Cellular stress in vitro and longevity in vivo

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Chapter 2

Rapid flow cytometric method for measuring Senescence Associated-β-galactosidase activity in human fibroblasts

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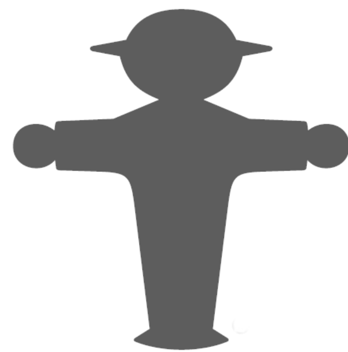

Summary

Senescence Associated-β-galactosidase (SA-β-gal) activity is a widely used marker for cellular senenescence. SA-β-gal activity is routinely detected cytochemically, manually discriminating negative from positive cells. This method is time-consuming, subjective and therefore prone to operator-error. We aimed to optimize a flow cytometric method described by other workers using endothelial cells to better differentiate between populations of fibroblasts in degrees of SA-β-gal activity. Skin fibroblasts were isolated from young (mean±SD age: 25.5±1.8) and very old (age 90.2±0.3) subjects. Different pH modulators were tested for toxicity. To induce stress-induced senescence, fibroblasts were exposed to rotenone. Senescence was assessed measuring SA-β-gal activity by cytochemistry (X-gal) and by flow cytometry (C_{12}FDG). The pH modulator Bafilomycin A1 (Baf A1) was found to be least toxic for fibroblasts and to differentiate best between non-stressed and stressed fibroblast populations. Under non-stressed conditions, fibroblasts from very old subjects showed higher SA-β-gal activity than fibroblasts from young subjects. This difference was found for both the flow cytometric and cytochemical methods (p=0.013 and p=0.056 respectively). Under stress-induced conditions the flow cytometric method but not the cytochemical method revealed significant higher SA-β-gal activity in fibroblasts from very old compared with young subjects (p=0.004 and p=0.635 respectively). We found the modified flow cytometric method measuring SA-β-gal activity superior in discriminating between degrees of senescence in different populations of fibroblasts.

Introduction

Cellular senescence can be induced by exhaustion of replicative capacity (1) or exposure to cellular stress (2). A variety of cellular markers of senescence have thus far been identified, amongst which there are cellular morphology (3), telomere length (4) and Senescence Associated-β-galactosidase (SA-β-gal) activity (5-8). β-Galactosidase is a collective name for enzymes which cleave non-reducing β-D-galactose residues from glycoproteins, sphingolipids and keratan sulfate in β-D-galactosides (9). These enzymes function optimally at pH 4. In senescent cells, β-galactosidase activity can also be detected at pH 6, although the function of SA-β-gal at this pH remains unknown (6).

SA-β-Gal activity can be cytochemically detected using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) as a substrate. Fibroblasts are stained, digitally recorded and SA-β-gal positive fibroblasts are manually counted and expressed as a percentage of total fibroblasts (6-8). Subjectivity, i.e. a low inter-rater reproducibility, is the main disadvantage of the method, and the procedure is highly time consuming. Kurz *et al.* (10) used a method based on flow cytometry to quantify SA-β-gal activity in endothelial cells (HUVEC). The flow cytometric method is not subjective and has a high throughput when compared with the cytochemical method. Kurz *et al.* (10) found the flow cytometric method to correlate well with the cytochemical method when using HUVECs.

Although the use of human fibroblasts was also exemplified in the paper by Kurz *et al.* (10), here we further extend the applications of the flow cytometric method in fibroblasts by performing the experiments under conditions of lysosomal alkalinisation and measuring stress induced premature senescence (SIPS). In this manuscript, we describe the optimization of the flow cytometric procedure, for use in human diploid fibroblasts and its comparison to the conventional cytochemical method. To investigate if the two methods are equally able to discriminate SA-β-gal activity between subpopulations of fibroblasts, we have compared fibroblast strains derived from young and very old subjects, both under non-stressed and stressed conditions.

Materials & methods

Fibroblast Cultures

Fibroblast strains were obtained from participants of the Leiden 85-plus Study, a population-based follow up study in which 599 inhabitants of Leiden, the Netherlands, aged 85 years took part (11). Skin biopsies were taken as described earlier (12) at the age of 89 or 90 years. In order to have a contrast in chronological age, skin biopsies were also obtained from young subjects (mean±SD age: 25.5±1.8 years).

