

A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*

(replicative senescence/tumor suppression/ β -galactosidase)

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Communicated by Arthur B. Pardee, Dana–Farber Cancer Institute, Boston, MA, June 12, 1995 (received for review March 28, 1995)

ABSTRACT Normal somatic cells invariably enter a state of irreversibly arrested growth and altered function after a finite number of divisions. This process, termed replicative senescence, is thought to be a tumor-suppressive mechanism and an underlying cause of aging. There is ample evidence that escape from senescence, or immortality, is important for malignant transformation. By contrast, the role of replicative senescence in organismic aging is controversial. Studies on cells cultured from donors of different ages, genetic backgrounds, or species suggest that senescence occurs *in vivo* and that organismic lifespan and cell replicative lifespan are under common genetic control. However, senescent cells cannot be distinguished from quiescent or terminally differentiated cells in tissues. Thus, evidence that senescent cells exist and accumulate with age *in vivo* is lacking. We show that several human cells express a β -galactosidase, histochemically detectable at pH 6, upon senescence in culture. This marker was expressed by senescent, but not presenescent, fibroblasts and keratinocytes but was absent from quiescent fibroblasts and terminally differentiated keratinocytes. It was also absent from immortal cells but was induced by genetic manipulations that reversed immortality. In skin samples from human donors of different age, there was an age-dependent increase in this marker in dermal fibroblasts and epidermal keratinocytes. This marker provides *in situ* evidence that senescent cells may exist and accumulate with age *in vivo*.

Normal, somatic cells do not divide indefinitely. This property, termed the finite replicative lifespan of cells, leads to an eventual arrest of cell division by a process termed replicative or cell senescence. Replicative senescence is generally studied in culture, where cells can be grown and monitored—in a controlled fashion—until most or all cells in a population senesce (1–4). Indirect evidence suggests that replicative senescence occurs *in vivo* (see refs. 2 and 4).

There are several salient features of senescence in culture (2–4). (i) It is exceedingly stringent in human cells, which, unlike many rodent cells, rarely if ever spontaneously immortalize. (ii) Senescent cells arrest growth with a G₁ DNA content and do not enter S phase in response to physiologic mitogens. Many genes remain mitogen-inducible, but some that are needed for cell cycle progression are repressed. (iii) Multiple, dominant-acting genes control the finite replicative lifespan and growth arrest of senescent cells. (iv) Senescent cells show selected, cell-specific changes in function. (v) Senescent cells remain metabolically active, and resist apoptotic death, for long periods of time.

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The idea that cellular senescence is tumor suppressive stems from molecular, cellular, and *in vivo* data. Immortality greatly increases the susceptibility to malignant transformation, in culture and *in vivo* (5, 6). Indeed, many tumors contain immortal cells or cells with an extended replicative lifespan (7). In addition, some oncogenes act primarily to immortalize or extend the lifespan of cells. Finally, Rb and p53, tumor suppressors that commonly suffer loss-of-function mutations in human cancers, are critical for cell senescence (see refs. 4 and 8).

The idea that senescence contributes to aging, by contrast, stems largely from studies in culture. Cells cultured from old donors tend to senesce after fewer population doublings (PD) than cells from young donors (9–11). Thus, cells in renewable tissues may deplete their replicative potential during aging. Moreover, cells from short-lived species tend to senesce after fewer PD than cells from long-lived species (11, 12), and cells from humans with hereditary premature aging syndromes senesce more rapidly than age-matched controls (13, 14). Thus, cellular replicative lifespan and organismic lifespan may be under overlapping genetic control. Together, the data support the idea that replicative senescence occurs *in vivo*. It is assumed that senescent cells accumulate *in vivo*, where their altered phenotype contributes to age-related pathology.

