Spontaneous senescence in the MDA-MB-231 cell line

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Abstract. Normal human somatic cells have a limited division potential when they grow *in vitro*. It is believed that shortening of telomeres, specialized structures at the ends of chromosomes, controls cell growth. When one telomere achieves a critical minimal length, the cell cycle control mechanism recognizes it as DNA damage and causes the cell's exit from the cycle in G1-phase. Because it is not possible to extend telomeres in normal cells, this non-dividing state is prolonged indefinitely, and is known as cellular senescence. The immortal cell line MDA-MB-231 has active telomerase, which prevents telomere shortening and allows cells' permanent divisions. However, there is a fraction of cells that do not divide over several days in culture as documented for some other tumour cell lines. Combination of methods has made it possible to isolate these non-growing cells and compare them with the fraction of fast-growing cells from the same culture. Although the non-growing fraction contains a significant percentage of typical senescent cells, both fractions have equal telomerase activity and telomere length. In this paper we discuss possible mechanisms that cause the appearance of this non-growing fraction of cells in cultures of MDA-MB-231, which indicate stress and genome instability rather than variation in telomerase activity or telomere shortening to affect individual cells.

INTRODUCTION

Normal human somatic cells undergo a limited number of divisions before entering an irreversible growth-arrest state defined as senescence (Hayflick & Moorhead 1961). Replicative senescence is thought to provide a barrier against unlimited cell proliferation and the occurrence of cancer (Campisi 2000). The best described counting mechanism for replicative senescence involves the telomere-shortening hypothesis. Due to DNA end replication and additional exonuclease degradation of the 5' strand at telomere ends, telomeres shorten with each subsequent cell division (Olovnikov 1973; Harley *et al.* 1990; Makarov *et al.* 1997). When one telomere reaches a critical length in normal cells, irreversible growth arrest is activated (Hemann *et al.*

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2001) and cells become senescent. However, these senescent cells are viable and metabolically active; they are stopped in the G1-phase of the cell cycle and cannot be stimulated to further division by growth factors (Cristofalo *et al.* 1992). They are resistant to apoptosis and can be kept in culture for years if maintained properly (Hayflick 1965). Senescent cells are enlarged and have a more granulated appearance than their dividing counterparts; they show activity of a lysosomal hydrolase (pH 6.0) which is called senescence-associated (SA)- β -Gal (Dimri *et al.* 1995).

In tumour cells, telomere maintenance is required to overcome cellular senescence and prevent chromosome degradation. In most cases this is achieved by the activation of telomerase (Counter *et al.* 1992), the enzyme responsible for telomere lengthening. Telomerase is not expressed in most somatic human tissues in which telomeres shorten with every cell division. However, introduction of the telomerase subunit hTERT into normal human cells results with telomere elongation and lifespan extension (Bodnar *et al.* 1998).

A subset of tumours, however, shows no telomerase activity. Cells maintain or increase their telomere length through an alternative mechanism based on recombination (ALT) (Bryan *et al.* 1995; Neumann & Reddel 2002).

Tumour cells can be readily induced to undergo senescence by genetic manipulation such as expression of cell cycle inhibitors, genes involved in senescence or telomerase inhibition as well as by treatment with chemotherapeutic drugs, radiation, or differentiating agents (Hahn *et al.* 1999; Roninson 2003).

This 'accelerated senescence', which does not involve telomere shortening, is also triggered in normal cells by the expression of mutant *Ras* or *Raf* genes (Serrano *et al.* 1997; Zhu *et al.* 1998) and by further forms of supraphysiological mitogenic signalling (Campisi 2001). The appearance of senescent cells has been documented in some tumour populations indicating spontaneous onset of senescence (Rubelj *et al.* 1997; Te Poele *et al.* 2002). To examine this phenomenon, we have detected and analysed the presence of senescent cells in the MDA-MB-231 cell line by tritiated thymidine labelling index and SA- β -galactosidase activity. Fractions of cells with high or low dividing capacities were separated from cycling cultures by DiI staining and subsequent separation on a high-speed flow cytometer–cell sorter. Isolated fractions were cultured once more, analysed, and telomere lengths and telomerase activity of both fractions were determined. The results obtained suggest stress and genome instability as the main cause of onset of senescence in a small fraction of MDA-MB-231 cells.