Biopsies were taken from the sun unexposed medial side of the upper arm and cultured under standardized conditions (12) at 37℃, 5%CO_2 and 100% humidity in D-MEM:F-12 supplemented with 10% fetal calf serum (FCS, Gibco, batch no. 40G4932F), 1 mM MEM sodium pyruvate, 10 mM HEPES, 2 mM Glutamax and antibiotics (100 Units/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B). This medium will be referred to as growth medium. All reagents were obtained from GIBCO, Breda, the Netherlands. When cultures reached 80% to 90% confluence, fibroblasts were subcultured using a 1:4 split ratio.

Experiments for optimization of the sample preparation were performed with two fibroblast strains from young subjects. Immunohistochemical double staining for p16 and SA-β-gal was performed using three randomly chosen strains under non-stressed and stressed conditions as well as one high passage strain that had undergone 79 population doublings. After optimization, fibroblast strains from ten young and ten very old subjects, which were randomly selected from the Leiden 85-plus Study, were compared.

Induction of cellular stress

Fibroblasts were seeded at a density of 2.6×10^4 fibroblasts per 25-cm^2 flask and in Permanox 2-chamber slides (Nunc, VWR, Amsterdam, the Netherlands) at 1.0×10^4 fibroblasts per chamber (4.2 cm^2/chamber). In pilot experiments, fibroblasts were stressed with 0.2 µM - 1 µM rotenone for three days (data not shown). Exposure to 0.6 µM rotenone induced considerable senescence after three days and this concentration was subsequently used to stress fibroblast strains from young and very old subjects.

Four hours after seeding, medium was replaced by growth medium supplemented with 0.6 μM rotenone. Fibroblasts were incubated for 72 hours in rotenone-supplemented medium, after which they were analyzed. All experiments were repeated.

Lysosomal pH adjustment

Lysosomal pH was increased to pH 6 by adding bafilomycin A1 (baf A1) or chloroquine (10;13) to the growth medium. Nigericin in a buffered solution was used to change the pH of all intracellular compartments (10;14;15).

Baf A1: Fibroblasts were incubated for one hour in growth medium supplemented with 100 nM baf A1 at 37℃, 5% CO_2, and one hour in growth medium with 100 nM baf A1 and 33 μM 5-Dodecanoylaminofluorescein-di-β-D-galactopyranoside ($C_{12}FDG$) prior to analysis.

Chloroquine: Fibroblast were incubated for two hours in growth medium containing 100 or 300 μM chloroquine at 37℃, 5% CO_2, and one hour in growth medium with 100 or 300 μM chloroquine and 33 μM $C_{12}FDG$.

Nigericin (10 μM) was added to the culture in the presence of a potassium-rich 2-(N-morpholino) ethanesulfonic acid (MES) buffer (150 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM Glucose, 10 mM MES buffer), pH 6, and incubated for 15 minutes at 37℃, not CO_2 enriched, after which $C_{12}FDG$ was added (33 μM) followed by one hour incubation at 37℃, not CO_2-enriched.

Measurement of toxicity pH-modulators

Toxicity of pH modulators was determined by measuring apoptosis and necrosis using the TACS Annexin V-FITC kit (R&D Systems, Abingdon, United Kingdom) and a FACSCalibur flow cytometer (BD, Oxford, United Kingdom). Events were gated into quadrants and Annexin V positive/propidium iodide (PI) negative (Annexin V+/PI-) and Annexin V positive/propidium iodide (PI) positive (Annexin V+/PI+) fibroblasts were analyzed as percentages of the total fibroblast population.

Immunohistochemical double staining for p16 and senescence-associated β-galactosidase

To show that rotenone induces senescence, double staining for p16 and SA-β-gal was performed as previously described by Itahana *et al.* (16). Fibroblasts were seeded in Permanox 4-chamber slides at 4000 cells/chamber and allowed to attach for four hours. After treatment with 600 nM rotenone for three days, fibroblasts were fixed with 4% paraformaldehyde in PBS for 4 minutes and stained for SA-β-gal activity as described in the subsequent paragraph. After permeabilization for 20 minutes in 0.2% Triton (Sigma, St Louis, MO, USA) in PBS, samples were blocked with blocking buffer (3% BSA in PBS) for one hour at room temperature and incubated for two hours with anti-p16 (JC8) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, USA), diluted 1/100 in blocking buffer. After three washes with PBS, coverslips were incubated with Alexa Fluor® 488 labeled anti-mouse antibody (Invitrogen, Breda, The Netherlands) diluted 1:1000 in blocking buffer for one hour. Slides were mounted with Vectashield Fluorescent Mounting Medium (Vector laboraties, Burlingame, CA, USA) and photographed with a Leica fluorescence microscope (Leica Microsystems, Rijswijk, the Netherlands). Per sample, 100 randomly chosen cells were assessed for SA-β-gal positivity and for p16 positivity.