Senescent cells are identified in culture by their failure to synthesize DNA under optimal conditions and exponential increase with passage. *In vivo*, however, cell growth is not easily manipulated or monitored, and DNA synthesis measurements do not distinguish senescent cells from quiescent or terminally differentiated cells. Senescence markers identified in culture (2–4) either are not specific or require single cell quantitation or manipulations (e.g., mitogen stimulation) that are difficult to do or control *in vivo*. Thus, the idea that senescent cells accumulate with age has remained speculative because senescent cells cannot be identified in tissues.

We describe a simple biomarker for replicative senescence that provides *in situ* evidence that senescent cells may persist and accumulate with age *in vivo*.

MATERIALS AND METHODS

Cells. WI-38, AG09602, AG06234, AG07720, AG0439, AG00780, and AG06300 were from the Coriell Institute (Camden, NJ). HCA2, HT1080, HeLa, TE85, and CMV-MJ were from O.P.-S., and 88-6 and MC17326 were from J. Oshima and G. Martin (University of Washington, Seattle).

Abbreviations: PD, population doublings; NHEK, neonatal human epidermal keratinocytes; dex, dexamethasone; β -Gal, β -galactosidase; SA- β -Gal, senescence-associated β -Gal.

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Cells were cultured, as described (15–17). Neonatal human epidermal keratinocytes (NHEK; Clonetics, San Diego) were cultured as per the supplier's instructions in 10% CO₂ and 10 mM HEPES (pH 7.4); differentiation was induced by CaCl₂ or phorbol 12-myristate 13-acetate (18). Microcell fusion was performed, as described (16, 17). IDH4 cells were grown in 1 μ M dexamethasone (dex) or arrested in 10% charcoal-stripped serum and medium lacking phenol red and dex, as described (19). Human endothelial cells (strain H3605) were from J. Wessendorf and T. Maciag (American Red Cross, Rockville, MD) (20), neonatal melanocytes were from Z. Abdel-Malek (University of Cincinnati), adult melanocytes were from shave biopsies (21), mammary cells were from V. Band (New England Medical Center, Boston) (22), and ovarian cells were from N. Auersperg (23). SV40-WI38, C33a, U2OS, SAOS, and HTB9 were from the American Type Culture Collection.

[³H]Thymidine Labeling. Sparse cells (1–5 \times 10³ per cm²) were given 10 μ Ci of [³H]thymidine (60–80 Ci/mmol; 1 Ci = 37 GBq) per ml for 48–72 hr, stained where indicated, washed in phosphate-buffered saline (PBS), rinsed twice in methanol, and processed for autoradiography, as described (15).

β -Galactosidase (β -Gal) Staining. Cells were washed in PBS, fixed for 3–5 min (room temperature) in 2% formaldehyde/0.2% glutaraldehyde (or 3% formaldehyde), washed, and incubated at 37°C (no CO₂) with fresh senescence-associated β -Gal (SA- β -Gal) stain solution: 1 mg of 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-Gal) per ml (stock = 20 mg of dimethylformamide per ml)/40 mM citric acid/sodium phosphate, pH 6.0/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150 mM NaCl/2 mM MgCl₂. Staining was evident in 2–4 hr and maximal in 12–16 hr. To detect lysosomal β -Gal, the citric acid/sodium phosphate was pH 4.0.

Skin Samples. Human skin from individuals undergoing Moh's micrographic surgery for skin cancer was rapidly frozen in liquid nitrogen, and mounted in OCT. Thin sections (4 μ m) were cut, mounted onto glass slides, fixed in 1% formalin in PBS for 1 min at room temperature, washed in PBS, immersed overnight in SA- β -Gal staining solution, counterstained with eosin, and viewed under bright field at 100–200 \times magnification.

RESULTS

β -Gal in Cultured Human Fibroblasts. Senescent human fibroblasts expressed a β -Gal that was detected in single cells by X-Gal, which forms a local blue precipitate upon cleavage (24), independent of DNA synthesis measurements. Early, middle, and late passage cultures (15) were given [³H]thymidine to label presenescent cells, stained for β -Gal, and processed for autoradiography. Thus, individual cells were monitored simultaneously for ability to synthesize DNA and β -Gal activity. Two human fibroblast strains, HCA2 (neonatal foreskin) and WI-38 (fetal lung), showed similar results.

