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Senescence-associated β-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells

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SUMMARY

Senescence-associated β-galactosidase is widely used as a biomarker of replicative senescence. However, it remains unknown whether this is a distinct enzyme active at pH 6, and differentially expressed in senescence, or a manifestation of an increase in the classic acid lysosomal β-galactosidase. Here we have investigated the origin of senescence-associated-β-galactosidase activity by modifying the intracellular and lysosomal pH of young and senescent human umbilical vein endothelial cells and examining the effect of these manipulations on the levels of activity, using a flow cytometric assay. Lysosomal alkalinisation with chloroquine or bafilomycin A_1, as well as equilibration of the intracellular milieu to pH 6 with nigericin, caused a profound (92-99%) inhibition of the total intracellular β-galactosidase activity. However, independent of pH alterations, senescent cells showed levels of β-galactosidase activity three- to sixfold higher than young cells. This increase in activity occurred in parallel to an increase in β-galactosidase protein levels. Acridine Orange staining revealed an increase in lysosomal content with replicative age, which correlated with the increase in β-galactosidase. These findings demonstrate that senescence-associated β-galactosidase is a manifestation of residual lysosomal activity at a suboptimal pH, which becomes detectable due to the increased lysosomal content in senescent cells.

Key words: Cell ageing, Biological marker, Beta-galactosidase, Vascular endothelium, Flow cytometry, Lysosome

INTRODUCTION

Acid β-D-galactosidase (EC 3.2.1.23) is a eukaryotic hydrolase localized in the lysosome (reviewed in Suzuki et al., 1995). It cleaves β-linked terminal galactosyl residues from a wide range of naturally occurring substrates, such as gangliosides, glycoproteins and glycosaminoglycans, as well as a number of artificial substrates. It has an acidic pH optimum (pH 4.0-4.5), which is close to the natural milieu of the lysosome. The mature form of this enzyme consists of a 64 kDa polypeptide, which is derived by post-translational processing from an 85 kDa precursor (d'Azzo et al., 1982). In the lysosome this 64 kDa species forms a functional multimeric aggregate of about 700 kDa (Hoeksema et al., 1979; Norden et al., 1974), stabilized through interactions with a glycoprotein known as the protective protein (Hoogeveen et al., 1983; Suzuki et al., 1995). Apart from lysosomal β-galactosidase, a β-galactosidase-related protein with no detectable enzymatic activity and a different subcellular localisation has been described in human fibroblasts (Morreau et al., 1989). This protein of unknown function results from alternative splicing of the lysosomal β-galactosidase mRNA precursor (Morreau et al., 1989). In addition, a neutral β-galactosidase has been described in human liver (Ho et al., 1973). This enzyme appears to be unrelated to the lysosomal acid β-galactosidase, based on its substrate specificity and immunoreactivity (Ho et al., 1973; Meisler and Rattazzi, 1974; Ben-Yosseph et al., 1977).

Lysosomal β-galactosidase activity can be detected in situ in most mammalian cells by means of a cytochemical assay, normally carried out at pH 4, using the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) (Dimri et al., 1995; van der Loo et al, 1998). Recently, Dimri et al. (1995) described a pH 6 β-galactosidase activity, which was found specifically in senescent human fibroblast cultures, but not in quiescent or terminally differentiated cells. Furthermore, this pH 6.0 activity enabled identification of senescent fibroblasts and keratinocytes in biopsies of aged human skin, and subsequently became known as senescence-associated β-galactosidase (SA-β-galactosidase). Various laboratories, including our own, have since used the SA-β-galactosidase assay on a variety of cells and tissues to demonstrate the onset of replicative senescence in culture (e.g. Reznikoff et al., 1996; Serrano et al., 1997; Bodnar et al., 1998; Van der Loo et al., 1998; Tsukamoto et al., 1998; Matsunaga et al., 1999) and in vivo (Sigal et al., 1999; Mishima et al.,

1999). However, the specificity and selectivity of this assay have been disputed by other groups. These have demonstrated β-galactosidase activity at pH 6 in immortalised cell lines following either serum starvation or phorbol ester-induced macrophage-like differentiation (Yegorov et al., 1998), or have detected pH 6 activity, using a sensitive high-pressure liquid chromatography assay, in various proliferating cell lines and in liver homogenates (Devarakonda et al., 1999). Moreover, despite its widespread use the nature of SA-β-galactosidase has remained somewhat obscure, its origin and function in senescence still being unknown. In particular, it has not been established whether a distinct enzyme active at pH 6 is expressed in senescent cells, or whether SA-β-galactosidase is a manifestation of an increased expression or activity of the classic acid lysosomal β-galactosidase, which under these conditions becomes detectable at pH 6, despite this normally being a suboptimal pH.

To address these issues, we have experimentally modulated the lysosomal and cytoplasmic pH of young and senescent human umbilical vein endothelial cell (HUVEC) cultures and monitored the influence of these manipulations on β-galactosidase activity in intact cells. β-galactosidase activity was monitored by flow cytometry, using 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG), in order to permit measurements in live cells and to increase the assay sensitivity. Our results demonstrate that the pH 6 β-galactosidase activity detected in senescent cells can be attributed to a rise in the level of the classic lysosomal enzyme. Furthermore, we provide evidence that this is a consequence of an increase in lysosomal mass in senescent cells.

MATERIALS AND METHODS

Materials

HUVECs and endothelial cell growth medium 2 (EGM-2) were purchased from Biowhittaker (Wokingham, UK). C_{12}FDG (Imagene Green™) and chloroquine were from Molecular Probes (OR, USA). X-Gal, bafilomycin A_1 (baf A_1), nigericin and Acridine Orange were from Sigma (Poole, UK). Stock solutions of C_{12}FDG (20 mM) and baf A_1 (0.1 mM) were made in dimethyl sulfoxide and stored at –20°C. Nigericin (5 mM) was dissolved in ethanol and stored at –20°C. Acridine Orange (0.5 mg/ml) was made fresh before each experiment in Hank's balanced salt solution (HBSS). Stock solutions were diluted in medium immediately before addition to the cultures. All other reagents were from standard suppliers or as listed in the text.

