PERGAMON

European Journal of Cancer 39 (2003) 230–239

European Journal of Cancer

www.ejconline.com

Chemopreventive agents induce a senescence-like phenotype in rat mammary tumours

K.T. Christov[a,*], A.L. Shilkaitis[a], E.S. Kim[a], V.E. Steele[b], R.A. Lubet[b]

[a]*Department of Surgical Oncology, University of Illinois, 840 South Wood Street, Chicago, IL 60612, USA*
[b]*Division of Cancer Prevention, National Cancer Institute, Bethesda, MD 20852, USA*

Received 1 July 2002; accepted 3 July 2002

Abstract

Terminal replicative senescence (TRS) is a physiological process associated with terminal differentiation, shortening of the telomere, and lack of proliferative activity. Immortalised and tumour cells have lost their differentiation potential and the ability to develop a senescence phenotype. Recently, others and we [11] have observed that some antitumour agents and radiation induce a senescence-like phenotype (SLP) in human immortalized and tumour cell lines. The main purpose of this study was to identify senescence-like cells (SLC) in mammary tumours of rats and assess whether chemopreventive agents that have been used for the prevention and/or treatment of breast cancer can induce a SLP in tumour cells. Sprague–Dawley rats with *N*-methyl-*N*-nitrosourea (MNU)-induced mammary tumours were randomised and treated with tamoxifen, vorozole, 4-(hydroxyphenyl)retinamide (4-HPR), or 9-*cis*-retinoic acid (9cRA). The SLC in mammary tumours were identified and characterised by: (a) SA-β-Gal staining method, which has been considered specific for human cells in TRS (b) staining for lipofuscin, which, although not specific, accumulates in the cytoplasm of cells in senescence; (c) lack of 5-Bromodeoxyuridine (BrdU) labelling after continuous (7 days) infusion of BrdU via osmotic pumps; (d) 90° side light scatter (9OLS) as evaluated by flow cytometry; and (e) decreased telomerase activity. We found that in control tumours, SA-β-Gal-positive cells were rare (below 1.0%) among the tumour cells, stroma fibroblast, myoepithelial and endothelial cells. SA-β-Gal-positive cells increased significantly in the tumours treated with chemopreventive agents and this was associated with a lack of proliferative activity, increased cell granularity, lipofuscin accumulation, and decreased telomerase activity. Thus, in this study we provide for the first time evidence that cells in replicative senescence are present in mammary tumours of rats and that chemopreventive agents can suppress tumor growth by a novel cellular mechanism, inducing a SLP in the tumor cells.

Keywords: Senescence; Mammary tumors; Telomerase activity; Chemoprevention

1. Introduction

Terminal replicative senescence (TRS) is a physiological process typical for normal cell populations, which is characterised by terminal differentiation, shortening of the telomere, and lack of proliferative activity [1]. Immortalised and tumour cells have lost their ability to develop a senescence-like phenotype (SLP) and that correlates with their high proliferative potential and high telomerase activity [2,3]. The cells in replicative senescence develop specific morphological and metabolic features associated also with an up- or down-regulation of specific genes/proteins [4,5]. Among them, the tumour suppressor genes *p16*, *p21*, *p53*, and RB appear to play principal roles [5–8].

Most studies on replicative senescence have been performed *in vitro* on fibroblast cell lines of human origin and under controlled growth conditions [4–7]. In these studies, SA-β-Gal staining has been used as specific for cells, which are in TRS [4,9]. β-Galactosidase belongs to the group of lysosomal enzymes and its activity at pH 6.0 has been associated with the disintegration of specific glycoproteins and complex glycolipids, which accumulate in pre-senescent and senescent cells (4,10). β-Galactosidase activity can be determined by using

Abbreviations: TRS, Terminal replicative senescence; MNU, *N*-Methyl-*N*-nitrosourea; 4-HPR, 4-(Hydroxyphenyl)retinamide; 9cRA, 9-*cis* Retinoic acid; RARs, Retinoic acid receptors, alpha, beta, gamma; RXRs, Retinoid X receptors alpha, beta, gamma; FCM, Flow cytometry; SA-B-Gal, Senescence Associated B-Galactosidase staining; SLC, Senescence-like cells; SLP, Senescence-like phenotype; BrdU, 5-Bromodeoxyuridene.

* Corresponding author. Tel.: 312-996-5347; fax: +312-996-9365.
E-mail address: christov@uic.edu (K.T. Christov).

0959-8049/03/$ - see front matter © 2002 Published by Elsevier Science Ltd.
PII: S0959-8049(02)00497-5

5-bromo-4-chloro-3-indolyl β-D-galactoside and other galactosides as substrates [10]. No data so far have been published as to whether SA-β-Gal staining can identify SLC in mammary gland tissues and tumours of rats and whether chemopreventive agents can induce a SLP in mammary tumours. In recent studies, we characterised the SLC in fibroblast and in various human tumour cell lines, including MCF-7 and MCF10AT cells treated with cytostatics or with retinoids [11]. We also observed that 4-HPR, a well-known inhibitor of mammary carcinogenesis in animal models and in premenopausal women, suppressed cell proliferation and decreased telomerase activity in mammary tumours [12]. The shortening of the telomere and/or decreased telomerase activity have been considered to be reliable markers for cells in replicative senescence [1,2].

In the present study, we further developed the SA-β-Gal staining method to identify SLC in mammary tissues and tumours of rats and provide for the first time data indicating that chemopreventive or antitumour agents can significantly increase SLC in tumours. MNU-induced mammary tumors were employed because they appear similar in pathogenesis, morphology, and (Oestrogen Receptor (ER) status to those of the human breast [13]. Chemopreventive agents were selected to affect specific, but different, cellular targets: tamoxifen inhibits ER signalling [14], (+) vorozole suppresses aromatase activity and thus decreases the estrogen circulation and tissue levels [15], 4-HPR induces cell differentiation and apoptosis [16], and 9-*cis* retinoic acid (9cRA) affects retinoic acid receptor (RAR α, β, γ) and/or retinoid X receptor (RXR α, β, γ) signalling and cell differentiation [17]. The above chemoprevention agents are currently used in various pre-clinical and clinical trials for the prevention and treatment of breast cancer.