Most cells express a lysosomal β -Gal that is optimally active at about pH 4 (25). Indeed, presenescent and senescent cells stained equally well when assayed at pH 4 (Fig. 1 *A* and *B*). Neither stained at pH 7.5, the optimum for the bacterial β -Gal reporter enzyme (not shown). At pH 6, only senescent cells stained (Fig. 1 *C* and *D*). We refer to this pH 6 activity as the SA- β -Gal.

Most early passage cells were labeled with [³H]thymidine and did not express SA- β -Gal (Fig. 1*C*). The occasional SA- β -Gal-positive cell almost invariably was unlabeled (Fig. 1*C*). With increasing PD, there was a striking inverse relationship between SA- β -Gal staining and radiolabeling (Fig. 2*A*). By late passage, most cells were unlabeled and strongly SA- β -Gal positive (Fig. 1*D*). The most intense staining was perinuclear and in late passage cultures (Fig. 1*D*). Upon replating, senescent cells retained SA- β -Gal and did not divide (not shown). Typically, radiolabeled cells were SA- β -Gal neg-

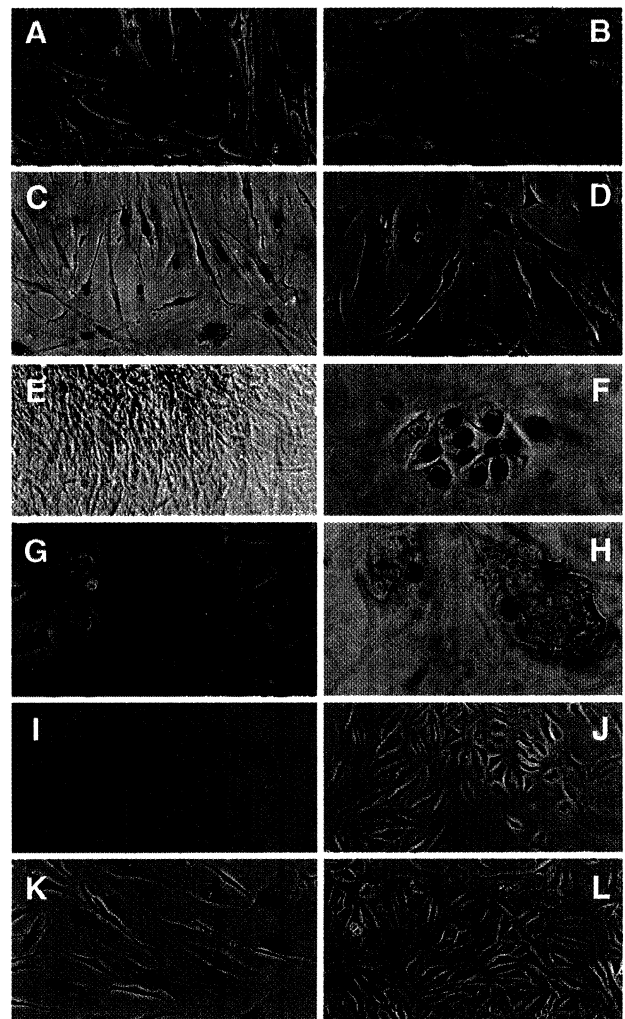


FIG. 1. β -Gal in cultured human cells. Cells were radiolabeled, stained, and photographed at 100 \times (*A–E* and *J–L*) or 200 \times (*F–I*) magnification (final magnifications: *A–E* and *J–L*, \times 60; *F–I*, \times 120). Most cells were either labeled and SA- β -Gal negative or unlabeled and SA- β -Gal positive. Double positive cells comprised <0.1% of early, and 5–8% of late, passage cultures. Such cells may have completed their last cell cycle and induced SA- β -Gal during the 3-day labeling or induced SA- β -Gal before the last cell cycle. Double negatives comprised 1–2% of early, and 20–25% of late, passage cultures. Some were slow-cycling cells, because labeling for 5–7 days reduced them from 22% to 16% and increased labeled cells from 15% to 20%. Others expressed low SA- β -Gal, since longer staining (24 vs. 8 hr) decreased them from 23% to 17% and increased SA- β -Gal positives from 69% to 75%. Ten