Cell culture

First passage cryopreserved HUVECs were grown in EGM-2, consisting of modified MCDB 131 medium supplemented with fetal calf serum, hydrocortisone, human basic fibroblast growth factor, vascular endothelial growth factor, R^3-insulin-like growth factor-1, human recombinant epidermal growth factor, heparin, ascorbic acid, gentamycin and amphotericin B, as supplied by the manufacturer. Cells were maintained in 25 cm^2 flasks (Falcon) and serially passaged until they reached senescence, as previously described (van der Loo et al., 1998). The number of population doublings (PD) was calculated using the formula: PD = (ln[number of cells harvested] – ln[number of cells seeded]) / ln2, as previously described (van der Loo et al., 1998). Early and late passage human mammary fibroblasts from a healthy young adult (kindly supplied by Dr A. Atherton, Breast Cancer Laboratory, Department of Surgery, University College London, UK) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS as previously described (Atherton et al., 1994).

For all the experimental procedures described below, cells at different cumulative population doublings (CPDs) were subcultured onto 6-well plates (unless otherwise indicated) and grown in parallel. In order to compensate for the decrease in the rate of growth that occurs as the cells advance towards senescence, seeding densities ($1.25\text{-}7.5\times10^3/cm^2$) and culture times (5-7 days for HUVECs and 14 days for fibroblasts) were adjusted so that cells reached 80-90% confluence by the day of harvesting. This allowed cultures at different CPDs to be harvested at the same time and measurements to be performed under identical conditions.

Modulation of intracellular pH

To induce lysosomal alkalinisation, cell monolayers were treated with 300 μM chloroquine for 2 hours or with 100 nM baf A_1 for 1 hour in fresh EGM-2 at 37°C under 5% CO_2. To alter the pH of all intracellular compartments (Negulescu et al., 1990; Escobales et al., 1990), cultures were washed with phosphate-buffered saline (PBS) and then incubated for 15 minutes at 37°C in a high [K^+], pH 6 equilibration solution consisting of 150 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM glucose and 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 6, in the presence of 10 μM nigericin.

Flow cytometric determination of β-galactosidase activity

To measure β-galactosidase activity by flow cytometry, we used the fluorogenic substrate C_{12}FDG. This compound is a membrane-permeable, non-fluorescent substrate of β-galactosidase, which after hydrolysis of the galactosyl residues emits green fluorescence and remains confined within the cell. Parallel cultures at various CPDs were pretreated to modulate intracellular pH as described above, or incubated with an equivalent amount of vehicle. C_{12}FDG (33 μM) was then added to the pretreatment medium and the incubation was continued for the indicated length of time. At the end of the incubation, cultures were washed with ice-cold PBS, resuspended by trypsinization, and analysed immediately using a FACScan flow cytometer (Becton Dickinson, Oxford, UK). Data were acquired and analysed with Cellquest software (Becton Dickinson). Light scatter parameters were used to eliminate dead cells and subcellular debris. The C_{12}-fluorescein signal was measured on the FL1 detector and β-galactosidase activity was estimated using the median fluorescence intensity (MFI) of the population. Autofluorescence at each CPD was assessed in parallel in cells not exposed to C_{12}FDG. In all cases these values were found to be negligible when compared to the fluorescence levels of equivalent samples incubated with C_{12}FDG, and therefore were not taken into consideration for the final estimation of β-galactosidase activity. Experiments were performed on duplicate cultures and the results averaged.

Assessment of lysosomal content with Acridine Orange

Morphological examination of lysosomal content was performed by vital fluorescence microscopy using the lysosomotropic fluorochrome Acridine Orange, essentially as previously described (Yoshimori et al., 1991). In brief, HUVECs at different CPDs were grown on coverslips coated with 0.5% gelatine and then pretreated with baf A_1 or an equivalent amount of vehicle, as described above. After washing with HBSS, cells were incubated for 10 minutes at 37°C with 5 μg/ml Acridine Orange in HBSS. After four washes with HBSS, coverslips were mounted onto glass microscope slides and viewed immediately under a Zeiss Axiophot fluorescence microscope with a 40×/0.75 NA Plan-Neofluar objective using a Zeiss longpass filter set (comprising a bandpass 450-490 nm exciter, an FT 510 nm dichroic mirror and a longpass 520 nm emitter). Representative fields of young and senescent cultures were photographed with identical shutter speeds using a 1600 ASA Kodak Ektachrome colour positive film.

Quantification of cellular lysosomal mass was carried out by flow cytometry. Cells were stained in 6-well plates with Acridine Orange as described above and resuspended by trypsinization. Photomultiplier settings were adjusted to detect the green fluorescence signal of Acridine Orange (mostly due to nucleic acid staining) on the FL1 detector, and the orange fluorescence signal (due to lysosomal staining) on the FL2 detector. Owing to this dual-wavelength emission of Acridine Orange, electronic compensation for the spillage of the nucleic acid fluorescence into the FL-2 channel was necessary. For this purpose we used cultures pretreated with baf A_1, taking advantage of the fact that these showed only green fluorescence.

Cytochemical staining for SA-β-galactosidase

Cytochemical staining for SA-β-galactosidase was performed at pH 6 as previously described (van der Loo et al., 1998). Stained cultures were viewed under an Axiovert 25CFL inverted microscope (Carl Zeiss, Germany) at 200× magnification, using a green conversion filter. The percentage of SA-β-galactosidase positive cells was determined by counting the number of blue cells under bright field illumination, and then the total number of cells in the same field under phase contrast. Representative fields were photographed using Kodak Ektachrome 64T color positive film.

Western blotting

To determine the levels of β-galactosidase protein expression, HUVECs at different CPDs were grown to confluence in sets of three 100 mm dishes. One dish was trypsinised for cell counting, while the remaining two were processed for western blotting as follows. After washing with ice-cold PBS, cells were harvested with a cell scraper, resuspended in approximately four volumes of ice-cold lysis buffer (10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes), pH 6.8, 1 μM phenylmethylsulfonyl fluoride (PMSF) and 5 μg/ml leupeptin), and disrupted by sonication (three cycles of 5 seconds at 22 Hz with intervals of 25 seconds) using a Soniprep 150 Ultrasonic Disintegrator (MSE Scientific Instruments, Nottingham, UK). The resultant lysates were centrifuged at 14000 ***g*** for 30 minutes at 4°C to remove particulate material. Proteins in samples of the supernatant fraction corresponding to 10^5 cells were separated by sodium dodecyl sulfate (SDS)-8% polyacrylamide gel electrophoresis and transferred to Immobilon™ P PVDF membranes (Millipore, MA, USA). Prestained molecular mass markers were run in parallel. Membranes were blocked for 1 hour at room temperature with 6% fat-free dried milk in PBS/0.1% Tween 20, and then incubated for 2 hours at room temperature with a rabbit anti-bacterial β-galactosidase polyclonal antibody (Chemicon, Harrow, UK) diluted 1:1000 in blocking buffer. This antibody has been previously shown to cross-react with the human lysosomal isoform (Hartz and Wilson, 1997). Duplicate membranes were blotted with a rabbit anti-actin polyclonal antibody (Sigma) at a 1:1500 dilution. Immunoreactive bands were detected using horseradish peroxidase-labeled donkey anti-rabbit IgG and enhanced chemiluminescence (ECL, Amersham Life Science, UK). The relative intensity of the bands was quantified