Flow cytometric measurement of SA-β-gal activity

C_{12}FDG is a substrate which, when hydrolyzed by SA-β-gal, becomes fluorescent and membrane impermeable (10,17). C_{12}FDG was added to the pH modulation medium/buffer. Fibroblasts were incubated with this solution for one hour at 37℃, 5% CO_2 (nigericin-buffer incubation not CO_2 enriched). After incubation, fibroblasts were trypsinized with trypsin EDTA (Sigma, St Louis, MO, USA), washed with PBS, resuspended in 200 µl PBS, and analyzed immediately using a FACSCalibur flow cytometer. Data were analyzed using FACSDiva software (BD, Oxford, United Kingdom). Cell debris was excluded on basis of light scatter parameters. C_{12}FDG was measured on the FL1 detector (500-510 nm wavelength). SA-β-gal activity was expressed as the FL1 median fluorescence intensity (MdFI, in arbitrary units) of the fibroblast population.

Cytochemical staining for SA-β-gal activity

The cytochemical method was conducted as described by Dimri *et al.* (6). A Senescence Cells Histochemical Staining Kit was used (Sigma, St Louis, MO, USA) and fibroblasts were processed according to the manufacturer's guidelines. Slides were washed twice with PBS, counterstained with Mayers-Hematoxylin staining solution (Sigma, St Louis, MO, USA) for 5 minutes at room temperature and washed twice again with PBS. Fibroblasts were then viewed by phase contrast on a Leica microscope (Leica Microsystems, Rijswijk, the Netherlands) and recorded at a 100x magnification by a digital color camera. Per sample, 500 randomly selected fibroblasts were photographed and counted. The number of positive, blue fibroblasts was divided by the total number of counted fibroblasts, resulting in the percentage of SA-β-gal positive fibroblasts.

Statistical analysis

All analyses were performed with the software package SPSS 14.0 (SPSS Inc., Chicago, IL). Differences between pH modulators were analyzed by one-tailed Student's *t* test. Differences between non-stressed and stressed fibroblasts as well as differences between fibroblast strains from young and very old subjects were analyzed by two-tailed Student's *t* test. Results of cytochemical SA-β-gal measurements were divided into tertiles and related to the flow cytometric results. Differences between tertiles were analyzed by ANOVA. Co-localization of p16 and SA-β-gal was described by calculating the specificity (p16-negative fibroblasts as percentage of SA-β-gal-negative fibroblasts) and sensitivity (p16-positive fibroblasts as percentage of SA-β-gal-positive fibroblasts). To determine the correlation between the flow cytometric and the cytochemical methods, we used Pearson correlation analysis.

Results

Toxicity

Unlike the cytochemical assay, in which cells are fixed with formalin, the flow cytometric method measures SA-β-gal activity in living cells. Therefore, the toxicity of the pH modulating agents was examined. Compared with fibroblasts without pH modification, the percentage of

annexin V+/PI- fibroblasts, reflecting early apoptotic cells, was only significantly increased after treatment with 300 μM chloroquine (Figure 1A). Rotenone treatment was used as a positive control for induction of apoptosis, and yielded a significant increase in percentage of early apoptotic cells (3.2±0.61%). Of the four pH modulating conditions tested, the increases in percentage of annexin V+/PI- fibroblasts compared with no pH modulation were smallest