MATERIALS AND METHODS

Cell lines and culture conditions

The human breast cancer MDA-MB-231 cell line was obtained from the American Type Culture Collection (ATCC). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) with 10% foetal bovine serum (FBS) in a humidified 37 °C incubator with 5% CO₂. Depending on experimental design, cells were subcultured at a ratio between 1 : 2 and 1 : 10.

SA- β -galactosidase staining and tritiated thymidine autoradiography

To determine the labelling index, cells were seeded into 30-mm Petri dish. After 24 h tritiated thymidine was added at a concentration of 10 μ Ci/ml and incubated for another 24 h, at 37 °C, 5% CO₂. Following this treatment, cells were fixed in 1% glutaraldehyde for 10 min, washed twice in PBS for 5 min and stained for SA- β -galactosidase activity at pH 6.0 over 16–18 h as previously described (Dimri *et al.* 1995). For autoradiography, cells were washed twice in PBS

for 5 min, twice in 70% ethanol for 5 min, and dried at room temperature for several hours. In a photography darkroom, fixed cells were overlaid with liquid photographic emulsion (Ilford Scientific Product, ILFORD Imaging UK Ltd), stored at 4 °C for 48 h processed in standard Kodak developer and universal fixer. More than 500 cells were scored for statistical analysis.

DiI staining and flow cytometry

An appropriate number of cells ($1-3 \times 10^6$) was trypsinized, spun at 200 g in a swing-rotor laboratory centrifuge, washed twice in PBS and resuspended in 10 ml of serum-free DMEM medium containing 5 µM solution of DiI [1,1'-dioctadecyl-3,3,3',3' tetramethylindocarbocyanineperchlorate ('DiI'; DiIC18(3)) catalogue no. D282, 100 mg, Molecular Probes, Eugene, OR, USA] and were incubated for 20 min at 37 °C. Following this treatment, cells were washed twice, and cultured in the dark, wrapped in aluminium foil. After 7 days, at ~80% confluence, cells were trypsinized, washed twice in freshly prepared washing/staining buffer [PBS (Sigma) containing 2% FBS (Sigma) and 0.02% EDTA (Sigma)], and were re-suspended in the same buffer at concentration of 2×10^7 /ml and kept on ice until sorting.

Control cells were stained for 20 mins and at the appropriate concentration placed on ice in the same buffer until sorting. Cells were analysed and sorted on a MoFlo high-speed cell sorter (DakoCytomation, Glostrup, Denmark) using SUMMIT software (DakoCytomation), 488 nm Argon-Ion laser tuned on 125 mW of power was used as a source of excitation. Three measured parameters, FSC (forward-scattered light) and SSC (side-scattered light) were detected with linear signal amplification (Fig. 2B) while DiI-specific fluorescence emission was detected on the FL2 channel using a 570/30 dichroic emission filter and logarithmic signal amplification. Two separate cell populations (L-fraction and R-fraction) were sorted simultaneously into 14-ml Falcon tubes filled with washing/staining buffer. Collected cells were centrifuged, re-suspended in culture medium and were further analysed.

Telomerase activity

Telomerase activity was assayed using the TRAPeze Telomerase Detection Kit (Intergen Company, CHEMICON International Inc., Temecula, CA, USA; Kim *et al.* 1994). Cells were lysed and lysates were mixed with primer and TRAPeze reaction mixture. This mixture was incubated for 30 min at 30 °C to allow telomerase-dependent elongation of TS primer. Elongated and amplified telomerase products were resolved on 12.5% polyacrylamide gel and were visualized with ethidium bromide (Fig. 4A). Intensities of internal standard and telomerase product bands were quantified in IMAGE MASTER VDS software, version 2.0 (Amersham Biosciences, UK). Telomerase activity in TPG (total product generated) units was calculated by comparing the ratio of telomerase products to an internal standard for each lysate, as described by Intergen. TPG is defined as the number of TS primers extended by at least four telomeric repeats through telomerase activity in an extract, during 10-min incubation at 30 °C.