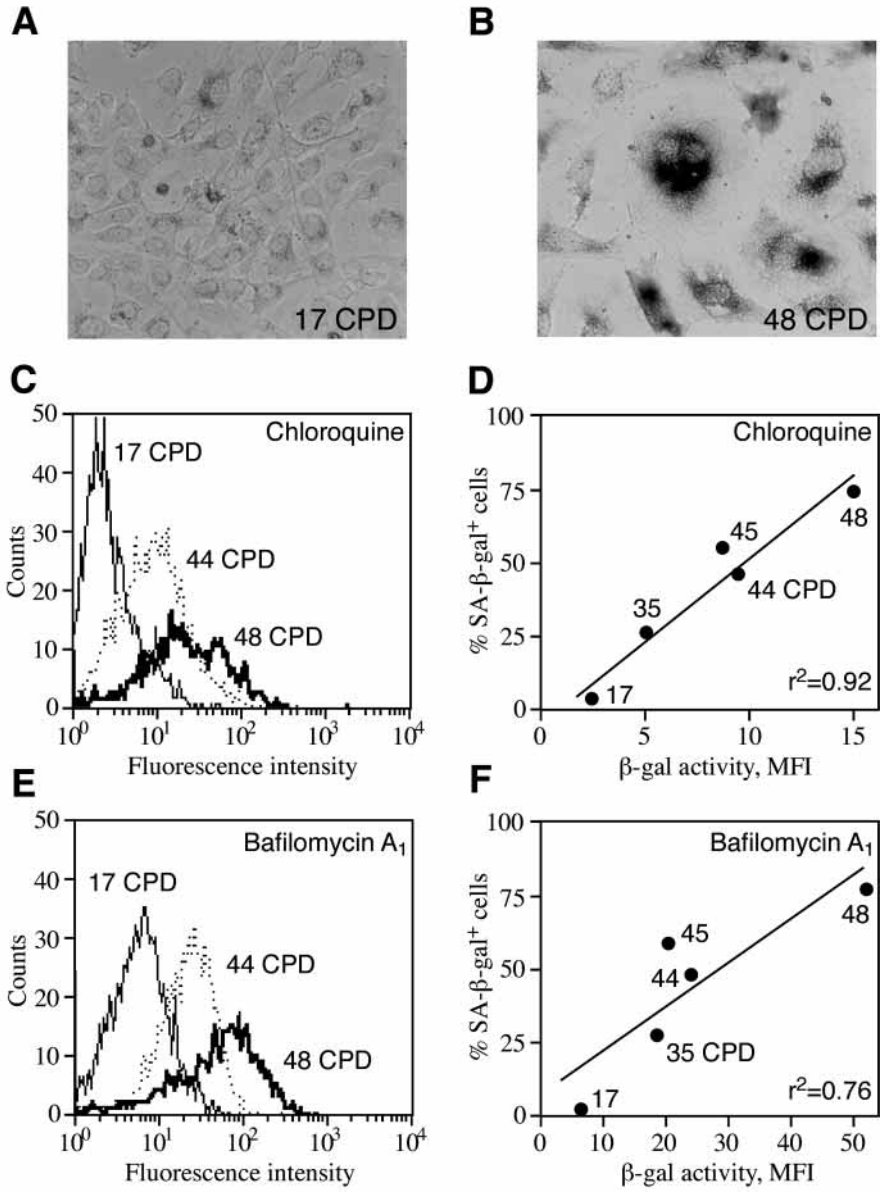

Fig. 1. Comparison of SA-β-galactosidase detection by cytochemistry and by flow cytometry in HUVECs. HUVEC monolayers were processed for β-galactosidase detection by cytochemistry at pH 6 (A,B), or by flow cytometry (C,E) after treatment with 300 μM chloroquine (C) or 100 nM baf A_1 (E), as described under Materials and Methods. (A,B) Photomicrographs of young (A) and senescent (B) cultures taken at the same magnification (135×). (C,E) Flow cytometric histograms of C_{12}-fluorescein fluorescence in young (thin tracing), intermediate (broken tracing) and senescent cultures (thick tracing); cultures were incubated with C_{12}FDG for 4 hours (C) or 1 hour (E) at 37°C prior to analysis. (D,F) Correlation between the percentage of SA-β-galactosidase positive cells measured by cytochemistry and the levels of β-galactosidase activity measured by flow cytometry in chloroquine (D) or baf A_1-treated cultures (F) at different CPDs; the square of the correlation coefficient (r^2) is indicated.

