed, that chronological age should not be considered as the only important factor in the decline of proliferative capacity (Maier et al., 2007). This criticism is valid also for the most frequently cited explanation of the limited capacity for continued proliferation of mitotic cells, which is related to telomere shortening with, however, some reservations (Macieira-Coelho, 2000). Quite logically the idea emerged that other markers of cell-aging might be more relevant for the characterization of cell-aging. One marker that emerged as a result of the work of Judith Campisi and her team is the expression of the SA-β-Gal (Dimri et al., 1995). One of the important underlying propositions is the existence of switch mechanisms enabling cells to enter senescence to escape malignant transformation (Campisi, 2001, 2003a,b, 2005a,b; Labat-Robert and Robert, 2007). This proposition is based on a number of observations and might well contribute to the understanding of the age-dependent variations of the acquisition of the senescent phenotype and also to the age-dependent variation of tumor-susceptibility (Macieira-Coelho, 2001). Some of the anti-oncogens, as p53, p16, pRb and others were proposed to play an important role in these switch mechanisms (for review see: Campisi, 2005b; Labat-Robert and Robert, 2007). One of the advantages of this concept is to propose a more practical experimental approach to the in vitro study of cell-aging and its modulation, than the fastidious and lengthy serial cell cultures. For these reasons we adapted the methodology proposed by the Campisi-team (Dimri et al., 1995) in order to explore the modulation of cell-senescence by AGEs and its inhibition by FROPs and RROPs. These experiments will be described.

2. Materials and methods

The fibroblast strain used came from Cambrex (Emerainville, France), normal human dermal fibroblasts derived from the skin biopsy of a 39 years old woman (NHDF-Adult, cryopreserved, Product Code CC-2511, Lot no. 4F1293). Cells were cultured in Dulbecco-modified Eagle's medium (DMEM-glutamax, Invitrogen) supplemented with 10% fetal calf serum (FCS, GIBCO), antibiotics: penicillin (100 U/ml, GIBCO), streptomycin (100 μg/ml, GIBCO), and an antifungic (Amphotericin B, 0.25 μg/ml, GIBCO). Cultures in

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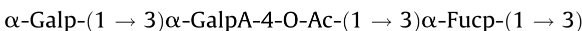
75 cm^2 ventilated flasks (Nun) were kept in a 37 °C O_2/CO_2 thermostat and subcultured after trypsinisation (0.05% trypsine, GIBCO). Culture medium was changed every 2–3 days, cells used between 8th and 14th passages, seeded in 12-well culture-plates (Nunc) at a density of 5×10^4 cells per well, in the above-described culture medium.

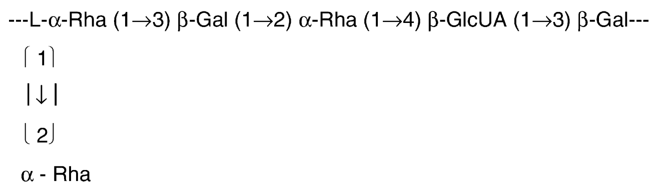
The preparation of AGE-products was described previously (Fodil-Bourahla et al., 2003; Andres et al., 2006). In the present experiments we used a preparation obtained by the incubation at 37 °C in sterile conditions and constant shaking, of BSA (Sigma–Aldrich, 1%), glucose 11 mM and $FeCl_2$ 0.01 M, for 4 weeks, followed by the purification on a Sephadex G25 column as described (Andres et al., 2006; Ravelojaona et al., 2006). Ten micrograms of this preparation was added to the cells in 12-well plates and incubated with or without the polysaccharides for 48 h.

Detection of SA-β-Gal-positive cells was done using the kit from OZ Biosciences (Marseilles, France) according to the indications provided with the kit. Cells were fixed and incubated with a solution containing the β-gal substrate, abbreviated X-gal (5-bromo-4-chloro-3-indoxyl-β-galactoside). After 24 h incubation the "blue" cells expressing SA-β-Gal were counted under the microscope.

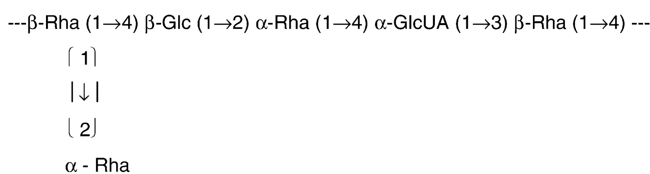
FROPs and RROPs were obtained from Solabia-BioEurope (Pantin, France) as sterile powders. They were dissolved in sterile PBS for further use at concentrations indicated in the tables. Their preparation and structure were described (Fodil-Bourahla et al., 2003; Péterszegi et al., 2003, 2006). Shortly, the FROPs came from the culture of a non-pathogenic strain of *Klebsiella pneumoniae*. The original polysaccharide (commercial name Fucogel®, FROP-1) had an average molecular weight of about 40 kDa. Its repetitive structure is