Southern blot analysis

Genomic DNA was isolated with DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) and digested with *RsaI/HinfI* (Roche, Indianapolis, IN, USA) restriction enzymes. Equal amounts (5 µg) of DNA were loaded on 0.8% agarose gel. DNA was transferred to positive nitrocellulose membranes (Roche) by capillary transfer, the membrane hybridized with digoxigenin-labelled terminal restriction fragment (TRF) telomere specific probe detected with CDPStar (Roche) using X-ray film (Kodak). The TRF telomere digoxigenin-labelled probe was prepared by PCR. Primers specific for the telomere sequence F: (CCCTAA)₄, R: (TTAGGG)₄ were amplified by non-template PCR (94 °C/1.5 min, 94 °C/45 s, 52 °C/30 s, 72 °C/1 min, 72 °C/10 min, 30 cycles).

Figure 1. Tritiated thymidine labelling index and SA-β-Gal activity in the MDA-MB-231 cell line. Four different phenotypes were observed (A): [3H]dT⁺/SA-β-Gal⁻ (→); [3H]dT⁺/SA-β-Gal⁺ (→); [3H]dT⁻/SA-β-Gal⁻ (→); [3H]dT⁻/SA-β-Gal⁺ (→). At least 500 cells were counted for statistical analysis (B).

RESULTS

Tritiated thymidine labelling index and SA-β-Gal activity of MDA-MB-231 cell line

Non-dividing senescent cells gradually accumulate over time in normal cultures (Hayflick 1965). Therefore, even young cultures have a fraction of senescent cells. The most obvious properties of senescent cells are their inability to incorporate tritiated thymidine, cell size enlargement and strong SA-β-Gal activity (Dimri *et al.* 1995; Rubelj *et al.* 2002). These cells should not be confused with non-dividing quiescent cells, which remain able to divide upon subculture, have a normal young phenotype and lack of SA-β-Gal activity (Cristofalo *et al.* 2004). A small percentage of cells demonstrating the typical senescent phenotype is also observed to spontaneously appear in some immortal cell lines (Te Poele *et al.* 2002). Although intriguing, this phenomenon has not been extensively studied. In order to detect and investigate the appearance of senescent cells in the immortal MDA-MB-231 cell line, we determined their tritiated thymidine labelling index and also SA-β-Gal activity along with the presence of senescence-specific morphology. The cells were first grown in the presence of tritiated thymidine for 24 h, fixed and stained for SA-β-Gal activity, after which liquid photographic emulsion was applied and cells were exposed for at least 48 h (see Materials and methods section). Four morphologies of cells were observed (Fig. 1A,B). Most cells showed the typical immortal phenotype [3H]dT⁺/

SA- β -Gal⁻ and were present at 73.5%. Cells with [3H]dT⁺/SA- β -Gal⁺ were the second largest group represented at 19.6%. Although dividing, these cells showed significant SA- β -Gal activity which can be explained as their sensitivity to stress during subculture rather than a genuine onset of cell senescence (see Discussion). However, 4.7% of cells were [3H]dT⁻/SA- β -Gal⁻ and they most probably represent cycling cells with delayed entrance into S-phase during the period of labelling, as has been observed previously (Rubelj *et al.* 2002). A fraction of cells with a typical senescent phenotype [3H]dT⁻/SA- β -Gal⁺ along with significant size enlargement was present at 2.2%. Although small, this fraction of slow or non-dividing cells along with spontaneously senescent cells is found in the MDA-MB-231 cell line. In order to isolate and analyse this fraction of cells we employed the recently developed technique of DiI staining and cell sorting (Ferenac *et al.* 2005).