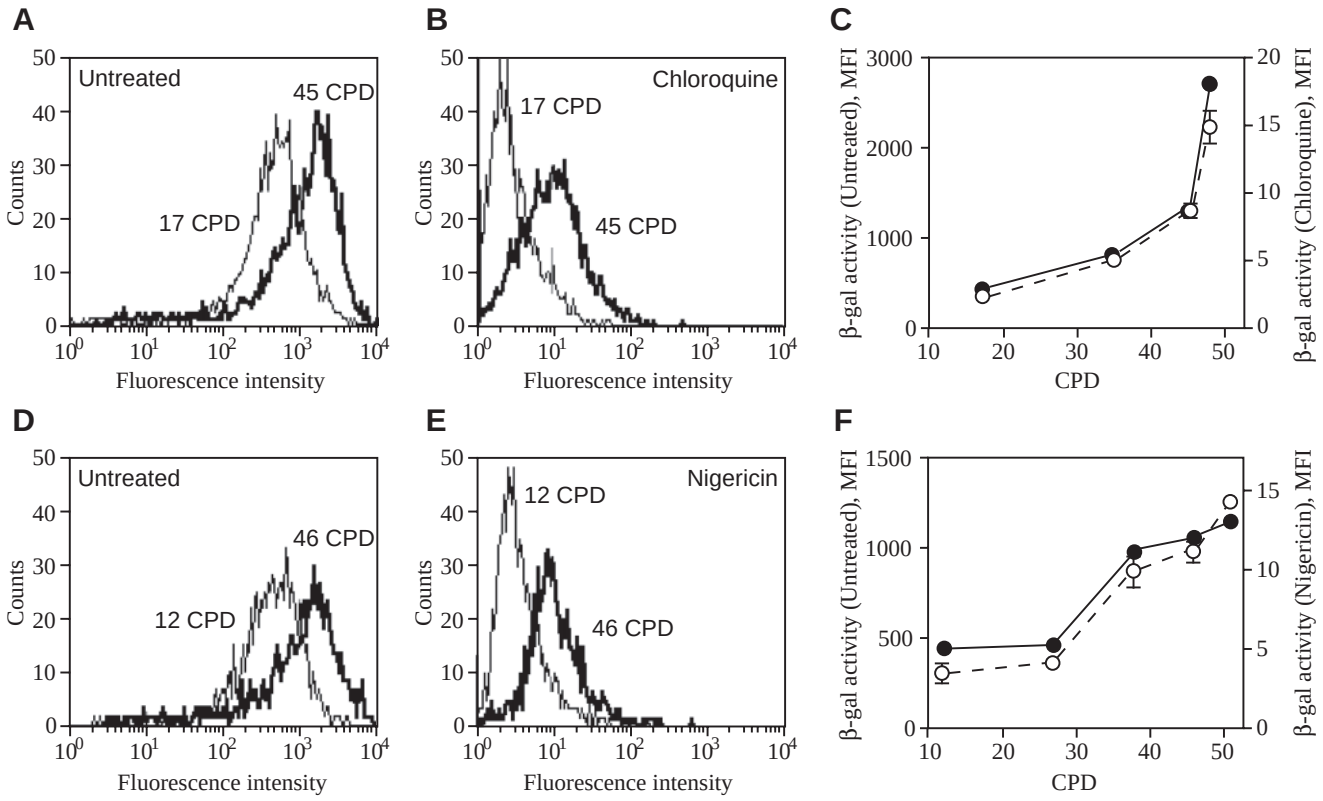

Fig. 2. Effect of lysosomal and cytoplasmic pH modulation on β-galactosidase activity of HUVECs at different replicative ages. (A-C) HUVEC monolayers were incubated with C_{12}FDG for 4 hours at 37°C in the absence (A) or presence (B) of 300 μM chloroquine prior to flow cytometric analysis of β-galactosidase activity. (A,B) Flow cytometric histograms of C_{12}-fluorescein fluorescence of cultures at the indicated CPDs. (C) C_{12}-fluorescein MFI of untreated (●, left *y*-axis) or chloroquine-treated (○, right *y*-axis) cultures at different CPDs; values are means ± s.d. of duplicate cultures; error bars smaller than the size of the symbol are not shown. (D-F) A similar experiment to that shown in A-C except that cells were incubated with C_{12}FDG for 1 hour in the absence (D) or presence (E) of 10 μM nigericin in a high [K^+], pH 6-buffered medium, as described in Materials and Methods.

by scanning densitometry analysis using the public domain NIH Image program (available at http://rsb.info.nih.gov/nih-image).

Statistical analysis

Experiments were performed at least three times and results from one representative experiment are shown. Where indicated, linear regression analysis was performed using the GraphPad Prism software package (version 2.01, GraphPad Software Inc., CA, USA).

RESULTS

SA-β-galactosidase can be measured by flow cytometry using lysosomal inhibitors

In order to measure endogenous β-galactosidase activity in intact HUVECs we adopted a flow cytometric technique that had been previously developed to monitor the expression of *E. coli* lacZ reporter gene constructs introduced into mammalian cells (Fiering et al., 1991). Our previous work (van der Loo et al., 1998), using the cytochemical assay at pH 4 and pH 6, suggested that the high levels of acid lysosomal β-galactosidase present in HUVECs at all replicative ages would mask the detection of a senescence-dependent activity in live cells. Therefore we reasoned that in order to carry out these experiments, it would be necessary to inhibit the lysosomal enzyme. To this end we used chloroquine, a weak base that concentrates in the lysosomes, raising their pH to approximately 6 (Yoshimori et al., 1991). Preliminary experiments showed that at a saturating loading concentration of C_{12}FDG (33 μM), and in the absence of chloroquine, there was an initial lag period of about 30 minutes, followed by a linear increase in the median fluorescence intensity of C_{12}-fluorescein for up to 5 hours. Experiments also showed that maximal β-galactosidase inhibition (>97%) was achieved by preincubation with 300 μM chloroquine for 2 hours before addition of C_{12}FDG (data not shown). Fig. 1 shows a comparison between flow cytometric measurements of β-galactosidase activity measured under lysosomal alkalinisation conditions and the conventional SA-β-galactosidase cytochemical assay, performed on cultures of different ages. Consistent with previous reports (van der Loo et al., 1998) the cytochemical assay revealed that most cells in young cultures (<20 CPDs) stained negative for SA-β-galactosidase (Fig. 1A), whereas in senescent cultures (48 CPDs) most cells were positive (Fig. 1B). As shown in Fig. 1C, flow cytometric histograms for HUVECs of different CPDs revealed in each case the presence of a single major cell population. Under chloroquine inhibition the median C_{12}-fluorescein fluorescence in young