To make sure that the SA-β-Gal-positive cells in mammary tumours do not have a proliferative activity, the animals were infused continuously for 7 days with BrdU [18]. The cells that do not accumulate BrdU were considered to be in replicative senescence. Flow cytometry for the assessment of cell granularity (90° light scatter, 90LS) [19] and lipofuscin staining [20] were also employed for the characterisation of SLC in mammary tumours. Tumour samples were also used for evaluation of telomerase activity, which is another marker of terminal replicative senescence and which has been reported to decrease in tumours treated with antitumour agents [1,2].

2. Materials and methods

2.1. Rats

Female, virgin Sprague–Dawley (Hsp: (SD/BR)) rats were obtained from Harlan Sprague–Dawley (Indianapolis, IN) aged 43 days and, after 1 week of quarantine, were randomised by weight and injected intraperitoneally (i.p.) with the carcinogen, *N*-methyl-*N*-nitrosourea (MNU) (Ash Stevens Inc., Detroit, MI). MNU was dissolved in sterile acidified saline (pH 5.0), and injected twice at a 50 mg/kg body weight, when the animals were 43 and 50 days old. Control animals at the same age received sterile saline only. Beginning 3 weeks after administration of the carcinogen, the animals were palpated twice weekly to monitor tumour appearance in the mammary gland. The animals were fed with a 4% Purina diet chow *ad libitum* and had free access to water. Animal experiments were approved by the University of Illinois Animal Care Review Board.

2.2. Tumour model

In MNU-treated animals, mammary tumours occurred between the 6th and 9th weeks after administration of the carcinogen. When tumours reached 8–10 mm/diameter (250–500 mm^3), the animals were randomised, and the administration of the chemopreventive agents was initiated. The animals were sacrificed by CO_2 narcosis, and tumours were removed and cut in two halves. One was fixed in 10% neutral formalin for histomorphology and immunohistochemistry (ICH); the other was frozen in liquid nitrogen for the SA-β-Gal assay and for the telomerase activity assay.

2.3. BrdU-labeling of proliferating cells

Proliferative activity of the mammary tumours was assessed by in vivo labelling with BrdU, (Sigma Chem. Co., St. Louis, MO). Animals were injected i.p. with BrdU, 50 mg/kg body weight (b.w.), 2 h before sacrifice. BrdU labelled cells were identified using an anti-BrdU monoclonal antibody (1:100) (Becton Dickinson, Palo Alto, CA) and the ABC kit (Vector, Burlingame, CA) [18]. The percentage of BrdU-labelled cells was evaluated after counting more than 1000 cells per tumour. To make sure that SLC are in replicative senescence, 5 animals with palpable mammary tumours were subcutaneously (s.c.) implanted with (Alzet osmotic pumps, Alza Co., Polo Alto, CA.), that delivered BrdU for 7 days [18]. The cells that remained unlabelled with BrdU were considered to be in TRS.

2.4. Identification of SLC in mammary tumours by SA-β-Gal staining

The SA-β-Gal activity assay at pH 6.0 has been considered specific for cells in TRS (4,8). SA-β-Gal cells were detected as previously described in Ref. [4], with some modifications. Frozen sections (5–7 μm thick) from control, and treated tumours were fixed in 3.0% formaldehyde for 5 min, washed in phosphate buffered solution (PBS) and stained in X-gal (5-bromo-4-chloro-

3-indolyl-β-D-galactoside) (Sigma Chem., Co. St. Louis MO) solution at pH 6.0 for 24 h at 37 °C. SA-β-Gal positive cells were stained in blue (Fig. 1a and b). The top sections of each slide were not incubated with SA-β-Gal staining solution and served as negative controls. To visualise the tumour architecture, the slides were counter-stained by neutral fast red or haematoxylin. The percentage of SA-β-Gal-positive cells was assessed in the areas of tumour disintegration by counting at least 1000 cells.

2.5. Lipofuscin assay

The deposition of lipofuscin in the cytoplasm has been also considered to be a marker of senescence [8]. Tissue sections from formalin-fixed, paraffin-embedded tumour samples were stained by the Ziel–Nielsen method to identify lipofuscin-positive cells [20]. By this method, the cytoplasm was stained purple and the nucleus red (Fig. 1c).

2.6. Flow cytometry (FCM)

FCM was used to evaluate heterogeneity in cell size and cell granularity. The latter appears to occur in cells in replicative senescence [20]. Tissue samples from control and treated tumours were disintegrated by mechanical and enzymatic treatment. The generated cell suspension was stained by propidium iodide and the cell scattering measured in Epix V flow cytometer (Coulter, Miami, FL). More than 10 000 cells were measured from each sample [21].

2.7. Telomerase activity assay

For telomerase detection, we used the polymerase chain reaction (PCR)-mediated telomere repeat amplification protocol (TRAP)[22]. As a positive control, an extract from cells with known telomerase activity (human breast cancer line MDA-MB-157, 50 cells equivalent) was used. As a negative control, the cell extract was substituted for lysis buffer [23]. As an additional control for the TRAP assay, we used an internal standard (ITAS) (a gift of Dr. J. Shay) that amplifies from the same primers. This internal standard, which consists of a 150-bp DNA product, allows the identification of false-negative tumour samples that might contain Taq polymerase inhibitors. Cell extracts were obtained, and TRAP was performed as previously described in Refs. [12,24]. Two microlitres of tissue extract (protein concentration 0.5 μg/μl) were used per assay. The CX primer, ITAS and Taq DNA polymerase (7 U per assay) were added to each sample after 5 min incubation at 90.8 °C to make a hot start. Aliquots (10 μl) of the PCR mixture were analysed on 0.4 mm, 8% non-denaturing acrylamide gels (20×40 cm), run in 0.5×TBE buffer until the xylene cyanol had migrated 17 cm from the origin. The gels were then dried and exposed for 20 h to hyperfilm MP films (Amersham, Arlington Heights, IL). Following autoradiography, gels were also analysed after overnight exposure with Molecular Dynamics PhosphorImager (Sunnyvale, CA). The area of integration of all peaks was normalised to the signal from the internal standard, then, after background subtraction, expressed relative to the positive control signal (100 cell equivalent) that was run with each experiment. The method described is only semi-quantitative, but it is sufficient for the comparative analysis of tumours relative to the same positive control cell extract.