Cell sorting

In order to isolate the fraction of non-dividing cells from MDA-MB-231 cell line, an appropriate number of cells was stained with DiI (Ledley *et al.* 1992; Ferenac *et al.* 2005), cultured for ~7 days until 80% confluence and was submitted to flow cytometry analysis, as described in the Materials and Methods section. The dye integrates into cell membranes and fluoresces orange-red under green light when incorporated (Fig. 2A). This has an absorption and fluorescence emission maximum separated by about 65 nm, facilitating fluorescent detection efficiency. DiI labelling does not affect cell viability or basic physiological properties. As labelled cells divide, each daughter cell inherits approximately 50% of the dye. This phenomenon allows flow cytometric analysis of labelling intensities of cultured cells enabling detection of individual cells having undergone different numbers of cell division during the period of growth. Depending on variation in cell size, fluorescence signals detected from successive generations of DiI-labelled cells could be broad and overlapped to some extent. However, MDA-MB-231 cells are uniform in size so it was possible to clearly distinguish non-labelled controls from maximum intensity stained cells (Fig. 2C). Also, cells that were cultured for 7 days clearly differed with regard to their DiI staining intensities according to the number of divisions they underwent. In Fig. 2D individual peaks of each dividing fraction of cells is clearly visible so that divisions from 0 to 3 can easily be distinguished. These properties of MDA-MB-231 cells and high quality sorting resulted in clear separation of two opposite fractions of non-dividing (R-fraction in Fig. 2D) or fastest growing cells (L-fraction in Fig. 2D). Gating was restricted to 10% of total number of sorted cells for each fraction (Fig. 2D). Positive control cells were cultured for the same period of time as DiI-labelled cells and were stained on the day of sorting while the unstained fraction of the same cells were used as a negative control.

Tritiated thymidine labelling index and SA- β -Gal activity of separated fractions

After separation of fast- and slow-growing fractions of cells from MDA-MB-231 cell line we examined their viability, dividing potential and presence of senescent cells. For this purpose a sample from each fraction was further subcultured to determine tritiated thymidine labelling index and SA- β -Gal activity (Fig. 3). As expected, overall the subpopulation of intensively dividing cells (L-fraction in Fig. 2E) demonstrated a high tritiated thymidine labelling index (90.4%). The subpopulation of non-dividing cells (R-fraction in Fig. 2E) showed surprisingly high overall tritiated thymidine incorporation (76.8%), although these cells divided 0–1 times during 7 days of culture. While a significant drop in the typical immortal phenotype [3H]dT⁺/SA- β -Gal⁻ was expected for the R-fraction which contained 11.7% of these cells, it was a surprise that the L-fraction contained only 32.6%. At the same time both fractions showed a great increase in the [3H]dT⁺/SA- β -Gal⁺ phenotype (L-57.8%; R-65.7%) compared to the untreated culture (19.6%,

Figure 2. DiI staining and sorting of MDA-MB-231 cells. Merged image of DiI and DRAQ5 stained cells observed under the confocal microscope (A). DiI is incorporated into cells' membranes (pseudo green), DRAQ5 is incorporated into nuclei (red). Gating strategy and sorted cell analysis. The polygonal region was drawn around cells according to their morphological properties on a FSC/SSC dot plot. Live cells were gated while dead cells and cell debris were excluded from further analysis and sorting (B). Fluorescence intensity of positive control cells stained on the day of experiment (solid blue) and unstained negative control cells (blue line) (C). Cell sorting (D). Sort decisions were made combining a live cell gate (B) with region L (fast-growing fraction; green) for one sort direction, and R (non-dividing cells; pink) for another sort direction. Each gate separated 10% of the entire population of cells. Clear separation of two cell groups is shown using an overlay histogram of analysed cells from both fractions sorted. Sort purity was $\geq 98\%$ in all sorting experiments performed (E).

Fig. 1A,B). Since SA- β -Gal activity can be induced in immortal cell lines as a result of oxidative stress or DNA-damaging agents (Robles & Adami 1998; Roninson 2003) the observed changes were probably a consequence of stress caused by high-speed cell-sorting treatment during which cells were exposed to intense aeration, high frequency vibrations and a strong electromagnetic

Figure 3. Tritiated thymidine labelling index and SA-β-Gal activity of L-sorted and R-sorted fractions. Blue arrows mark senescent cell phenotype [3H]dT⁻/SA-β-Gal⁺ (A). At least 500 cells were counted for statistical analysis (B).

field (see Discussion section). Nevertheless, it should be noticed that the great majority of 'non-dividing' cells retained their ability to divide when subcultured after separation, although typical senescent cells with [3H]dT⁻/SA-β-Gal⁺ and enlarged morphological phenotype increased in this population to 15.6% (Fig. 3A,B) vs. 2.2% in whole culture. This meant that there was at least ~1.56% of truly senescent cells per PD in the entire cell population, which could be described as spontaneous senescence. Percentage of cells with [3H]dT⁻/SA-β-Gal⁻ phenotype did not significantly change in both fractions (R, 7.5%; L, 5.5%) compared to the untreated population (4.7%).