to 15% of cells in senescent cultures were unlabeled and SA- β -Gal negative for unknown reasons. (*A* and *B*) Early passage (*A*) and senescent (*B*) WI-38 cells stained for lysosomal β -Gal. (*C*) Early passage HCA2 cells; labeled, SA- β -Gal staining. An unlabeled SA- β -Gal-positive cell is in the lower right. (*D*) Senescent HCA2; labeled, SA- β -Gal staining. A labeled SA- β -Gal-negative cell is in upper left. (*E*) Presenescent, confluent WI-38; SA- β -Gal staining. Staining disappeared 2 days after replating. (*F*) Early passage NHEK; labeled, SA- β -Gal staining. (*G*) Middle passage NHEK; labeled, SA- β -Gal staining. (*H*) Early passage differentiated NHEK; labeled, SA- β -Gal staining. (*I*) CMV-MJ cells 10 passages after receiving human chromosome 1; labeled, SA- β -Gal staining. Unlabeled SA- β -Gal-positive cells were not seen in the parent culture. (*J*) IDH4 cells growing in dex; SA- β -Gal staining. (*K*) IDH4 minus dex for 17 days; SA- β -Gal staining. (*L*) IDH4 minus dex for 20 days, then plus dex for 3 days; SA- β -Gal staining.

ative, and unlabeled cells were SA- β -Gal positive (see legend to Fig. 1).

SA- β -Gal Is Not Induced Quiescence or Terminal Differentiation. Presenescent fibroblasts were made quiescent by

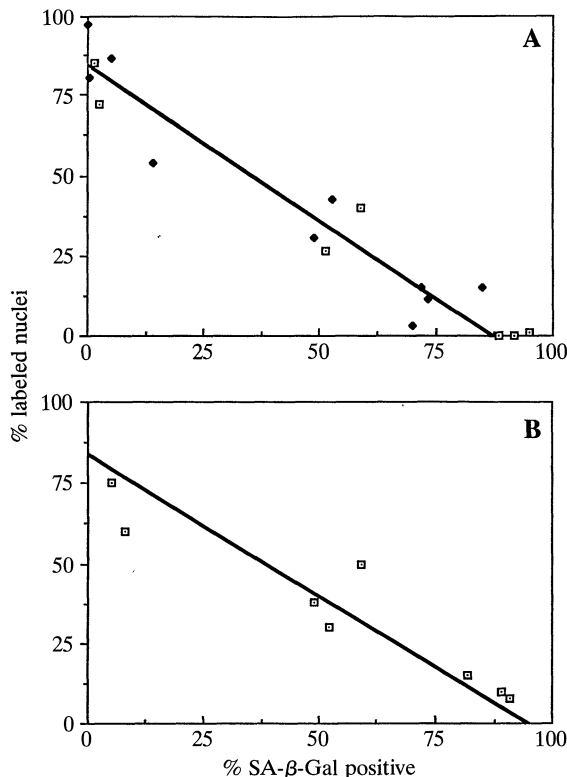


Fig. 2. SA- β -Gal and replicative capacity in culture. Cells were radiolabeled for 72 hr and stained, and 100–500 cells were scored for labeled nuclei and SA- β -Gal staining. (A) SA- β -Gal and replicative capacity during serial passage. Open symbols, WI-38 cells; closed symbols, HCA2 cells. (B) SA- β -Gal, replicative capacity, and donor age. The name, genotype, donor age, and percent labeled nuclei of five normal (N) and three Werner (W) skin fibroblast cultures are as follows: AG07720, N, 24 yr, 75%; AG0439, N, 73 yr, 30%; AG06234, N, 17 yr, 15%; AG09602, N, 92 yr, 60%; 88-6, N, 52 yr, 10%; AG00780, W, 59 yr, 50%; AG06300, W, 36 yr, 38%; MC17326, W, 57 yr, 8%.