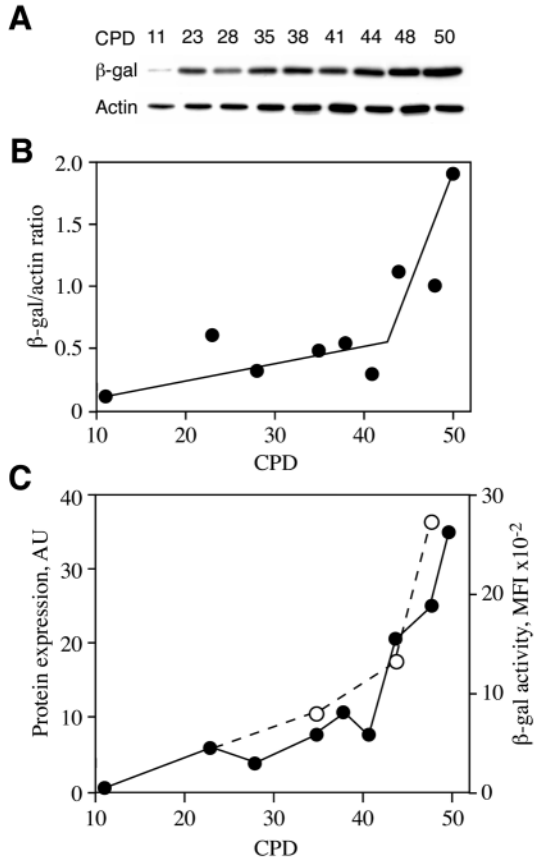

Fig. 3. Correlation between β-galactosidase protein expression and activity in HUVECs of different replicative ages. HUVEC monolayers at different CPDs were processed for immunoblotting and flow cytometric analysis of β-galactosidase activity. (A) Immunoblots showing levels of β-galactosidase (β-gal) and actin in protein extracts from 10^5 cells at the indicated CPDs. (B) Immunoreactive bands in A were quantified by scanning densitometry; the ratio of β-galactosidase to actin protein level is shown against the replicative age of the culture. (C) β-galactosidase protein levels (●, left *y*-axis) and activity (○, right *y*-axis) in cultures at different CPDs. AU, arbitrary units.

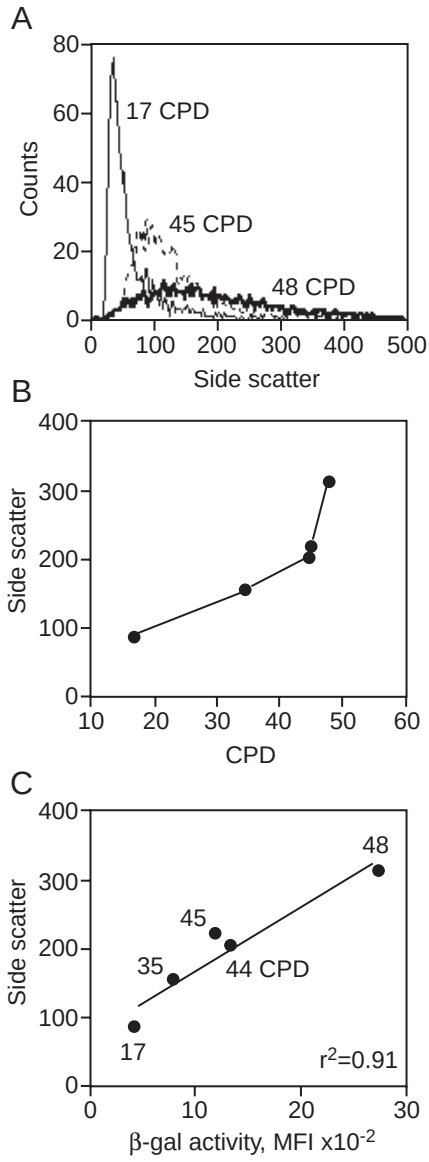

Fig. 4. Correlation between side scatter and β-galactosidase activity in HUVECs of different replicative ages. Light scatter profiles of HUVEC monolayers at different CPDs were obtained by flow cytometry. β-galactosidase activity was measured in parallel samples following incubation with C_{12}FDG for 4 hours at 37°C. (A) Flow cytometric histograms of SSC in young (thin tracing), intermediate (broken tracing) and senescent cultures (thick tracing); (B) mean SSC of cultures at different CPDs. (C) Correlation between the mean SSC and the levels of β-galactosidase activity at different CPDs; the square of the correlation coefficient (r^2) is indicated.

cultures (thin tracing) was found to be very low. In contrast, in senescent cultures median C_{12}-fluorescein fluorescence increased more than fivefold (thick tracing) while middle-aged cells (35-45 CPDs) displayed intermediate levels of fluorescence (broken tracing). The results obtained by flow cytometry were compared with those from parallel cultures using the cytochemical assay (Fig. 1D). At different replicative ages the median fluorescence intensity of C_{12}-fluorescein correlated closely (r^2=0.92) with the percentage of cells staining positively for SA-β-galactosidase. Similar results were obtained when lysosomal alkalinisation was induced via a different mechanism involving the use of baf A_1, a specific inhibitor of vacuolar H^+-ATPase (Yoshimori et al., 1991) (Fig. 1E,F). Taken together, these experiments clearly indicated that under conditions in which the lysosomal pH is raised, flow cytometry can reliably measure an elevation of β-galactosidase activity in live cells associated with replicative senescence.

A senescence-dependent increase in β-galactosidase activity can also be detected without lysosomal inhibition

In order to examine whether SA-β-galactosidase is a distinct entity differentially expressed in senescence or, alternatively, a consequence of an increase of the lysosomal enzyme, we measured β-galactosidase activity under normal conditions, that is when the lysosomal pH was preserved. Fig. 2A shows representative flow cytometric histograms of young and