2.8. Chemopreventive agents

Tamoxifen was supplied by Sigma Chem. Co., St. Louis, MO. It was mixed with sesame oil and injected s.c. every day at 1.0 mg/kg b.w. for 10 or 20 days. (+) Vorozole (R-83842) was obtained from Janssen Research Foundation, Springfield, PA. Vorozole was given at 2.5 mg/kg b.w. by gavage for 2, 4 or 10 days [25]. 4-HPR was obtained from R.W. Johnson Pharmaceutical Research Institute (Spring House, PA) and was added to the diet at 782 mg/kg diet. Animals with mammary tumours were randomised and treated for 1 or 2 weeks with 4-HPR [26]. 9-*cis* Retinoic acid (9cRA) was purchased from Sigma Sci., Co., St. Louis, MO, and was given at 60 mg/kg diet for 10 days.

2.9. Statistical analysis

The significance of differences in the values between various groups was evaluated by ANOVA with Fisher's LSD test [27]. The correlation analysis between the values of SLC and BrdU-labelled cells was performed by SAS computer program. Values for $P<0.05$ were considered significant.

3. Results

3.1. Senescence-like cells are rare in rat mammary tumours

The first question we asked was whether there are SA-β-Gal-positive cells in control mammary tumours and what their location is among the tumour parenchyma (Table 1). Since replicative senescence is associated with a lack of proliferative activity, parallel tumour sections were treated with anti-BrdU antibody to show where the SA-β-Gal-positive cells are located. In this study forty four tumours; 12 from controls, i.e. that were not treated with chemopreventive agents, and 32 from animals treated with various chemopreventive

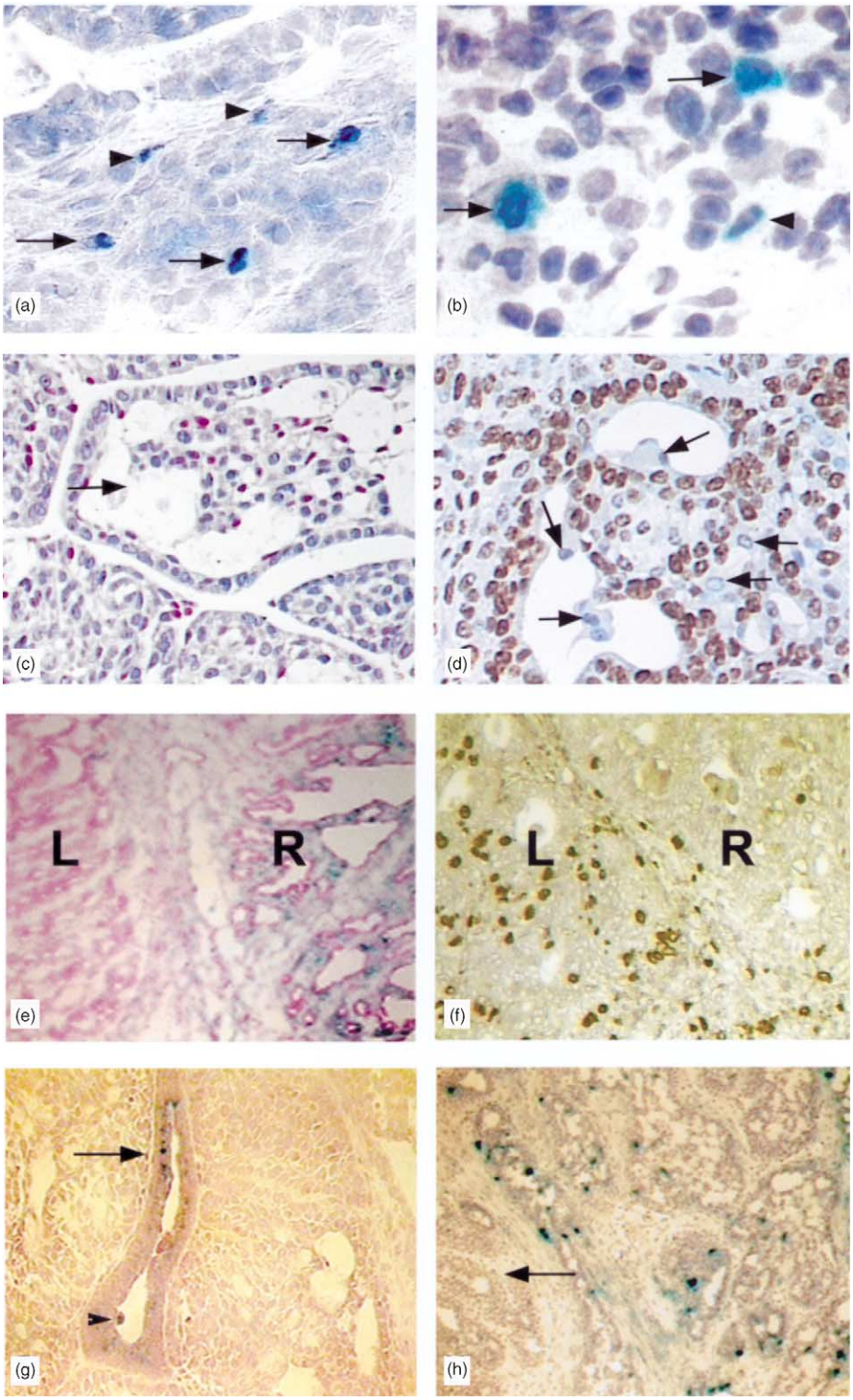

Fig. 1. (a). SA-β-Gal-positive cells (blue-stained, arrow) in a control mammary tumour. Frozen tissue sections were cut and stained by the SA-β-Gal method as described in the Materials and methods. SA-β-Gal-positive cells (blue-stained) are rare and dispersed among the tumour cells. SA-β-Gal-positive cell with arrow head is most probably a stroma cell. The slide is counter-stained by haematoxylin for visualisation of the tumour morphology (×400).