This polysaccharide was degraded by endoglycosidases to yield oligosaccharides. The one used in the here described experiments, designed FROP-3, consisted essentially of hexasaccharides and octasaccharides. The RROPs were also obtained from bacterial strains. The RROP-1 compound (commercial name Rhamnosoft®) of about 45 kDa molecular weight and the other polysaccharide RROP-2 (commercial name BEC 291®) of about the same molecular weight were obtained from a *Klebsiella planticola* strain. Both RROP-1 and RROP-2 were degraded by mild acid hydrolysis (HCl) to oligosaccharides. A 5 kDa average molecular weight oligosaccharide designated RROP-3 was obtained from RROP-2, and other two oligosaccharides, RROP-4 of 14.5 kDa, and RROP-5 of 5 kDa molecular weight derived from RROP-1. The structure of RROP-1 is



The structure of RROP-2 is



For more details, see Fodil-Bourahla et al. (2003), Péterszegi et al. (2003, 2006), and Andres et al. (2006). The EROB-mixture contains the following polysaccharides: 30% of FROP-3, 30% of RROP-1 and 20% of RROP-3.

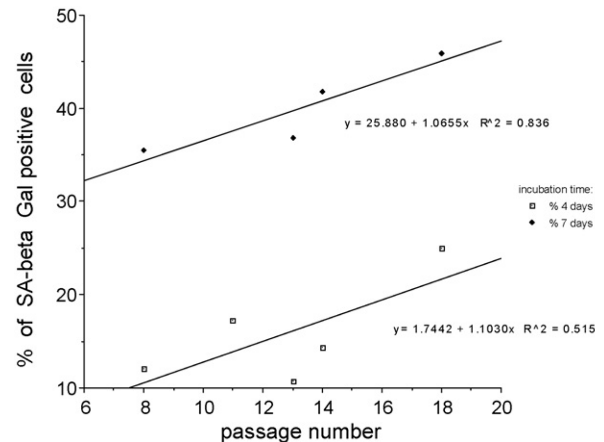


Fig. 1. Increase with passage number of SA-β-Gal expressing cells in human skin fibroblast cultures after 4 and 7 days of culture.

The following protocol was used for the incubation of cells with AGE-products and/or polysaccharides. Cells (4×10^4) were seeded per well in the Nunc-plates and left for 24 h to adhere to the culture dish. AGEs and/or polysaccharides were added as indicated in the tables and incubated for 48 h. After this period, 4 days of incubation, cells were stained for SA-β-Gal. Other cultures were further incubated for 3 more days, 7 days altogether before staining.

3. Results

3.1. SA-β-GAL expression in fibroblasts, effect of an AGE-preparation

Fig. 1 shows the proportion of SA-β-Gal-positive cells at successive passages of the fibroblasts, at 4 and 7 days of incubation. After an increased culture period from 4 to 7 days the average proportions of SA-β-Gal-positive cells increased from 15.0 ± 0.53% to 39.75 ± 4.98% ($p < 0.000072$). This shows that during an extended culture period the proportion of SA-β-Gal-positive cells increased considerably. Moreover, at both incubation times, the proportion of SA-β-Gal-positive cells increased with passage number.

Table 1 shows the effect of the AGE-product tested on SA-β-Gal expression at increasing passages. After 2–4 days of incubation SA-β-Gal expression increased considerably, in the five consecutive experiments. No evident explanation could be found for the differences. The decreasing tendency at 4 days of incubation with increasing passage number might be attributed to a decreasing susceptibility to AGE-effects with increasing passage number and/or a slow evolution of the composition of the AGE-preparation used (the glycoxidation products are unstable and change with time). After a longer incubation time, 7 days, the increase of positively

Table 1
Effect of an AGE-product on SA-β-Gal expression in human dermal fibroblast cultures at different passages

Passage number	4 days culturing % increase blue cells	$p<$	7 days culturing % increase blue cells	$p<$
8	232.26 ± 5.33	0.00026	132.62 ± 9.72	0.049
8	131.62 ± 2.13	0.017		
13	173.97 ± 6.92	0.012	159.95 ± 6.73	0.38 (N.S.)
14	129.69 ± 2.46	0.0012	129.80 ± 2.50	0.02
18	103.82 ± 2.20	0.44 (NS)	115.31 ± 5.97	0.075 (NS)

The AGE-product was added at a concentration of 1.5 μM. The proportion of positively staining cells is expressed as a percentage above the control value taken as 100%. For other details see Section 2 (mean ± S.D.).

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