Telomerase activity and telomere length

Telomere shortening is a well-known mechanism of cell-growth control. In order to continue indefinite divisions all immortal cells, single-cell eukaryotes and stem cells must maintain their telomeres above a critical length, which mostly they do through telomerase expression (Kim *et al.* 1994; Wright *et al.* 1996). Like most immortal cell lines, MDA-MB-231 cells also have

Figure 4. TRAP assay. (A) Polyacrilamide gel of TRAP reaction: L, reaction from fast dividing fraction; R, reaction from non-dividing fraction; Lhi, heat inactivated protein extract from fast dividing fraction; Rhi, heat inactivated protein extract from non-dividing fraction; -C, negative control; +C, positive control; Ma, pBR322 HaeIII, Roche marker V. (B) TPG-unit values: L, fast dividing fraction; R, non-dividing fraction. TPG is defined as the number of TS primers extended by at least four telomeric repeats through telomerase activity in an extract, during 10-min incubation at 30 °C.

an active telomerase (Aldous *et al.* 1999). Since previous results have demonstrated that cultures immortalized by telomerase expression contain a fraction of senescent cells (Gorbunova *et al.* 2003), which surprisingly have increased telomerase activity while maintaining unchanged telomere lengths, we decided to examine these two parameters in both fast- and slow-dividing fractions of MDA-MB-231. Telomerase activity was measured by the very sensitive TRAP method (see Materials and methods section). We found that telomerase activity in both isolated fractions was about equal; 136 TPG units in L-fraction vs. 147 in R-fraction (Fig. 4). Similar results were obtained for telomere lengths so that both fractions showed equal telomere lengths as a whole cell population (Fig. 5). These results point to stress as the main cause of the onset of cell senescence in MDA-MB-231 cells.

Figure 5. TRF analysis of sorted fractions. L, fast-growing fraction; R, nongrowing fraction; M, untreated MDA-MB-231 cells. MW marker λ HindIII on the left.

DISCUSSION

It has been known for some time that amongst spontaneous or virus-induced immortalized tumour cell lines, there is a small subpopulation of cells ($\leq 10\%$) with limited division potential (Pereira-Smith & Smith 1981). Depending on the tumour cell line this fraction could vary between approximately 3% and 20% and in addition to spontaneous exit from further divisions, cells show typical senescence phenotypes including SA- β -galactosidase staining and flattened enlarged morphology (Te Poele *et al.* 2002). Although the exact explanation for senescence in tumour cell lines is still obscure, it is considered to occur as a consequence of genomic instability in those cells by which they lose an important part of their cell cycle mechanism (Elmore *et al.* 2002).

To examine the subpopulation of non-dividing cells in the immortal cell line MDA-MB-231, we assayed labelling index with tritiated thymidine. Although a great majority of MDA-MB-231 cells incorporated tritiated thymidine, $\sim 7\%$ of cells stayed unlabelled. Inability of DNA synthesis does not demonstrate the difference between senescent cells from quiescent or terminally differentiated cells, so we combined SA- β -galactosidase assay as the senescence biomarker.

Although the percentage of SA- β -Gal positive cells is relatively high in relation to the whole population ($\sim 22\%$), the majority of them incorporate tritiated thymidine as well. Only $\sim 2.2\%$ of cells showed the typical senescent $[3H]dT^-/SA-\beta-Gal^+$ phenotype.