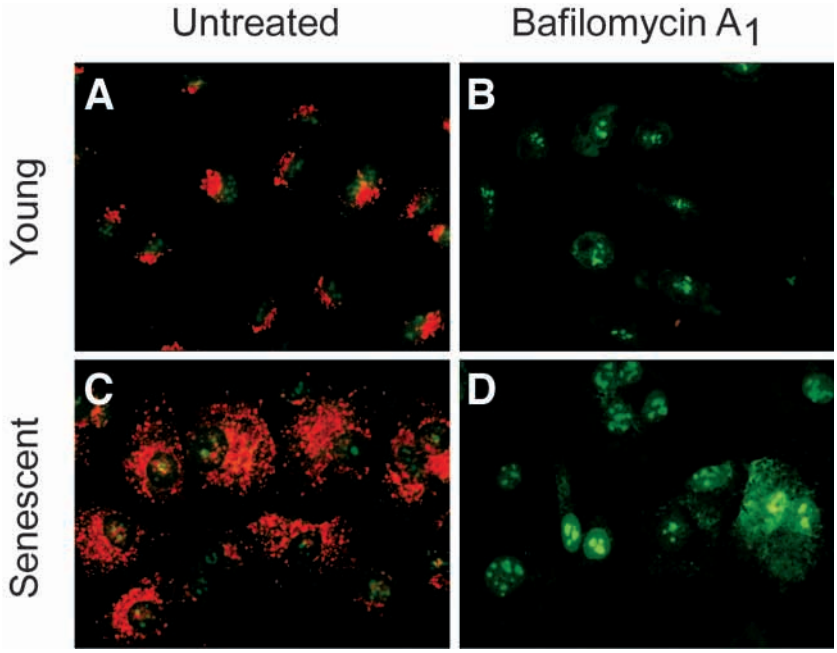

Fig. 5. Fluorescence micrographs of HUVECs stained with Acridine Orange. Young (A,B) and senescent (C,D) HUVECs were grown on coverslips, pre-treated with baf A_1, (B,D) or an equivalent amount of vehicle (A,C) and then stained with Acridine Orange as described under Materials and Methods. Photomicrographs were taken at the same magnification (225×) with exposure times of 0.5 seconds (A,C) and 4 seconds (B,D), respectively.

senescent cells incubated with C_{12}FDG in the absence of lysosomal inhibitors. As in the case of chloroquine-treated cultures (Fig. 2B), we found in untreated cultures that senescent cells showed substantially higher levels of β-galactosidase activity than young cells. Furthermore, even though the absolute levels of β-galactosidase activity in untreated cultures were much higher than in chloroquine-treated cultures (average increase in five independent experiments was 66-fold; data not shown), the relative increase in activity as a function of replicative age was comparable to that seen in the treated cultures of the same age (Fig. 2C). Overall, results from different experiments using various serially passaged endothelial cell cultures, showed that, independent of the lysosomal pH, the rate of C_{12}FDG hydrolysis was about 3-6 times higher in senescent than in young HUVECs.

Lack of evidence for an extralysosomal pH 6 β-galactosidase activity in senescent cells

The above results strongly suggested that the detection of activity at pH 6 by cytochemistry was due to a senescence-associated increase in lysosomal β-galactosidase. On the other hand, the possibility of an extralysosomal β-galactosidase active at pH 6 had not been completely ruled out. To investigate this alternative, cultured cells were exposed to the K^+/H^+-ionophore nigericin in a high $[K^+]$, pH 6-buffered medium before adding C_{12}FDG to the solution. These conditions allow both intracytoplasmic and lysosomal pH to equilibrate with the pH of the medium (Negulescu et al., 1990; Escobales et al., 1990), and therefore resemble those of the cytochemical assay carried out at pH 6. Comparison of Fig. 2D and E shows that this treatment reduced β-galactosidase activity to very low levels (average inhibition in 3 independent experiments was 99%; data not shown), similar to those seen after lysosomal alkalinisation with chloroquine (Fig. 2B). As in the case of chloroquine (Fig. 2C), the relative increase in activity in senescent cultures was similar in both the absence and presence of nigericin (Fig. 2F), indicating that the appearance of SA-β-galactosidase is not caused by an extralysosomal pH 6 activity.

β-galactosidase protein content increases with replicative age

Western blot analysis was performed to explore the cause of the increase in β-galactosidase activity with replicative age. Since general increases in protein levels and cell size are known to occur during serial passage in culture (reviewed in Hayflick, 1980), the contribution of these factors was addressed by comparing β-galactosidase protein levels with those of actin. As shown in Fig. 3A, the content of β-galactosidase protein per cell increased gradually until approx. 41 CPDs, after which, as the cultures approached senescence, it increased sharply (see also Fig. 3C). In contrast, actin levels increased steadily up to approx. 41 CPDs, but remained relatively constant thereafter. The ratio of β-galactosidase to actin protein levels (Fig. 3B) followed approximately the β-galactosidase pattern, showing a similar sharp increase (15-fold compared to young cells) as the cells approached the end of their replicative life span. Fig. 3C shows that the increase in β-galactosidase protein levels (left *y*-axis) was closely matched by an increase in activity (right *y*-axis).

Lysosomal mass increases in senescence

We then investigated whether the senescence-associated increase in β-galactosidase resulted from changes in lysosomal mass. A preliminary indication of the relationship between β-galactosidase activity and lysosomal mass was found in the side scatter (SSC) characteristics, an indicator of cellular granularity, of HUVEC populations at different CPDs. In all flow cytometric profiles the SSC of these cells increased as a function of replicative age (Fig. 4A,B). Furthermore, this increase correlated closely with the increase in β-galactosidase activity (Fig. 4C). Further evidence that the increase in β-galactosidase activity reflected a rise in the lysosomal mass

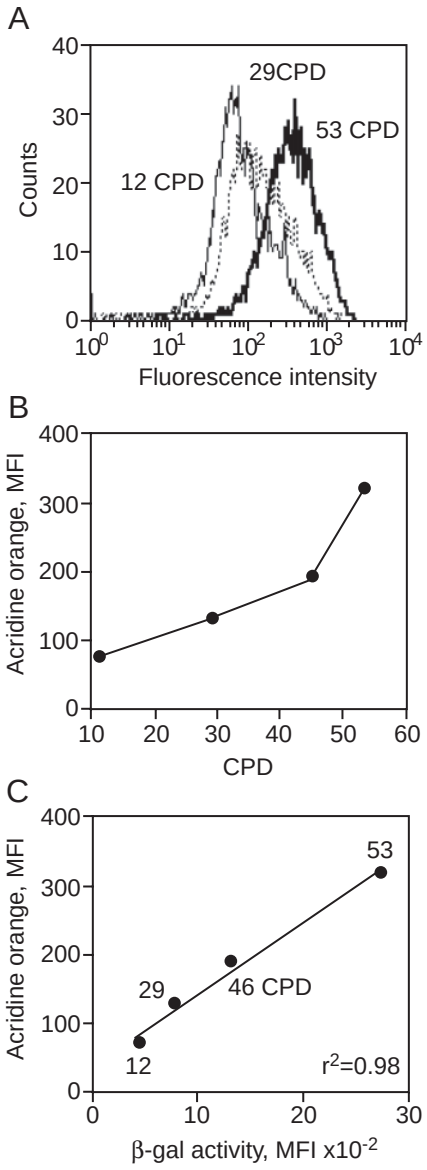

Fig. 6. Correlation between Acridine Orange staining and β-galactosidase activity in HUVECs of different replicative ages. Acridine Orange fluorescence profiles of HUVEC monolayers at different CPDs were obtained by flow cytometry as described under Materials and Methods. β-galactosidase activity was measured in parallel cultures following incubation with C_{12}FDG for 4 hours at 37°C. (A) Flow cytometric histograms of Acridine Orange fluorescence (orange emission) in young (thin tracing), intermediate (broken tracing) and senescent cultures (thick tracing); (B) Acridine Orange MFI of cultures at different CPDs. (C) Correlation between Acridine Orange MFI and the levels of β-galactosidase activity at different CPDs; the square of the correlation coefficient (r^2) is indicated.