serum deprivation. Growth declined markedly over 3 days, but SA- β -Gal was not induced, even after 7 days (Table 1). Similar results were obtained with cells made quiescent by confluence, except that positive staining was evident where cells were dense and overlapped (Fig. 1E). This staining was generally less intense than that seen in senescent cells and was lost within 2 days of replating.

Early passage human keratinocytes (NHEK) were also largely SA- β -Gal negative (Fig. 1F; Table 1) but stained for lysosomal β -Gal (not shown). After 8–10 PD, [3 H]thymidine labeling declined, and unlabeled SA- β -Gal-positive cells rose (Fig. 1G; Table 1). Thus, senescence and SA- β -Gal were also linked in keratinocytes. Moreover, early passage terminally differentiated NHEK did not express SA- β -Gal, despite a decline in proliferation (Fig. 1H; Table 1).

SA- β -Gal Correlates with Replicative Age. Skin fibroblasts from two young and two old adult donors were tested for SA- β -gal and replicative capacity. Each culture had undergone 15–20 PD when tested. Two had replicative potentials typical of their age (9–11), and two were unusual in this regard. In all cases, SA- β -Gal varied inversely with replicative age, not donor age (Fig. 2B). We also tested skin fibroblasts from donors with the Werner syndrome, a hereditary disorder showing accelerated aging *in vivo* and accelerated senescence in culture (13, 14, 26). Werner syndrome cultures accumulated SA- β -Gal-positive cells coincident with the loss of replicative capacity (Fig. 2B). Thus, SA- β -Gal reflected the replicative or physiologic age of cells, not necessarily the chronological age of the donor.

SA- β -Gal in Other Cell Types. SA- β -Gal and replicative senescence were correlated in several other cell types. Human umbilical vein endothelial cultures at early passage (65% labeled) had few SA- β -Gal-positive cells (11%), but positive cells increased (32% and 45%) at later passages (36% and 24% labeled). SA- β -Gal also correlated with replicative capacity in human mammary epithelial cells, rising from 2% at early passage to 30% at middle passage. Finally, neonatal human melanocytes were <1% SA- β -Gal positive at early passage but >90% positive at senescence. An exception was melanocytes from adult donors. Early passage adult melanocytes grew well in culture, yet >90% expressed pH 6 β -Gal activity.

Immortal Cells Do Not Express SA- β -Gal. SA- β -Gal was undetectable in immortal cells, including large tumor antigen (T-antigen)-immortalized WI-38 cells and human carcinoma, fibrosarcoma, and osteosarcoma cells (HT1080, HeLa, C33a, U2OS, SAOS, TE85, HTB9, CMV-MJ). These cultures were >90% [3 H]thymidine labeled and <0.1% SA- β -Gal positive.

Genetic manipulations that induced immortal cells to senescence induced SA- β -Gal. Human chromosome 1 causes CMV-MJ cells to senesce after 5–10 PD (17). Eight PD after chromosome 1 was introduced into these cells, 39% were SA- β -Gal positive and mostly unlabeled (Fig. 1I). Similar results were obtained with HeLa cells, in which chromosome 4 causes senescence after very few doublings (16). Virtually all cells in hybrid clones of <60 cells were SA- β -Gal positive. Chromosome 11 does not cause HeLa to senesce and did not induce SA- β -Gal (not shown).