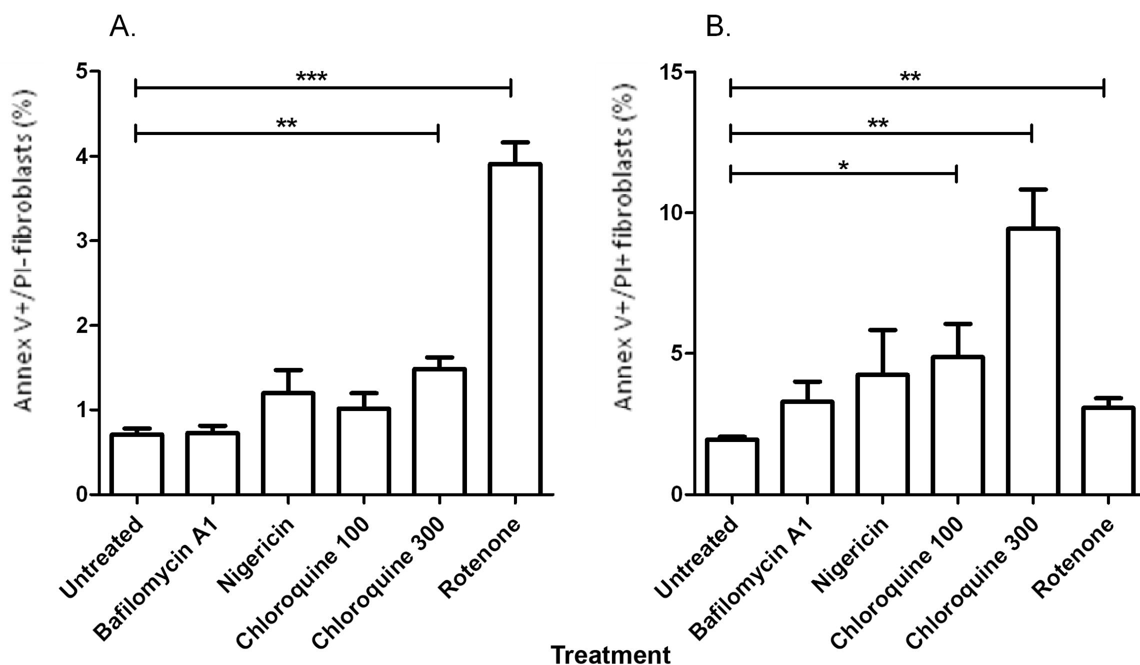

Figure 1. Apoptosis and cell death induced by pH modulation. A, Mean percentage of annexin V+/PI- fibroblasts, reflecting early apoptotic cells. B, Mean percentage of Annexin-V positive/PI positive fibroblasts, reflecting cell death. Mean ± SEM of repeated experiments in two strains. *p≤0.05 **p≤0.01 ***p≤0.001"

for bafilomycin A1 (mean increase ± SD: 0.02±0.27%) compared with treatment with nigericin (0.49±0.63%), 100 μM chloroquine (0.31±0.47%) and 300 μM chloroquine (0.77±0.47%). In Figure 1B, increases in the percentage of annexin V+/PI+ fibroblasts, followed a similar trend as shown in Figure 1A. All pH-modulating agents yielded an increase in the percentage of annexin V+/PI+ fibroblasts. This increase was not significant for bafilomycin A1 and nigericin (1.34±1.35% and 2.29±3.2% respectively). Chloroquine treatment induced a significant

increase in percentage of annexin V-positive/ PI-positive fibroblasts (100 µM: 2.92±2.37%, 300 µM: 7.48±2.88%) as did rotenone treatment (1.13±0.87%).

C_{12}FDG conversion

To determine if the pH modulators would affect the ability to discriminate between degrees of SA-β-gal activity, senescence was induced with rotenone and SA-β-gal activity was assessed using C_{12}FDG. Figure 2 shows representative histograms of both non-stressed and rotenone-stressed fibroblast strains. Discrimination between non-stressed and stressed fibroblasts was best when the pH was modulated using Baf A1 (Median Fluorescence Intensity [MdFI] in arbitrary units, non-stressed: 1475±154, stressed: 2405±260, p=0.008). Other pH modulators also showed changes in MdFI (nigericin: non-stressed: 502±16, stressed: 698±7, p=0.0001; 300 µM chloroquine: 350±48 vs. 482±28, p=0.012), except for 100 µM chloroquine (997±68 AU vs. 866±74, p=0.29).

