Fibroblasts cultured from venous ulcers display cellular characteristics of senescence

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Purpose: **A well-recognized characteristic of venous ulcers is impaired healing. Fibroblasts cultured from venous ulcers (wound-fb) have been shown to have reduced growth rates and are larger than normal fibroblasts (normal-fb) from the ipsilateral limb. Reduced growth capacity and morphologic changes are 2 well-known traits of cellular senescence. Other molecular changes are overexpression of matrix proteins, such as cellular fibronectin (cFN), and enhanced activity of β-galactosidase at pH of 6.0 (senescence associated β-Gal, or SA-β-Gal). Senescence, an irreversible arrest of cell proliferation with maintenance of metabolic functions, may represent in vivo aging and thus may be related to impaired healing.**

Methods: **Cultured normal-fb and wound-fb from 7 venous ulcer patients (average age, 51 years) were obtained by taking punch biopsies of the perimeter of the ulcer and from the ipsilateral thigh of the same patient. Growth rates, SA-β-Gal activity, and level of cFN protein (immunoblot) and message (Northern blot) were measured.**

Results: **In all patients, wound-fb growth rates were significantly lower than those of normal-fb ($P = .006$). A higher percentage of SA-β-Gal positive cells were found in all wound-fb (average, 6.3% vs. 0.21%; $P = .016$). The level of cFN, was consistently higher in all wound-fb tested. Also, in 4 patients, the level of cFN messenger RNA (mRNA) was increased.**

Conclusion: **Fibroblasts cultured from venous ulcers exhibited characteristics associated with senescent cells. Accumulation of senescent cell in ulcer environment may be associated with impaired healing. (J Vasc Surg 1998;28:876-83.)**

Venous ulceration is the final outcome of lower extremity chronic venous insufficiency in most cases. It is a debilitating recurrent complication, with an estimated prevalence of 0.1% to 0.2% of the population in developed countries.[1] It has been estimated that $1 billion is spent annually in the treatment of chronic wounds.[2] In a case control study done in our institution, the average age of patients with venous ulcers was approximately 20 years older than those with uncomplicated varicose veins, demonstrating, in a multivariate analysis, that age is an independent risk factor.[3] Also, chronic lower extremity ulcers develop in 50% to 60% of patients with premature aging syndromes, such as Werner's syndrome.[4,5] Fibroblasts isolated from Werner's syndrome patients exhibit a striking reduction in growth potential, with a 70% reduction in the patient's expected life span and premature senescence.[5]

The exact pathophysiologic mechanisms leading to ulcer formation and impaired ulcer healing have not been elucidated. In previous experiments in our laboratory, we demonstrated that fibroblasts cultured from venous ulcers have reduced growth rates and are larger than normal fibroblasts from the ipsilateral limb of the same patient.[6] Reduced growth capacity and morphologic changes are 2 well-known traits of cellular senescence.[7] The hallmark of cellular senescence is an irreversible arrest of cell proliferation and altered cell functions.[8–10] The reduction in proliferative capacity of cells from old donors and patients with premature aging syndromes[11–14] implicate cellular senescence in aging and age-related pathologies.[8,9]

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Senescent cells acquire altered patterns of gene expression, remain metabolically active, and resist apoptotic death for long periods,[6] thus accumulating in vivo upon aging.[15,16] Other characterized cellular and molecular changes of senescence are overexpression of the extracellular matrix protein fibronectin[7] and induction of β-galactosidase activity at a pH of 6.0 (senescence associated β-Gal activity, or SA-β-Gal).[16]

We hypothesized that accumulation of senescent fibroblasts within the venous ulcer environment plays a significant pathophysiologic role in the mechanism associated with impaired healing of these lesions.