Table 1
Location of SLC in various mammary cell populations

Cell type	Frequency*
Normal mammary structures	
Ductal epithelial cells	–[a]
Alveolar epithelial cells	±[b]
Myoepithelial cells	±
Fibroblast	±
Endothelial cells	–
Adipocytes	–
Desquamated cells in the lumenal structures	+[c]
Tumours	
Adenocarcinomas	+
Papillary carcinomas	+
Solid carcinomas	+
Tumours: Proliferation areas	–
Areas close to necrosis	++
Tumour cells in cystic structures	+++[d]
Myoepithelial cells	+
Stroma fibroblasts	+
Endothelial cells	–

[a] –, Lack of SA-β-Gal-positive cells.
[b] ±—Single SA-β-Gal-positive cells in at least 5 high-power (×400 magnification) microscopic fields.
[c] ±+—several (<10) SA-β-Gal-positive cells in 5 high-power (×400) microscopic fields.
[d] ++ or +++—SA-β-Gal-positive cells in groups comprising isolated tumour areas.

agents (Table 2). In all of the control tumours, single tumour cells, stroma fibroblasts, and myoepithelial cells were stained for SA-β-Gal (Fig. 1a and b). The cytoplasm of the SA-β-Gal-positive cells was blue-stained whereas in the areas occupied by the nucleus there was no staining (Fig. 1b, arrowhead). Mitotic figures, which were frequent in the proliferating tumour areas, were absent in SA-β-Gal-positive cells when counterstained by haematoxylin. In addition to SA-β-Gal staining, parallel tumour sections were stained for lipofuscin (Fig. 1c). The lipofuscin-positive cells (red-stained nucleus and purple cytoplasm) were also found among the tumour-, stroma-, and myoepithelial cells in the same tumour areas where SA-β-Gal-positive cells were frequent.

In order to assess whether or not there were cells in replicative arrest among the tumour parenchyma, rats with palpable mammary tumours were s.c. implanted with osmotic pumps filled up with BrdU-solution. Osmotic pumps were kept for 7 days when the animals were sacrificed. All proliferating cells during the above period of time should accumulate BrdU and their nuclei should be positively stained by the anti-BrdU-antibody. Cells that lack BrdU-staining should be considered to be in terminal replicative arrest (Fig. 1d, arrow). SA-β-Gal-positive cells were located on the tips of papillary structures or within the most internal cell layer of alveolar or cystic tumour formations where the cells usually do not proliferate and the only alternative for them is to desquamate and die. Cells within the cystic structures or ductal lumen always contained SA-β-Gal-positive cytoplasmic granules.

3.2. Chemopreventive agents induce SLC in mammary tumors

The main goal of this study was to find out whether chemopreventive agents, which have different mechanisms of action, could induce SLC in mammary tumours. Animals with palpable (8–10 mm) tumours were treated for different time intervals with tamoxifen, vorozole, 4-HPR or 9cRA, and the percentage of SA-β-Gal-positive cells was evaluated (Table 2). Based on our recent data on the effects of chemopreventive agents on cell proliferation, apoptosis and telomerase activity in mammary tumours [12,25,26], various time points were selected. Histological examination of the tumours treated with the chemopreventive agents revealed that, at the early time points (4 and 10 days), a disintegration of tumour parenchyma occurred in the restricted tumour areas only. With extension of the treatment to 20 days (tamoxifen), the areas of tumour disintegration increased and occupied most of the tumour parenchyma. SA-β-Gal cells were found mostly in the areas of tumour response, where tumour parenchyma was replaced by cystic formations (Fig. 1e). As shown in the figure, the tumour was composed of two cell populations; the left one (L) was apparently resistant to tamoxifen, because the tumour architecture was preserved, and there were no SA-β-Gal cells, the right one (R) was sensitive to tamoxifen, tumour parenchyma was composed of cystic structures and there were plenty of SA-β-Gal-positive cells (Fig. 1e, blue-stained). On a parallel slide, BrdU-labelled cells were evaluated (Fig. 1f). Among the resistant cell population (L), there were plenty of BrdU-labelled cells (brown stained, whereas among the responsive cell population R, where the SA-β-Gal cells were frequent (Fig. 1e,R) there was a lack of BrdU-labelled cells. In another tumour treated for 10 days with tamoxifen, SA-β-Gal cells were rare among the tumour cells, but frequent within a ductal hyperplastic lesion preserved among the tumour parenchyma (Fig. 1g, arrow). In the animals treated with tamoxifen, SA-β-Gal cells increased from less than $0.4\pm0.1\%$ in control tumours to $3.8\pm2.0\%$ in the 10-day-treated and to $2.7\pm1.9\%$ in the 20-day-treated animals ($P<0.01$ and $P<0.05$, respectively) (Table 2). In the tumour areas where SLC were most frequent, apoptotic cells were also frequent (data not shown).

Another group of animals was treated for 2, 4 or 10 days with 2.5 mg/kg b.w. vorozole (Table 2). In a recent study, we found that the above dose increased apoptotic cell death and decreased cell proliferation in mammary tumours [25]. Although the number of tumours examined was small, a progressive increase in SA-β-Gal cells