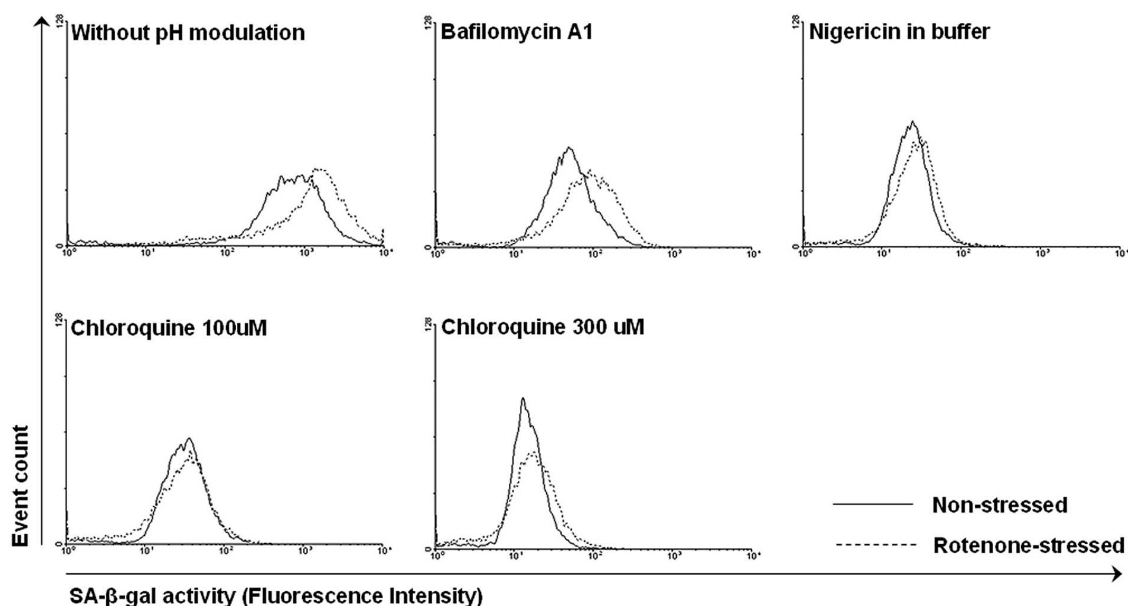

Figure 2. Change in SA-β-gal activity measured by the flow cytometric method in fibroblast strains after induction of stress-induced premature senescence. Histograms are representative examples of SA-β-gal activity in non-stressed and rotenone-stressed fibroblast strains (n=2, duplicate experiments). Samples were treated with pH modulating agents prior to analysis. Scales are equal.

Even though pH modulation appeared not to be essential to SA-β-gal activity quantification with the flow cytometric method as shown in Figure 2, it is a necessary step for the cytochemical method. When no pH modulation is applied all cells would stain blue, making it impossible to distinguish senescent from non-senescent cells. Modulation of the lysosomal pH was thus applied in further experiments, to better compare the flow cytometric method with the cytochemical method. Based on toxicity and SA-β-gal activity, we concluded that Baf A1 was the agent of choice for pH modulation and was thus used in all subsequent experiments.

Rotenone induced senescence increases SA-β-gal activity and p16 expression

Figure 3 displays a representative photograph of fibroblasts positive for both SA-β-gal activity and p16. The absence and presence of SA-β-gal activity and p16 expression were assessed for three fibroblast strains, both under non-stressed and stressed conditions.

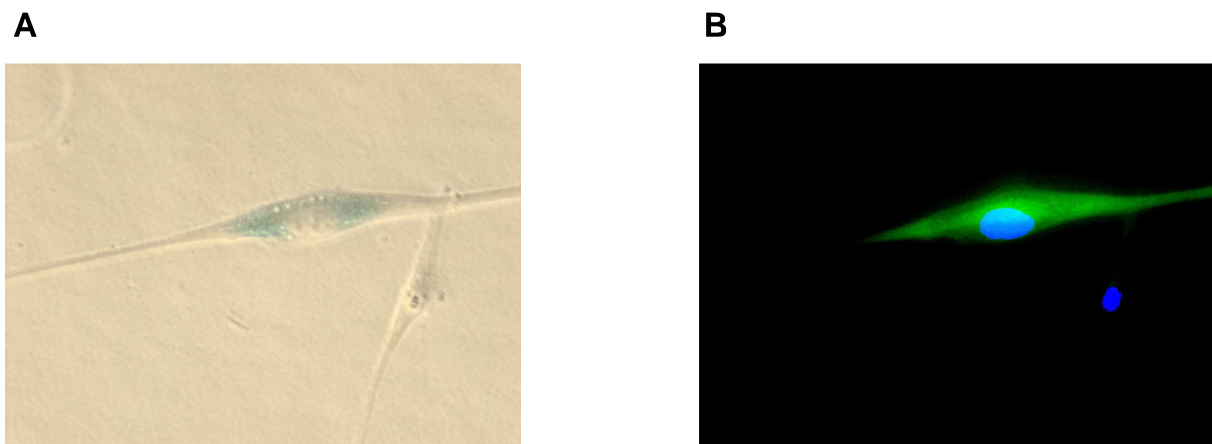

Figure 3. SA-β-gal activity and p16 expression at 3 days after rotenone treatment. A, Phase-contrast photograph showing a SA-β-gal positive (blue) and a SA-β-gal negative fibroblast. B, Immunofluorescent photograph showing a p16 positive (green) and a p16 negative fibroblast. Blue: DAPI.

The combined results for these three strains are presented in Table 1. Under non-stressed conditions, 54% of the fibroblasts were double-negative for SA-β-gal activity and p16 expression (66%, 47% and 50% for the three strains respectively) whereas under rotenone-stressed conditions, 43% of the fibroblasts were double-positive (33%, 40% and 55%). High passage fibroblasts were included in the experiment as positive control (47%). Despite the

considerable concordance, discordance remained present. Specificity and sensitivity were calculated to be 78% and 37% respectively for non-stressed fibroblasts and 62% and 73% respectively for stressed fibroblasts.

Table 1. SA-β-gal activity and p16 expression of three fibroblast strains, under non-stressed and rotenone stressed conditions and in one strain at high passage.