in the ageing cell was obtained by vital staining of HUVECs with Acridine Orange. This dye is a weak base, which accumulates in lysosomes after being protonated in the acid environment, emitting orange fluorescence (Darzynkiewicz and Kapuscinski, 1992). In addition, Acridine Orange binds to nucleic acids, emitting green fluorescence when used at low concentrations. Under these conditions fluorescent microscopy revealed a marked increase in the number and size of lysosomes in senescent cells (Fig. 5A,C). Pre-treatment of parallel cultures with baf A_1 had a dramatic effect on the orange fluorescence, which disappeared completely, confirming its selective lysosomal localization (Fig. 5B,D). As shown in Fig. 6A,B, flow cytometric analysis of Acridine Orange-stained HUVECs at different CPDs confirmed the increase in lysosomal content as a function of replicative age. Fig. 6C shows that, similar to the increase in SSC, the increase in Acridine Orange fluorescence correlated closely with the increase in β-galactosidase activity. In summary, these results indicate that the senescence-associated rise in the level of β-galactosidase activity reflects an increase in lysosomal content in ageing cells.

Comparison between β-galactosidase activity and lysosomal mass in human fibroblasts

In order to extend our findings to cell types other than endothelial cells, flow cytometric analysis of β-galactosidase activity and cellular lysosomal content were also performed on early and late passage human fibroblasts and compared with the results of the SA-β-galactosidase staining of the respective cultures. The results of these experiments are summarised in Fig. 7. Both β-galactosidase activity (Fig. 7C,D) and lysosomal content (Fig. 7E,F) increased to the same extent (two- to threefold) in late-passage (approx. 32 CPDs) fibroblasts compared to early passage (approx. 8 CPDs) cultures.

DISCUSSION

Replicative senescence is a permanent non-dividing state, which ensues in most somatic cells following a predetermined number of cell divisions. Classically manifested upon serial passage in culture (Hayflick and Morehead, 1961; Hayflick, 1965), it results from the shortening of chromosomal telomeres (Bodnar et al., 1998), and entails an irreversible arrest of the cell cycle, concomitant characteristic changes in gene expression and morphology, and the alteration of cellular functions (reviewed in Smith and Pereira-Smith, 1996; Campisi, 1996). Replicative senescence is thought to be one of the mechanisms involved in physiological ageing (Finch, 1990) and in the development of certain pathological conditions (Goldstein and Harley, 1979; Rudolph et al., 2000). Until recently, identification of senescent cells, both in culture and in tissue, relied primarily on morphological characteristics. For this reason, since its first description (Dimri et al., 1995) SA-β-galactosidase has been readily embraced as a biomarker of senescence by many researchers, despite the fact that its function and origin remain unknown. The findings presented here demonstrate that this enzymatic activity is solely due to a replicative age-dependent increase in the cellular content of the classic lysosomal β-galactosidase.

SA-β-galactosidase has been operationally defined as a pH 6 hydrolytic activity manifested in situ when senescent cells are incubated with the chromogenic substrate X-Gal (Dimri et al., 1995). In the present study we adopted an alternative approach to measure this activity, namely a flow cytometric assay that detects the hydrolysis of a fluorogenic β-galactosidase substrate in living cells. This method featured a number of advantages over the cytochemical assay. First, the