Table 2
Chemopreventive agents induce SLC in mammary tumors

Agent/tumour	Dose mg/kg	Days of treatment	SLC[a]	BrdU[b]
Control tumours	*N/A*	*N/A*		
1. Adenocarcinoma			0	8.4
2. Adenocarcinoma-cribriform			0.2	12.8
3. Adenocarcinoma			0	15.5
4. Papillary carcinoma			0	7.2
5. Papillary carcinoma			0.4	9.0
6. Adenocarcinoma			0	14.3
7. Papillary carcinoma			0.2	7.4
8. Adenocarcinoma			0	5.8
9. Adenocarcinoma-cribriform			0.5	11.0
10. Adenocarcinoma			0.4	17.8
11. Papillary carcinoma			0.7	8.9
12. Papillary carcinoma			0.3	10.2
			0.4±0.1	12.1±3.4
Tamoxifen	*1.0 mg/kg b.w.*			
13. Adenocarcinoma		10	4.0	1.3
14. Adenocarcinoma		10	7.2	0.8
15. Adenocarcinoma-solid		10	1.8	7.8
16. Adenocarcinoma		10	3.2	2.2
17. Papillary carcinoma		10	2.8	4.4
			3.8±2.0	*3.3±2.8*
18. Adenocarcinoma		20	5.6	2.1
19. Adenocarcinoma		20	1.5	3.3
20. Adenocarcinoma-cribriform		20	1.8	2.2
21. Adenocarcinoma		20	2.0	3.5
			2.7±1.9	2.8±0.7
(+) Vorozole	*2.5 mg/kg b.w.*			
22. Adenocarcinoma		2	0.5	6.2
23. Adenocarcinoma		2	1.2	2.4
24. Adenocarcinoma		2	3.7	2.1
			1.8±1.6	3.5±2.2
25. Adenocarcinoma			4.3	3.2
26. Adenocarcinoma		**4**	5.2	1.5
27. Adenocarcinoma		4	2.8	2.4
28. Adenocarcinoma		4	3.7	2.8
			4.0±1.0	2.5±0.7
29. Adenocarcinoma		10	10.4	2.5
30. Adenocarcinoma		10	15.3	1.8
31. Adenocarcinoma		10	5.8	4.2
			10.5±4.8	2.8±1.2
4-HPR	*782 mg/kg diet*	**7**		
32. Adenocarcinoma-solid		7	0	12.4
33. Adenocarcinoma		7	2.0	5.0
34. Adenocarcinoma-comedo		7	0	9.8
				9.0±3.7
35. Adenocarcinoma-comedo		14	3.8	6.6
36. Adenocarcinoma		14	1.3	4.4
37. Adenocarcinoma		14	2.6	3.3
38. Adenocarcinoma-cribriform		14	10.2	2.0
39. Adenocarcinoma-solid		14	0.0	12.5
			4.4±3.9	5.7±4.1
9-cis RA	60 mg/kg diet			
40. Adenocarcinoma		10	18.3	3.2
41. Adenocarcinoma		10	4.2	6.5
42. Adenocarcinoma-solid		10	2.6	3.6
43. Adenocarcinoma		10	5.0	9.7
44. Adenocarcinoma		10	10.2	3.2
			7.8±6.4	5.0±2.8

b.w., body weight; N/A, not applicable.

[a] SLC, senescence-like cells as identified by SA-β-Gal staining.

[b] Percent of BrdU-labelled cells from the same tumours where SA-β-Gal staining was performed; From each animal, a single tumour was examined. The treatment with the above chemopreventive agents was initiated when the tumour size was in the range of 8–10 mm/diameter. Chemopreventive agents and the doses used are given in bold.

with treatment duration was found: 1.8±1.6% in the 2-day group, 4.0±1.0% in the 4-day group, and 10.5±4.8% in the 10-day-treated animals. The increase of SA-β-Gal cells was associated with a decrease in BrdU-labelled cells (Table 2). In the animals treated for 10 days with vorozole, a disintegration of tumour parenchyma occurred and in the same tumour areas SA-β-Gal cells increased (Fig. 1h, blue-stained cells). In other tumour areas apparently resistant to vorozole, there was lack of SA-β-Gal cells (Fig. 1h, arrow).

4-HPR and 9cRA, which appear to have different mechanisms of action at the molecular level, also induced SLC in the mammary tumours. Both agents affected restricted areas of the tumour parenchyma only, predominantly the tumour periphery. 4-HPR was given in the diet for 7 or 14 days and 9cRA for 10 days (Table 2). In 2 of 3 tumours treated for 7 days and in 1 of 5 tumours treated for 14 days, 4-HPR did not induce SLC in tumour cells. In the tumours treated for 14 days, SA-β-Gal cells increased up to 4.4±3.9% in the restricted areas. In the animals treated with 9cRA, SA-β-Gal cells also increased, from 0.4±0.1% in the control animals to 7.8±6.4%; ($P<0.001$} in the animals treated with 9cRA. 9cRA increased SA-β-Gal cells not only among tumour cells, but also among myoepithelial cells and the stroma fibroblasts surrounding the tumour cells.

Since the development of a SLP in normal and tumour cell populations is associated with a loss of proliferative activity, a correlation analysis was undertaken between the percentage of SA-β-Gal cells and BrdU-labelled cells in the tumours treated with chemopreventive agents. BrdU-labelled cells were from tumours pulse labelled with BrdU for 2 h. A significant negative correlation was found between SLC and BrdU-labelled cells (Fig. 2) ($R=-0.53563$, $P<0.004$) indicating that the occurrence of a SLP in tumour cells is associated with a loss of proliferative activity.

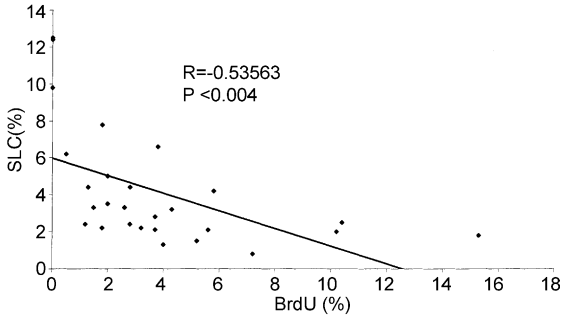

Fig. 2. Correlation analysis between the percentage of SLC and BrdU-labelled cells (BrdU) in the tumours treated with chemopreventive agents ($N=31$). The values of SA-β-Gal cells (ordinate) were compared with those of BrdU-labelled cells (abscise).

Table 3
Effects of chemopreventive agents on telomerase activity in mammary tumours[a]

Treatment/agent	Treatment days	Tumours/animals	Telomerase activity	P value
Control	0	5	575±230	
Vorozole, 2.5 mg/kg b.w.	4	10	490±104	N.S.
Vorozole, 2.5 mg/kg b.w.	10	9	226±90	0.05
4-HPR, 782 mg/kg diet	14	13	125±22	0.01

[a] Telomerase activity was assessed by the TRAP method (see Materials and Methods). The P values compare the differences between control and vorozole-treated or 4-HPR treated tumours. From each animal, a single tumour was examined.