Simian virus 40 (SV40) T antigen extends replicative lifespan (4, 8, 21) and delayed SA- β -Gal induction. Human ovarian epithelial cells senesce after 6–8 passages, but SV40-infected cultures proliferate for 20 passages (23). Normal cultures at fourth passage were 20% [3 H]thymidine labeled and 80% (mostly unlabeled) SA- β -Gal positive; fourth passage SV40-infected cultures were 80% labeled and 1% SA- β -Gal positive (not shown). T-antigen-expressing cultures eventually enter crisis, from which rare immortal cells emerge (8, 19). Less than 1% of SV40-infected human fibroblasts expressed SA- β -Gal prior to crisis, 95% were positive during crisis, and <0.1% were positive after immortal cells overgrew the culture (not shown). SA- β -Gal was also examined in IDH4 human fibroblasts, in which a dex-inducible T antigen reversibly regulates senescence (19). IDH4 cells growing in dex were <0.1% SA- β -Gal positive. After dex withdrawal, growth ceased and 88% of the cells expressed SA- β -Gal. SA- β -Gal

Table 1. SA- β -Gal in senescence, quiescence, and terminal differentiation

Cells	Passage condition	% labeled nuclei	% SA- β -Gal positive
WI-38	Early/prolif	72	3
	Early/quies, 3 days	9	2
	Early/quies, 7 days	2	2
	Middle-late/prolif	27	52
	Middle-late/quies, 4 days	1	56
NHEK	Early/prolif	77	<0.1
	Early/diff (Ca, 4 days)	28	<0.1
	Early/diff (PMA, 4 days)	21	3
	Middle/prolif	53	23
	Middle/diff (Ca, 5 days)	12	2

WI-38 cells growing in 10% serum (prolif) were radiolabeled for 72 hr and stained for SA- β -Gal or were shifted to 0.2% serum for 3 or 7 days (quies) and stained, and parallel cultures were labeled for 48 hr thereafter. NHEK in growth medium (prolif) were similarly labeled and stained or were given 2 mM CaCl₂ (Ca) or 80 nM phorbol 12-myristate 13-acetate (PMA) for 4 or 5 days (diff), labeled for the last 48 hr, and stained. For each determination, 100–500 cells were counted.

declined to <0.1% when dex was resupplied and growth resumed (Fig. 1 *J-L*).

SA- β -Gal in Human Skin. To explore the idea that senescent cells accumulate *in vivo*, skin samples from 20 human donors, aged 20–90 yr, were sectioned and stained for β -Gal. Most cells in the sections stained well for lysosomal β -Gal (not shown). SA- β -Gal-stained sections were examined blind by a dermatological pathologist for the staining frequency and identity of positive cells (Fig. 3; Table 2). Two staining patterns were evident.

First, positive staining was always seen in the hair follicles, and frequently in associated sebaceous glands (Fig. 3*A*), and in eccrine glands and ducts (Fig. 3*B*). This staining was independent of donor age. A second pattern was seen in the dermis and epidermis (Fig. 3 *C-H*). This staining increased with age in frequency and intensity (Table 2).

In the dermis, SA- β -Gal-positive cells were tentatively identified as fibroblasts, and they were sparsely and randomly distributed. None of the young donors (<40 yr) showed dermal staining. By contrast, all but one old (>69 yr) donor had