In order to examine truly spontaneously senescent cells, our interest was focused on the subpopulation of cells that did not divide during a longer period of growth (~7 days) in culture and for that purpose we used the DiI staining technique (Ferenac *et al.* 2005). DiI is a fluorescent dye which incorporates into outer and inner cell membranes but does not affect cell viability or basic physiological properties (Ledley *et al.* 1992; Ferenac *et al.* 2005). It is homogeneously spread by lateral diffusion along the cell membrane so that during cell division every daughter cell will inherit an equal amount of the dye, reducing its amount to 50% per cell. These characteristics make DiI ideal for sorting and isolation of cells with different frequency of divisions from whole culture populations. Upon separation of proliferating (L) and non-proliferating (R) fractions of cells we were able to examine their division potential and phenotype as well as telomere lengths and telomerase activity. The latter play a crucial role in control of cell proliferation in culture (Rodier *et al.* 2005).

The L-fraction of cells showed an expected high degree of tritiated thymidine labelling (~90%), but it was surprising that the labelling index of the R-fraction was also relatively high (~77%), although they divided 0–1 times during 7 days in culture. Both sorted fractions showed a remarkably high percentage of SA- β -Gal positive cells (L, 61.9%; R, 81.3%) in comparison with the untreated whole cell population (21.8%). While an increase in stress in some tumour cell lines has led to the end of further proliferation and intense SA- β -Gal activity (Te Poele *et al.* 2002), we observed increased SA- β -Gal activity in both separated fractions while they resumed most of their dividing potential. This could be explained with a form of sensitivity to stress of MDA-MB-231. It is known that some normal and immortal cell lines respond to various forms of stress by positive SA- β -Gal activity (Robles & Adami 1998; Roninson 2003), called stress-associated β -Gal (Ramirez *et al.* 2001). In this case stress-induced damage caused injury and partial leakage through lysosome membranes, and the presence of β -Gal activity in cytoplasm along with cell cycle arrest. We speculate that a large fraction of MDA-MB-231 cells may have suffered mild membrane damage caused by the experimental procedure we performed but still retained their dividing capacity. Since the R-fraction of cells contained a high percentage that did not incorporate tritiated thymidine and show high SA- β -Gal activity (15.5% against 4.1% in L-fraction) we assume that most of them (or at least ~11.5%) represent genuine senescent cells. Constant telomere maintenance over a certain minimal length is necessary for unlimited cell divisions or immortality (De Lange 2002). Tumour cell lines achieve this by telomerase activation or by a mechanism of alternative lengthening of telomeres (ALT) (Bryan *et al.* 1995; Neumann & Reddel 2002). Since MDA-MB-231 cells express telomerase, its inactivation could cause the appearance of cells with limited dividing potential. Therefore we tested telomerase activity in both isolated fractions by the very sensitive PCR-based TRAP (Telomere Repeat Amplification Protocol) assay, and contrary to our expectations, both fractions showed equal telomerase activity. Thus, we can rule out changes in telomerase activity as a cause of the appearance of senescent cells. Similar results were obtained in studies of individual clones of further immortal cell lines which showed that there is heterogeneity in telomere lengths among populations with stable overall telomere lengths (Bryan *et al.* 1998). Among cells with active telomerase there are clones which in some part of their life lose telomerase activity and have shorter telomeres than the clones with unchanged telomerase activity. A small fraction of these cells exhibit the senescence phenotype, which is not related to the loss of telomerase activity. Also, among clones which stopped dividing there were those with short and long telomeres. In experiments in which normal human fibroblasts were immortalized by stable expression of telomerase, it has been observed that there is a fraction of senescent cells. These cells were isolated and telomere lengths and telomerase activity determined. Surprisingly, it was found that they had increased telomerase activity but their telomere lengths were unchanged (Gorbunova

et al. 2003). Following these observations we decided to examine telomere lengths in the non-/slow-growing fraction of MDA-MB-231 cells. Telomere lengths were determined in both isolated fractions as well as a whole population as a control. Similar to the above experiment, both fractions showed equal telomere lengths. These results point to stress and genome instability rather than variation in telomerase activity or telomere shortening among individual cells as the cause of the appearance of senescent cells.

Besides the arrest of further divisions and changed morphology, a characteristic of senescent cells is arrest in the G1-phase of the cell cycle. For future experiments it would be interesting to check DNA contents of both sorted fractions of the MDA-MB-231 cell line and compare them with normal senescent cells. This would indicate the part of cell cycle control machinery responsible for spontaneous senescence in this immortal cell line.

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