3.3. The occurrence of SLC in mammary tumours is associated with an increased cell granularity

In previous studies, we [11] and others [5,8,19] have reported that the development of a senescent phenotype in cells cultured *in vitro* and treated with antitumour agents is associated with an increased cell size and cell granularity. The latter appears to reflect the accumulation of lipofuscin and of other lipoprotein granules in the cytoplasm, which have also been considered characteristic for cells in TRS [19]. In this study, we employed flow cytometry (FCM) to assess the forward light scatter (FS), which reflects the cell size and the 90° light scatter (90LS) which gives information about cell granularity in control ($N=20$) and tamoxifen-treated ($N=10$) tumours. In Fig. 3a and b the distributions of the individual values for FS (abscise) and 90LS (ordinate) are given for the control and tamoxifen-treated tumours, respectively. FS was apparently similar in the control and tamoxifen-treated tumours, whereas the 90LS was increased in the latter (Fig. 3b). These data strongly support the increase of SA-β-Gal cells in tamoxifen-treated tumours and the relevance of both cellular phenomena to SLP in mammary tumours.

3.4. The increase of SLC in mammary tumours is associated with a decreased telomerase activity

We also examined whether the increase in SLC in tumours treated with chemopreventive agents is associated with a decreased telomerase activity. Three groups of tumours were examined: control, treated with (+) vorozole for 4 or 10 days, and treated with 4-HPR for 14 days (Table 3). From the results presented in Table 3, it is evident that (+) vorozole given for 4 days did not significantly affect the telomerase activity.

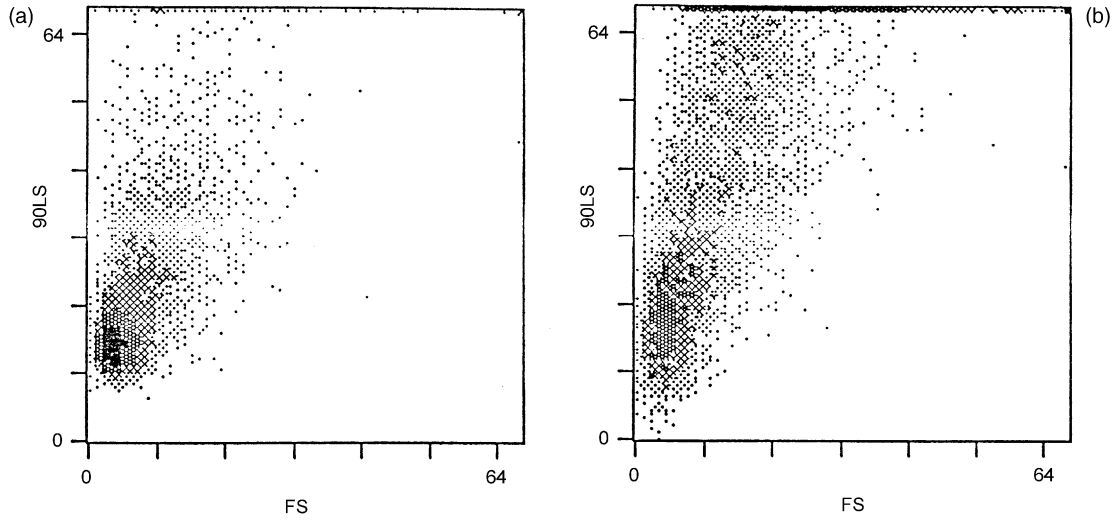

Fig. 3. (a) and b. Effects of tamoxifen on forward light scatter (FS) and 90 light scatter (90LS). Animals were treated for 20 days with tamoxifen, 1.0 mg/kg b.w. Frozen samples from mammary tumours were disintegrated mechanically and enzymatically, the generated cell suspension was stained by propidium iodide, and the FS and 90LS were assessed by flow cytometry. (a) represents FS/90LS values of a control tumour, in (b) are given the values of FS/90LS in a tamoxifen (20 days) treated tumour. An equal number of cells (5000) were measured from each tumour.

However, with extension of (+) vorozole administration to 10 days, telomerase activity significantly decreased ($P<0.05$). These data correlate with much higher values of SLC in the 10-day vs. 4-day (+) vorozole-treated tumours (see also Table 2). 4-HPR given for 14 days also decreased telomerase activity ($P<0.01$) in the mammary tumours (Table 3). In a separate study (data not presented), we found that 1.0 mg/kg tamoxifen given s.c. for 10 or 20 days also decreased telomerase activity in a subset of mammary tumours. In the same tumours, a high percent of SA-β-Gal-positive cells was also found. Thus, it appears that chemopreventive agents differentially suppressed telomerase activity in mammary tumours and that this was associated with an increase in SLC.

4. Discussion

The main goal of this study was to identify and characterise cells that are in TRS in mammary tumours and to assess whether chemopreventive agents, that are currently used in various breast cancer prevention and therapeutic studies, can induce TRS in these tumours. To identify SLC in rat mammary tissue and tumours, we modified the original SA-β-Gal staining method, which has been considered specific for humen cells in replicative senescence [4] and identified SLC in mammary tumours of control and treated rats. In almost all previous studies, the SA-β-Gal staining has been mainly used to identify cells in TRS on *in vitro* growing cell lines [1,5–10]. To make sure that the cells in TRS do not proliferate, animals with mammary tumours were infused for 7 days with BrdU, via osmotic pumps. Since the cell cycle time of the rat's mammary tumor cells is around 18 h [28], the lack of BrdU labelling in individual tumour cells suggests that they have been out of the cell cycle for at least 7–8 consecutive cell divisions. Therefore, the BrdU-negative tumour cells might be considered to be in replicative senescence. However, we can not exclude the possibility that some BrdU-negative cells might also be in a G_0 or in a long G_1 phase and will re-enter the cell cycle after proper stimulatory signals [1,8,9]. A third direct indicator that SA-β-Gal positive-cells do not proliferate is the lack of mitotic figures in these cells, as estimated by counterstaining of the slides with a haematoxylin after completing the SA-β-Gal staining. Lipofuscin accumulation in the cytoplasm also suggests that the tumour cells have developed a SLP. Although not specific like SA-β-Gal staining, the lipofuscin staining has been also used to characterise cells in TRS [6,8]. Additional evidence that chemopreventive agents may induce a SLP in mammary tumours was obtained from our experiments that showed an increased side 90° light scatter (90LS), has also been others by [20] and a decreased telomerase activity, which is another marker of replicative senescence [1,2].