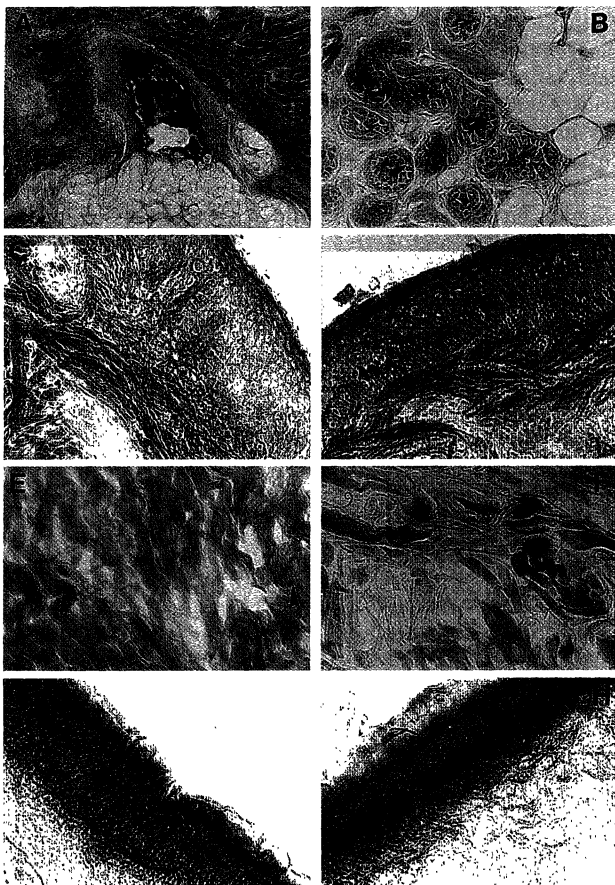


FIG. 3. SA- β -Gal in human skin. Human skin samples were sectioned, stained for SA- β -Gal, counterstained, and photographed at 100–400 \times magnification (final magnification given below). Sections were scored blind for SA- β -Gal frequency (Table 1). Scores for the sections shown are given in parentheses. (*A*) Age-independent staining in hair follicle and sebaceous gland. ($\times 60$; 38-yr-old female.) Note dense dermal collagen typical of young skin and absence of staining outside the follicle. (*B*) Age-independent staining in eccrine gland. ($\times 120$; 76-yr-old male.) (*C*) Young dermis and epidermis. (–, $\times 120$; 38-yr-old female.) (*D*) Young dermis and epidermis. (–, $\times 120$; 38-yr-old female.) Note sun damage (basal epidermal cell melanization) but absence of staining. (*E*) Old dermis. (++, $\times 120$; 70-yr-old male.) Staining in single cells, indicated by arrowheads, was present in adjacent sections. (*F*) Old dermis. (+++, $\times 240$; 76-yr-old male.) (*G*) Old epidermis. (++, $\times 120$; 73-yr-old female.) (*H*) Old epidermis. (+++, $\times 120$; 73-yr-old male.)

Table 2. SA- β -Gal activity in human skin from donors of different age

Donor	Age, yr	Sex	Site	SA- β -Gal staining	
				Epidermis	Dermis
Young					
1	37	♀	Cheek	+	–
2	38	♀	Shoulder	–	–
3	37	♀	Nose	±	–
4	20	♀	Lip	–	–
5	39	♀	Lip	++	–
6	31	♂	Scalp	–	–
7	31	♀	Scalp	–	–
8	38	♂	Cheek	±	–
9	33	♂	Nose	+	–
10	31	♂	Temple	±	–
Old					
1	78	♀	Nose	+++	++
2	69	♂	Temple	±	++
3	73	♀	Nose	++	++
4	76	♂	Arm	+++	+++
5	81	♀	Lip	+++	–
6	70	♀	Lip	+++	++
7	81	♂	Neck	+++	+++
8	73	♂	Temple	+++	+++
9	75	♂	Scalp	++	+++
10	90	♂	Scalp	±	+++

Human skin samples from the indicated donors and sites were sectioned and stained. For each sample, at least five sections were viewed in a blinded fashion and assessed for SA- β -Gal. –, No staining; ±, one positive cell; +, two to four positive cells; ++, several positive cells in the dermis, clusters in the epidermis; +++, positive cells in all sections of dermis, multiple clusters in all sections of epidermis (see Fig. 3).

positive dermal staining (Table 2). In the epidermis, SA- β -Gal-positive cells were in the basal layers, randomly distributed in some sections, clustered in others. The upper differentiated layers were negative. About half of the young donors showed some epidermal staining, typically in one or a few cells. One young donor sample was from obviously sun-damaged skin yet was devoid of staining (Fig. 3*D*). By contrast, SA- β -Gal-positive cells were always seen in the epidermis of old donors. Two old donors had staining in isolated cells; the remainder had clusters of positive cells (Fig. 3 *G* and *H*).