	SA-β-gal negative # cells (%)	SA-β-gal positive # cells (%)	Total # cells (%)
Low passage, non-stressed,			
p16 negative	163 (54)	57 (19)	220 (73)
p16 positive	47 (16)	33 (11)	80 (27)
Total	210 (70)	90 (30)	300 (100)
Low passage, rotenone-stressed			
p16 negative	79 (26)	45 (15)	124 (41)
p16 positive	48 (16)	128 (43)	176 (59)
Total	127 (42)	173 (58)	300 (100)
High passage			
p16 negative	23 (23)	10 (10)	33 (33)
p16 positive	20 (20)	47 (47)	67 (67)
Total	43 (43)	57 (57)	100 (100)

For each strain, 100 cells were counted. High passage strain was stained after 79 population doublings.

Comparison of flow cytometric- and cytochemical method

Twenty fibroblast strains, ten fibroblast strains from young and ten from very old subjects, were used to determine the relation between the flow cytometric and the cytochemical method. The two methods were performed simultaneously for each fibroblast strain, both under non-stressed and stressed conditions. Rotenone induced a significant increase in SA-β-gal activity for both methods as shown in Figure 4A and B. Figure 4C and D show the three tertiles of percentages of SA-β-gal positive fibroblasts detected by the cytochemical method

plotted against the corresponding SA-β-gal MdFI values determined by the flow cytometric method.
Under non-stressed conditions (Figure 4C), significant increases in flow cytometric SA-β-gal activity between tertiles of the cytochemical method were found (trend: p=0.01). When analyzed continuously, the MdFI correlated significantly with the percentages of SA-β-gal positive fibroblasts (r^2=0.31, p=0.014). Under stressed conditions (Figure 4D) no relation was found between the flow cytometric method and cytochemical method (trend p=0.82). Even though both methods detect a significant increase of SA-β-gal activity upon rotenone treatment, we found no relation between the two methods under stressed conditions.

To clarify which of the two methods is the method of choice, both methods measuring SA-β-gal activity were used to discriminate fibroblasts from young and old donors. Figure 5A shows the significant difference in SA-β-gal activity that was found in fibroblasts from young and very old subjects under non-stressed conditions using the flow cytometric method (MdFI in arbitrary units, young: 2161±643, old: 3125±903, p=0.013). When analyzed with the cytochemical method (Figure 5B), the same difference in SA-β-gal activity in young and very old subjects was found, though this difference was not statistically significant (young: 18.7±5.84%, old: 28.3±13.65%, p=0.056). Upon induction of senescence with rotenone, the difference in SA-β-gal activity between young and very old subjects remained by using the flow cytometric method (MdFI in arbitrary units, 3438±689 vs. 4617±880, p=0.004), but was absent when the cytochemical method was performed (44.3±10.7% vs. 47.3±16.1%, p=0.634).