In control tumours, very few SLC were identified and most of them were on the tips of papillary structures or among the most internal cell layers of various alveolar or cystic tumour formations (Fig. 1). In these cellular structures apparently the only alternative of tumour cells is to desquamate and die. SLC in mammary tumours were also observed among the stroma fibroblsts, myoepithelial cells and endothelial cells, data suggesting that tumour cells may produce cytokines that potentiate the development of TRS in the above cell types and thus affect tumour development and progression. In fact, most studies on TRS *in vitro* have been performed on fibroblast cell lines, and the development of replicative senescence in the latter has been related to specific alterations in *TP53*, *p21* $^{waf1/cip1}$ or $p16^{ink4}$ genes [3,5–8].

To induce SLC in mammary tumours, chemopreventive agents were used which previously have shown inhibitory effects on mammary carcinogenesis [13–16]. We found that tamoxifen, (+) vorozole, 4-HPR, and 9cRA induced TRS in mammary tumours and this was associated with a decreased cell proliferation (Table 2 and Fig. 2) and increased apoptotic cell death (data not shown). The above chemopreventive agents were given for short time intervals in order to assess early cellular markers of response in mammary tumours, which could eventually be used as intermediate endpoints in efficacy studies, including those aiming to evaluate the response of breast cancer cells in pre-surgical clinical trials. Continuous administration of chemopreventive and/or antitumour agents may result in the development of resistance in tumour cells and thus affect their response [14].

As shown in Table 2, there were substantial differences in the values of SA-β-Gal-positive cells among the various chemopreventive agents and at the different time points examined. However, because of the small number of tumours in the individual groups, we are not going to make a detailed statistical analysis of the differences in the values of SLC between the various chemopreventive agents. All our data indicate that chemopreventive agents, independent of their mechanism of action, significantly increased the cells in replicative senescence in the mammary tumors.

An interesting observation was that the occurrence of SLC in the tumours treated with chemopreventive agents was associated with an increase in 90LS, parameter that reflects cell granularity [20]. These data support previous studies [5,7], including ours [11], where *in vitro* growing cells have been treated with doxorubicin or with other antitumour agents and an increase in cell size and cell granularity have been observed. The latter parameter appears to be associated with the deposition of lipofuscin granules in the cytoplasm and has been considered to be a marker of replicative senescence [7,8].

We also found that the increase in SLC in mammary tumours treated with (+) vorozole and 4-HPR was

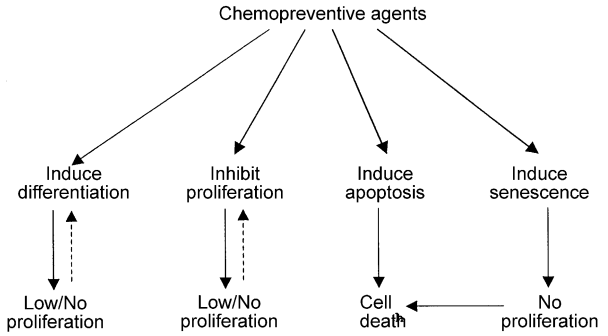

Fig. 4.

associated with a decreased telomerase activity (Table 3). These data support our recent study where a progressive decrease in telomerase activity was found in mammary tumours of animals treated with 4-HPR for 1, 2 or 4 weeks [12]. However, the decrease in telomerase activity in mammary tumors treated with chemopreventive agents should be considered a complex phenomenon, because, in addition to increased SLC, decreased cell proliferation and increased apoptotic cell death were also observed [12,25]. Since, telomerase activity is relatively high in normal mammary epithelial cells as well, the development of tumours should not be directly related to an increased telomerase activity [29]. A correlation has been observed between the inhibition of cell proliferation and decreased telomerase activity in cell lines treated with various antitumour agents [30] and our data, from vorozole- and tamoxifen- treated tumours, supports the above observations. In recent clinical trials, patients with advanced breast carcinomas, who have been treated with chemotherapy, had a significant reduction of telomerase activity in all treated tumours compared with non-treated tumours [31,32].

Based on the data obtained in this study, as well as on the results from previous studies on the mechanism of action of chemopreventive agents [33–35], we postulated that chemopreventive agents may suppress mammary carcinogenesis and tumor growth by at least four cellular mechanisms (Fig. 4): (i) inhibiting cell proliferation, (ii) inducing differentiation that is usually associated with a decreased cell proliferation, (iii) increasing cell death (apoptotic and non-apoptotic) (iv) inducing replicative senescence in tumour cells. The first two cellular events might be reversible, and after removal of chemopreventive agents from the diet, tumour cells may re-enter the cell cycle (Fig. 4, dashed lines). SLC are in a terminal replicative arrest, and the only alternative for them is to die by non-apoptotic cell death.

In conclusion, we provide for the first time evidence that cells with a SLP are present in mammary tumours of rats and that tamoxifen, vorozole, 4-HPR or 9cRA can increase the percentage of these cells. Thus, our data support the hypothesis that replicative senescence is an independent tumour suppressor mechanism, which could potentially be used for assessing the efficacy of various chemopreventive and antitumour agents and for developing new strategies for the prevention and treatment of cancer.

Acknowledgements

The authors wish to thank Kevin Greenfield for editing the manuscript. Supported by contract CN-55179 and CN-65123 from the National Cancer Institute, Bethesda, MD.