DISCUSSION

SA- β -Gal and Senescence. Cells from all three embryonic layers expressed SA- β -Gal upon senescence in culture. Still, some cells—adult melanocytes and sebaceous and eccrine gland cells—expressed the activity independent of senescence or age. In addition, we have not detected SA- β -Gal in senescent fibroblasts from two mouse strains, although senescent rat fibroblasts were positive. Thus, SA- β -Gal is not a universal marker of replicative senescence, which is not surprising. The age- or senescence-independent expression by some cells suggests that induction in otherwise nonexpressing cells may reflect senescence-associated changes in differentiation (see ref. 4). SA- β -Gal must have cell-specific functions, and its induction upon senescence may be limited to certain cell types. Nonetheless, SA- β -Gal was a good marker of senescence in some human cells. Moreover, its induction depended on replicative or physiologic age, suggesting that it could serve as a biomarker of aging. We suggest that the age-dependent rise in SA- β -Gal in human skin reflects an accumulation of senescent fibroblasts and keratinocytes *in vivo*.

Origin and Function. We do not yet know the origin or function of SA- β -Gal. A neutral β -Gal activity has been described in mammalian tissues (27). Senescence might entail

the induction of this or a related enzyme. Alternatively, because some lysosome activities are elevated in senescent cells (28, 29), lysosomal β -Gal may increase such that its activity is detectable at pH 6. Senescent cells might also express the alternately spliced (short) form of lysosomal β -Gal (25), which may encode pH 6 activity, or localization might alter the lysosomal β -Gal pH optimum. Preliminary data (G.P.D., B. Huang, and J.C., unpublished) favor the second possibility. *In situ* staining, which is not quantitative, showed little difference between presenescent and senescent cells at pH 4, but soluble assays showed 5- to 10-fold more pH 4 activity in senescent cells. The pH vs. activity profile was too broad to discern a distinct pH 6 peak. Senescent cells also expressed 3- to 5-fold more lysosomal β -Gal mRNA. Finally, when the long-form, but not short-form, cDNA was overexpressed in SA- β -Gal-negative cells, staining was detectable at pH 6. These data are consistent with lysosomal β -Gal overexpression giving rise, at least in part, to the activity in senescent cells.

It is unlikely that SA- β -Gal causes the growth arrest of senescent cells. Indeed, cells positive for SA- β -Gal and [³H]thymidine were consistently, albeit infrequently, observed. SA- β -Gal most likely reflects the change in cell function that invariably accompanies senescence.

Senescence Genes and Pathology. SA- β -Gal provides a simple assay for chromosomes that induce senescence and should facilitate the cloning of senescence genes. It also provides *in situ* evidence that senescent cells may accumulate *in vivo* at sites that show age-related pathology. Dermal thinning and collagen breakdown are hallmarks of aging skin that may be due to senescent fibroblasts, which overexpress collagenase and underexpress collagenase inhibitors (30). Similarly, endothelial cells lining vessels subject to hemodynamic stress may senesce early in life (C. Harley and M.L., unpublished). The altered functions of senescent endothelial cells (31) may initiate or exacerbate atherosclerosis. Further studies are now needed to determine how SA- β -Gal relates to other physiologic signs of age and to show that senescent cells indeed contribute to age-related pathology. A biomarker for replicative senescence should facilitate these studies.

We thank Drs. J. Wessendorf and T. Maciag for endothelial cells, J. Oshima and G. Martin for normal and Werner fibroblasts, N. Auersperg for ovarian cells, Z. Abdel-Malek for neonatal melanocytes, V. Band for mammary cells, W. Wright for IDH4 cells, D. Grande for access to patient specimens, and D. d'Azzo for helpful discussions. We also thank J. Rogoeczi and X. Xie for help with the IDH4 cells and frozen sections. This research was supported by National Institute on Aging Grants AG09909, AG11658 (J.C.), AG09927 (M.P.), AG05333, AG07123 (O.P.-S.), and AG00594 (E.E.M.).

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