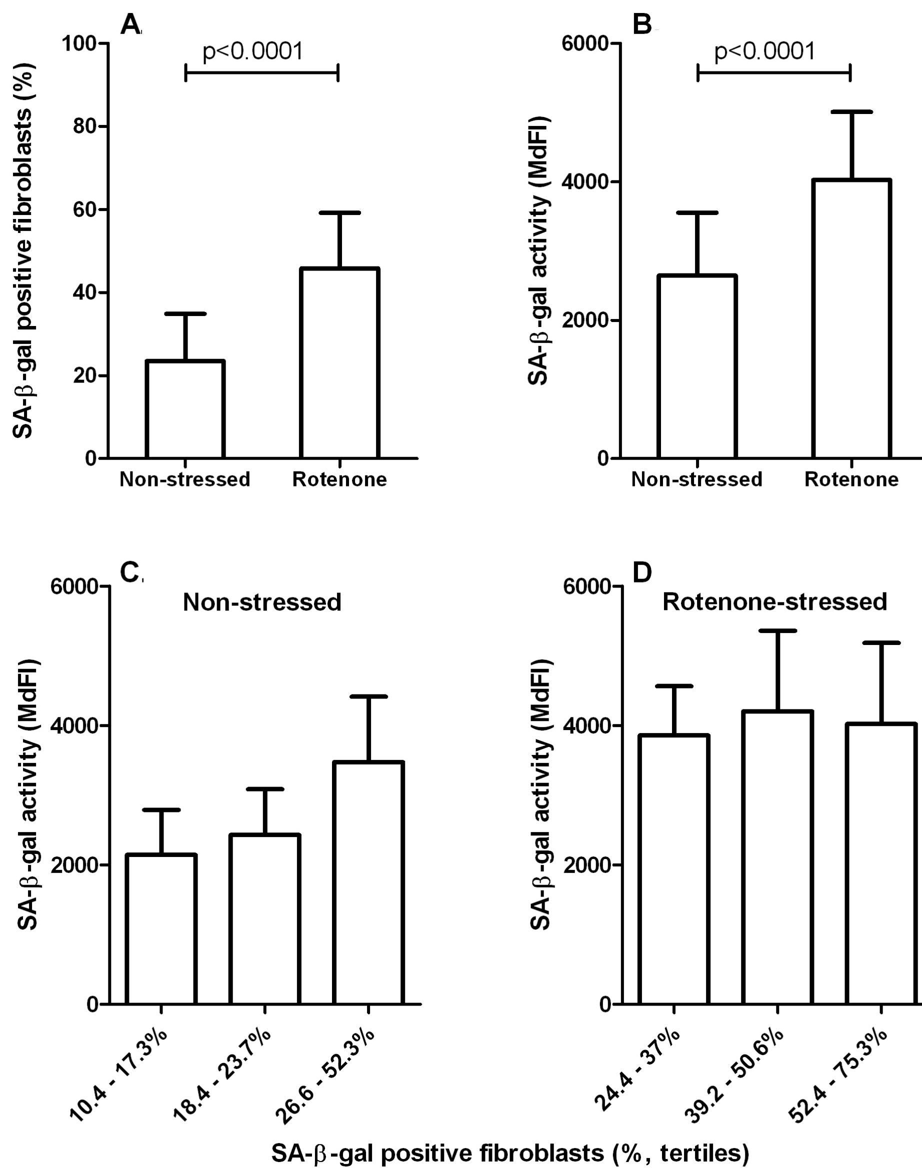

Figure 4. A/B, SA-β-gal activity measured with the cytochemical and flow cytometric methods under non-stressed conditions and after stress with rotenone. A, cytochemical method. B, flow cytometric method. C/D, correlation between SA-β-gal activity measured by the flow cytometric method and cytochemical method. C, Non-stressed fibroblasts, ANOVA for trend p=0.01. D, rotenone-stressed fibroblasts, ANOVA for trend p=0.82. N=20 fibroblast strains. MdFI: Median Fluorescence Intensity, bars: mean±SD.

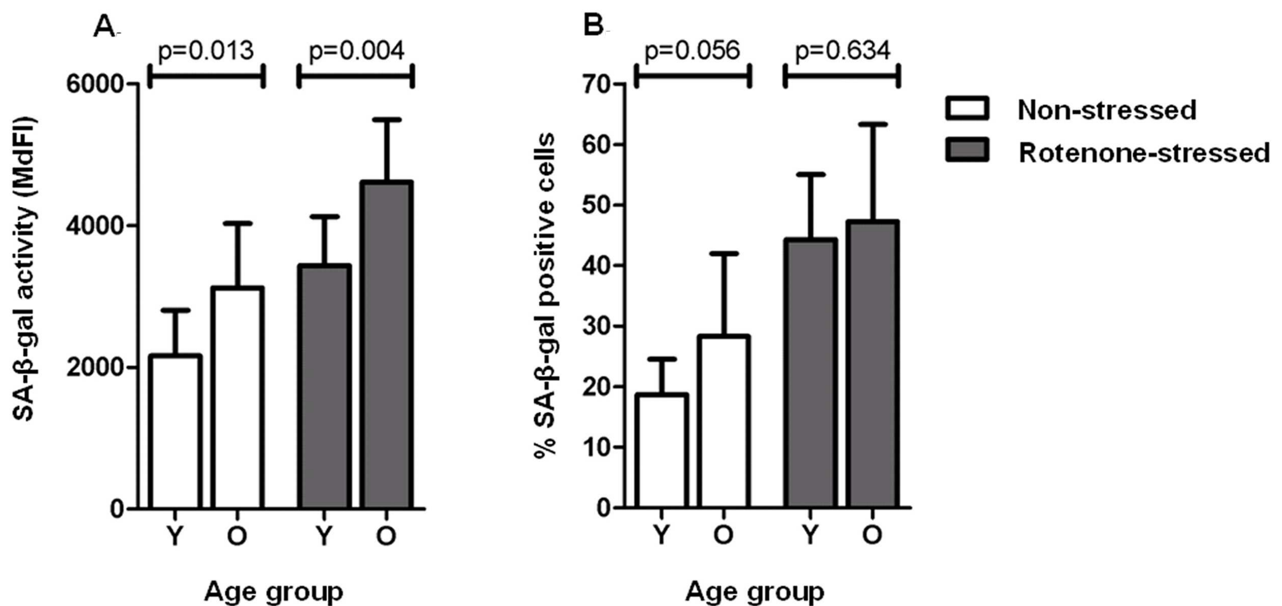

Figure 5. SA-β-gal activity of fibroblast strains from young and very old subjects measured by: A, the flow cytometric method and B, the cytochemical method. Bar-charts display SA-β-gal activity of fibroblast strains from young (Y, n=10) and very old (O, n=10) subjects, in non-stressed and rotenone-stressed conditions. MdFI: Median Fluorescence Intensity, bars: mean±SD.