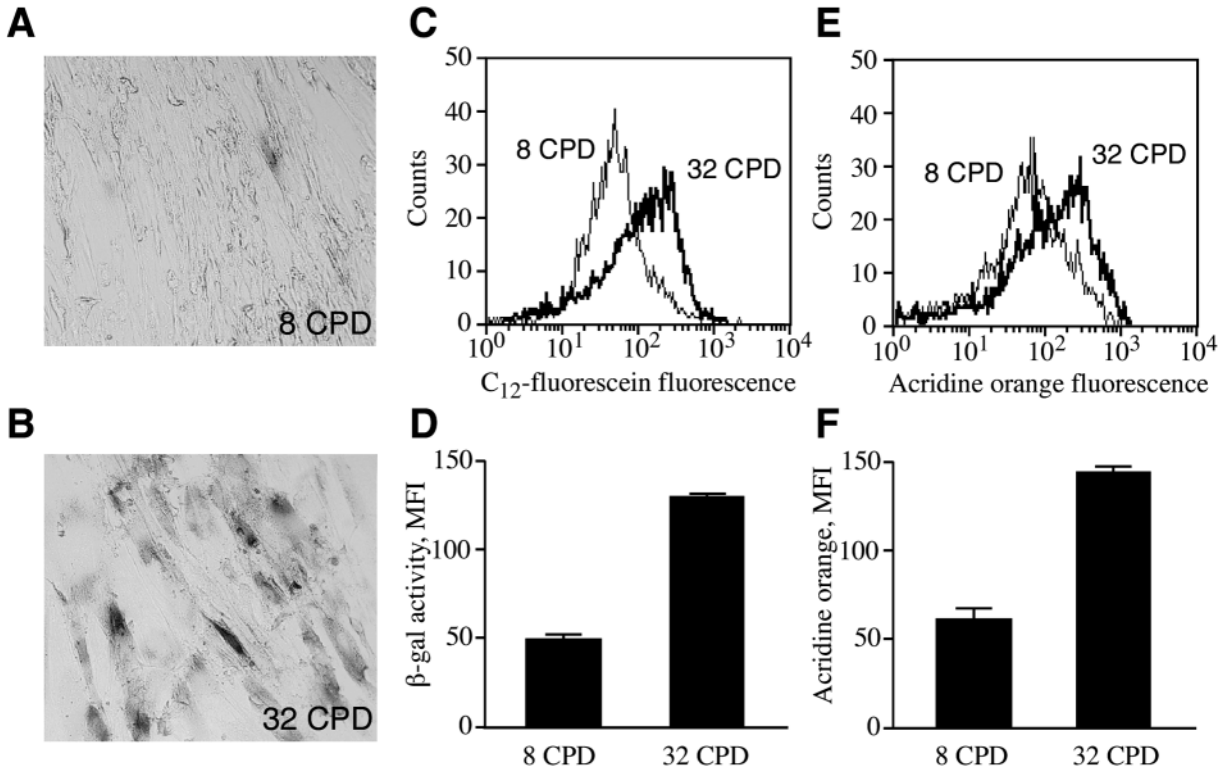

Fig. 7. β-galactosidase activity and lysosomal content in fibroblasts. Mammary fibroblast monolayers were processed for cytochemical detection of β-galactosidase at pH 6 as in Fig. 1 (A,B) and for flow cytometric determination of β-galactosidase activity (C,D) and lysosomal content (E,F) as described in Fig. 6. (A,B) Photomicrographs of third (A) and fifteenth (B) passage cultures. (C,E) Flow cytometric profiles of C_{12}-fluorescein fluorescence (C) or Acridine Orange fluorescence (E) in third (thin tracing) and fifteenth passage cultures (thick tracing). (D,F) MFI values (means ± s.d. of duplicate cultures) of the corresponding flow cytometric profiles.

use of a fluorogenic substrate greatly enhanced the sensitivity of the assay. Second, by studying living cells we were able to alter the pH of the lysosomal compartment selectively, and in this way we could evaluate the contribution of the lysosome to the activity detected at pH 6. Finally, flow cytometry provided a quantitative measure of β-galactosidase activity for each cell in the population, enabling a more accurate evaluation of differences in activity within the same culture and also between cultures of different replicative ages. Using this method under conditions of lysosomal alkalinisation, we examined serially passaged endothelial cells until the onset of senescence. We found that there was a linear correlation between the relative levels of β-galactosidase activity measured in this way, and the results from conventional cytochemistry, hence validating the flow cytometric approach.

The characteristics of the flow cytometric assay mentioned above enabled us to investigate in more detail the origin of SA-β-galactosidase. Our results showed that, with or without lysosomal alkalinisation (chloroquine and baf A_1 experiments), or equilibration of the intracellular environment to pH 6 (nigericin experiments), the relative increase of β-galactosidase activity with age, when compared to that present in young cultures, was equivalent under all intracellular pH conditions. Taken together these findings demonstrated that SA-β-galactosidase and lysosomal β-galactosidase could not be dissociated, suggesting that SA-β-galactosidase is not the result of a separate isoform differentially expressed or activated during senescence. In contrast, our findings endorsed the notion that detection of SA-β-galactosidase is due to a rise in the lysosomal enzyme and results from its residual activity at a suboptimal pH. The origin of the age-related increase in β-galactosidase activity was further investigated by western blot analysis. This analysis substantiated the flow cytometric findings, demonstrating that the rise in activity was due to an increase in enzyme mass. Furthermore, it also showed that this rise cannot be accounted for solely by the general increase in protein or cell size found in ageing cells. These results also suggest that upregulation of enzyme activity does not play a relevant role in this age-related increase.

A major finding of this study is the demonstration that the senescence-associated rise in β-galactosidase activity could be entirely explained by an equivalent increase in lysosomal mass, confirming that the origin of SA-β-galactosidase is the lysosome. It could be argued that these findings merely reflect a general increase in cytoplasmic constituents, characteristic of the cellular hypertrophy shown by senescent cells. However, the fact that β-galactosidase protein content increased even when measured relative to actin, a cytoskeletal component, ruled out this possibility. Thus, the rise in lysosomal content appears to be a more specific dysfunctional change of senescent cells. It has been proposed that the increase in cellular lysosomal content in ageing cells is caused by the accumulation of non-degradable intracellular macromolecules and organelles in autophagic vacuoles (Brunk and Termann, 1999). In vivo, this process is manifested by the accumulation of lipofuscin in ageing post-mitotic cells (Yin, 1996). It has

been further suggested that these secondary lysosomes loaded with non-degradable material are not available for further digestion of macromolecules, forcing cells to synthesise more primary lysosomes in an attempt to continue with normal cellular function. However, most of the newly formed primary lysosomes appear to fuse with these lipofuscin-containing acidic vacuoles, contributing further to their increase in size and content of hydrolytic enzymes (Brunk and Termann, 1999). This description of events is entirely consistent with our findings showing that senescent cells are filled with large and numerous lysosomes and have considerably higher levels of β-galactosidase.

Cellular lysosomal content was reported to increase in senescence more than 20 years ago (Robbins et al., 1970; Brunk et al., 1973). Similarly, elevated activities of lysosomal enzymes (Turk and Milo, 1974; Cristofalo and Kabakjian, 1975; Bosmann et al., 1976) have been associated with in vitro ageing. Despite these early findings, a relationship between lysosomal content and SA-β-galactosidase activity has not been demonstrated until now. Here we have shown a strong correspondence between the increases in cellular lysosomal mass, β-galactosidase protein mass, β-galactosidase activity under various intracellular pH conditions, and SA-β-galactosidase cytochemical staining with replicative age. These findings rule out the existence of a distinct enzyme responsible for the cytochemical detection of SA-β-galactosidase in senescent cells. SA-β-galactosidase can therefore be explained as a threshold phenomenon of the assay under unfavourable pH conditions (pH 6). Thus, when performed at pH 4, a condition in which the lysosomal enzyme displays maximal activity, all cells generate a strong stain irrespective of replicative age, making identification of senescent cells impossible. In contrast, at pH 6 the specific activity of this enzyme is very low, as a result of which young cells fall below the level of detection of the cytochemical assay, whereas old cells with an increased lysosomal β-galactosidase content show positive staining. This conclusion is consistent with findings in the present study showing that at pH 6 (in the presence of nigericin) β-galactosidase activity is reduced by 99%.

Our findings should not diminish the attractiveness of the SA-β-galactosidase stain as a valid marker of cellular senescence, in particular when dealing with tissue biopsies. Nonetheless, if, as our results demonstrate, positive staining is due to an increase in lysosomal content, care should be taken when assessing senescence on the basis of this marker alone. Finally, the present study should help to reconcile the use of SA-β-galactosidase as a biomarker of senescence with other reports describing the detection of β-galactosidase activity at pH 6 in certain non-senescent cells (Yegorov et al., 1998; Devarakonda et al., 1999).

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