References

1. Smith JR, Pereira-Smith OM. Replicative senescence: implication for in vivo aging and tumor suppression. *Science* 1988, **273**, 63–67.
2. Holt SE, Shay JW, Wright WERefining the telomere-telomerase hypothesis of aging and cancerNature Biotech141996836839
3. Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelhutz AJ. Both Rb/p16ink4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature* 1998, **396**, 84–88.
4. Dimri GP, Xinhua L, Basile G, *et al*. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci, USA* 1995, **92**, 9363–9367.
5. Duncan IL, Reddel RR. Genetic changes associated with immortalization. *Biochemistry* 1997, **62**, 1263–1274.
6. Chang BD, Watanabe K, Broude EV, *et al*. Effects of p21waf1/Cip1/Sdi1 on cellular gene expression: implications for carcinogenesis, senescence, and age-related diseases. *Proc Nat Acad Sci USA* 2000, **97**, 4291–4296.
7. Xu HJ, Zhou Y, Ji W, *et al*. Reexpression of the retinoblastoma protein in tumor cells induces senescence and telomerase inhibition. *Oncogene* 1997, **15**, 2589–2596.
8. Sugrue MM, Shin DY, Lee SW, Aaronson SA. Wild-type p53 triggers a rapid senescence program in human tumor cells lacking functional p53. *Proc Natl Acad Sci USA* 1997, **94**, 9648–9653.
9. Campisi J. The biology of replicative senescence. *Eur J Cancer* 1997, **33**, 703–709.
10. Miller AL, Frost RG, O'Brian JS. Purification of human liver acid b-D-galactosidase using affinity chromatography. *Analyt Biochem* 1976, **74**, 537–545.
11. Chang DC, Broudy E, Dokmanovic D, *et al*. A senescence-like phenotype distinguishes tumor cells that undergo terminal proliferation arrest after exposure to anticancer agents. *Cancer Res* 1999, **59**, 3761–3767.
12. Bednarek A, Shilkaitis A, Green A, *et al*. Suppression of cell proliferation and telomerase activity in 4-(hydroxyphenyl) retinamide treated mammary tumors. *Carcinogenesis* 1999, **20**, 879–883.
13. Russo J, Russo IH, van Zwieten MJ, Rogers AE, Gustersson B. Classification of neoplastic and non-neoplastic lesions of the rat mammary gland. In Jones TC, Mohr U, Hunt RD, eds. *Integument and Mammary Glands, Monographs on Pathology of Laboratory Animals*. New York, Berlin, Heidelberg, Springer, 1989, 275–340.
14. Jordan VG, Morrow M. Tamoxifen, reloxifene, and the prevention of breast cancer. *Endocrine Reviews* 1999, **20**, 253–278.
15. Lubet RA, Steele VE, DeCoster R, *et al*. Chemopreventive effects

of aromatase inhibitor vorozole (R83842) in the methylnitrosourea-induced mammary cancer model. *Carcinogenesis* 1998, **19**, 1345–1351.
16. Moon RC, Mehta RG, Rao KVN. Retinoids and cancer in experimental animals. The Retinoids: Biology, Chemistry, and Medicine. In *Sporn MB, Roberts, AB, Goodman DS2nd ed.*. , New York, 1994, Raven Press.
17. Gottardis M, Lamph WW, Shalinski DR, Wellstein DR, Heyman R. Efficacy of 9-*cis* retinoic acid in experimental models of cancer. *Breast Cancer Res Treat* 1996, **38**, 85–96.
18. Christov K, Swanson S, Guzman R, Thordarson G, Talamantes F, Nandi S. Kinetics of mammary epithelial cell proliferation in pituitary isografted Balb/c mice. *Carcinogenesis* 1993, **16**, 101–106.
19. Sherwood SW, Rush D, Elsworth JL, Schimke RT, Defining cellular senescence in IMR. 90 cells: a flow cytometric analysis. *Proc Natl Acad Sci USA* 1988, **85**, 9086–9090.
20. Pearse AGE. Histochemistry, vol.I, 4th Ed. London, 1985.
21. Vindelov LL, Christiansen IJ. A review of techniques and results obtained in one laboratory by an integrated system of methods designed for routine clinical flow cytometric DNA analysis. *Cytometry* 1990, **11**, 753–770.
22. Piatyszek MA, Kim NW, Weinrich SL. Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). *Methods Cell Sci* 1995, **17**, 1–15.
23. Bednareck A, Sahin A, Brenner AJ, Johnston DA, Aldaz CM. Analysis of telomerase activity levels in breast cancer: positive detection at the in situ breast carcinoma stage. *Clinic Cancer Res* 1997, **3**, 11–16.
24. Bednarek AK, Chu Y, Aldaz CM. Constitutive telomerase activity in cells with tissue-renewing potential from oestrogen regulated rat tissues. *Oncogene* 1998, **16**, 381–385.
25. Christov K, Shilkaitis A, Green A, *et al*. Cellular responses of mammary tumors to aromatase inhibitors, effects of Vorozole. *Breast Cancer Res Treat* 2000, **60**, 117–128.
26. Green A, Shilkaitis A, Christov K. 4-HPR selectively suppresses the development and progression of ductal hyperplastic lesions and carcinoma in situ in mammary carcinogenesis. *Carcinogenesis* 1999, **20**, 1535–1540.
27. Snedecor GJW, Cochran WG. *Statistical Methods, vol. 11*, 8th edn. IA, Ames, Iowa State Univ Press, 1998.
28. Russo J, Russo IH. Influence of differentiation and cell kinetics on the susceptibility of rat mammary gland to carcinogenesis. *Cancer Res* 1980, **40**, 2677–2687.
29. Varon D, Jiang C, Hedican C, *et al*. Telomerase activity in the normal and neoplastic rat mammary gland. *Cancer Res* 1997, **57**, 5605–5609.
30. Herbert BS, Wright AC, Passons CM, *et al*. Effects of chemopreventive and antitelomerase agents on the spontaneous imortalization of breast epithelial cells. *J Nat Cancer Inst* 2001, **93**, 39–45.
31. Wang X, Wong SC, Pan J, *et al*. Evidence of cisplatin-induced senescence-like growth arrest in nasopharingeal carcinoma cells. *Cancer Res* 1998, **58**, 5019–5022.
32. Hoos A, Hepp HH, Kaul S, Ahlert T, Bastert G, Wallwiener D. Telomerase activity correlates with tumor aggressiveness and reflects therapy effect in breast cancer. *Int J Cancer* 1998, **79**, 8–12.
33. LeBourhis X, Toilon RA, Boilly B, Hondermark H. Autocrine and paracrine growth inhibitors of breast cancer cells. *Breast Cancer Res Treat* 2000, **60**, 251–258.
34. Kelloff GJ, Hawk ET, Crowell JA, *et al*. Perspectives on chemoprevention agent selection and short-term clinical prevention trials. *Eur J Cancer Prev Suppl* 1996, **2**, 79–85.
35. Rables SJ, Adami JR. Agents that cause DNA double strand breaks lead to p16INC4A enrichment and premature senescence of normal fibroblasts. *Oncogene* 1998, **16**, 1113–1123.