Discussion

The cytochemical detection of SA-β-gal activity is a widely used marker for cellular senescence and was firstly described by Dimri *et al.* (6). Although the cytochemical method is widely used, it is subjective, prone to inter-rater variability and time-consuming. Kurz *et al.* (10) described a method to measure SA-β-gal activity in HUVECs by flow cytometry, using a fluorogenic substrate. Using this method, cells are neither strictly negative nor positive for SA-β-gal activity but each cell in a population is quantified separately, resulting in more accurate evaluation of differences in SA-β-gal activity within and between populations of cells. This method was validated with the conventional cytochemical method as described by Dimri *et al.* (6). Because cellular senescence is widely studied in human diploid fibroblasts, we aimed to optimize the flow cytometric method using this cell type.

Rotenone was chosen as senescence-inducing agent because it acts on mitochondrial complex I, leading to increased levels of intracellular Reactive Oxygen Species (ROS) (17), which is supposed to mimic the physiological process of ROS induced damage that underlies the aging process (18). Armstong *et al.* showed the apoptotic effect of rotenone when applied in high concentrations (19). When used in lower concentrations, we found that rotenone induces senescence as measured by colocalisation of increased p16 expression and increased SA-β-gal activity. As a positive control, we also observed colocalisation of increased p16 expression coinciding with increased SA-β-gal activity in high passage fibroblasts as was previously observed by Itahana *et al*. (16). In line with these results, Duan *et al*. (20) showed that antisense p16 postponed senescence and decreased SA-β-gal activity, indicating that SA-β-gal activity is indeed a good marker of senescence. However, the discordance in SA-β-gal activity and p16 expression as described in Table 1 shows that increased p16 expression is not a prerequisite for an increase in SA-β-gal activity and that SA-β-gal activity does not always depend on p16 expression. Although various markers have been described that identify senescent cells, the current consensus is that none of these markers is specific for senescence only (21). One of the hallmarks of senescence is growth arrest, which can be mediated by two main pathways: the p53/p21 and the p16/pRB pathways. Although p16 is expressed by many senescent cells, it is not exclusive for senescence (16;22), which is a possible explanation for the SA-β-gal negative/p16 positive fibroblasts observed in our experiments. The SA-β-gal positive/p16 negative fibroblasts could well be the result of senescence induced by pathways other than p16, for example p53. Additionally, examples of senescence have been described that are independent of p16 and p53 (23;24).

Both the flow cytometric and the cytochemical method showed significant increases in SA-β-gal activity after exposure to stress. Under non-stressed conditions, both methods were able to detect a difference between fibroblast strains derived from young subjects compared with strains from very old subjects. When these fibroblast strains were exposed to stress, only the flow cytometric method was able to identify differences in SA-β-gal activity between fibroblasts from young and very old subjects. This could be due to the marked differences between the two methods, the most notable being that, using the cytochemical method, SA-

β-gal activity of individual fibroblasts is assessed dichotomously, whereas the flow cytometric method measures SA-β-gal activity in the fibroblasts on a continuous fluorescence scale. It may thus be well conceivable that two samples measured cytochemically might yield similar percentages of SA-β-gal positive fibroblasts while the intensity of the stained fibroblasts is different between the samples. This difference between samples would be detected by the flow cytometric method since every fibroblast is measured on a continuous scale. Another important difference is that the cytochemical method is performed on fixed fibroblasts whereas flow cytometry is applied to living fibroblasts. Enzyme activity depends on a proper configuration of the protein involved. It is likely that in fixed fibroblasts, enzyme activity will be affected by protein cross-linking and it may thus be hypothesized that measuring SA-β-gal activity with the flow cytometric method in living fibroblasts may better reflect biology.

Since the flow cytometric method described relies on living fibroblasts, we tested the pH modulators for toxicity. We found that Baf A1 was not toxic for fibroblasts whereas chloroquine and nigericin were. Kurz *et al.* (10) show that the SA-β-activity is indeed lysosomal, so we preferred to selectively change lysosomal pH (Baf A1 and chloroquine), as opposed to cytosolic pH (nigericin buffer). Since Baf A1 was virtually non-toxic, whereas chloroquine was not, Baf A1 was our pH-modulator of choice.

Our results suggest that under most conditions, both methods show changes in SA-β-gal activity, but we found the flow cytometric method superior to discriminate between populations of fibroblasts, showing different levels of induced SA-β-gal activity being associated with senescence. Taking into account that the flow cytometric is also less labor-intensive and more time- and cost-effective than the cytochemical method, we strongly recommend flow cytometry for measuring SA-β-gal activity in cultured fibroblasts.

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