METHODS

Patients

Patients with chronic venous ulcers were interviewed and examined. Nine patients agreed to enroll in the study, but only 7 completed all stages. The severity of venous disease in enrolled patients was determined by a trained vascular surgeon using the guidelines prepared by the Executive Committee of the American Venous Forum (Clinical Classification and Grading of Chronic Venous Disease).[18] Patients in whom concomitant arterial disease was suspected, as evidenced by absent pulses, were excluded from the study. Patients who were taking immunosuppressive medication were excluded from the study. All patients with diabetes, patients with known HIV-positive status, and patients with active infection were excluded. The Institutional Review Board at Boston Medical Center approved this study, and informed consent was obtained from all enrolled patients.

Materials

Dulbecco's modified Eagles's medium (DMEM), trypsin, and Trizol reagents were purchased from GibcoBRL Life Technologies (Gaithersburg, Md). Monoclonal anti-cellular fibronectin (cFN) antibody was purchased from Sigma (St Louis, Mo). An ECL Western blotting detection system kit was purchased from Amersham Life Science (Arlington Heights, Ill). Bovine calf serum (CS) was obtained from Hyclone Laboratories (Logan, Utah). Nitrocellulose and Nylon membranes were purchased from Amersham (Arlington Heights, Ill), and the oligolabeling kit was from Pharmacia LKB Biotechnology (Piscataway, NJ). Radiolabeled nucleotide (d-CTP) was from New England Nuclear (Boston, Mass). X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) was purchased from Sigma (St Louis, Mo).

Fibroblasts isolation and culture

A 4 mm punch biopsy specimen was obtained from each patient from (1) the uninvolved skin of the thigh and (2) the ulcer edge. All ulcer biopsies were obtained from epithelialized areas. Techniques used to isolate and culture dermal fibroblast are described in detail elsewhere.[6] In brief, biopsies were treated with a 5-minute povidone-iodine bath, followed by a 5-minute bath in 70% ethanol. Overnight trypsinization was then performed at 4°C (trypsin solution = 1 μg/mL), allowing separation of the dermal tissue from the epidermal and adipose tissue. The dermal tissue was isolated under sterile conditions, cut sharply into 1 to 2 mm fragments, and placed in etched plastic tissue culture dishes (Fisher). Complete medium (CM) that consisted of DMEM with 10% CS and antibiotic solution (penicillin, methicillin, Fungizone, and streptomycin) was added to support cell growth. Two fibroblast populations were then obtained, normal fibroblasts (normal-fb) and wound fibroblasts (wound-fb). Cells were then passed into 2 separate tissue culture dishes (passage 1). Once 80% to 90% confluence was reached, 1 plate was used to freeze fibroblasts using dimethyl sulfoxide solution, and then cells were stored at –140°C in liquid nitrogen. From the other plate, fibroblasts were harvested and used to generate growth curves (passage 2). All other analyses were performed on fibroblasts previously frozen (passage 3 cells).

Growth curves

Growth curves were assessed by plating fibroblasts at 1500 cells per plate and determining total cell number per plate, by means of a Coulter Counter, on days 7, 10, 13, and 16. A combination of Trypsin/ethylenediaminetetraacetic acid (EDTA) solution was used to detach cells from tissue culture dishes, and detachment was monitored by means of light microscopy. Trypsin/EDTA action was neutralized with media containing 10% CS. Cells were then harvested and transferred to plastic vials containing Isoton. Complete harvesting of cells was reassessed by examining plates under light microscopy. Plotting total cell number against harvesting days generated growth curves. Growth rate was calculated by means of the slope of a best-fit line.

Assessment of senescence in vitro

Frozen fibroblasts were thawed following standard technique and placed in 100 mm tissue culture dishes in the presence of CM. When 80% to 90% confluence was obtained, cells were divided in 3

groups: (1) fibroblasts plated for determination of SA-β-GAL activity, (2) fibroblasts plated for extracellular matrix protein extraction and quantification of fibronectin protein, and (3) fibroblasts plated for total RNA extraction to evaluate fibronectin mRNA content.

SA-β-GAL activity. SA-β-GAL staining was performed according to a protocol described previously. Briefly, third passage fibroblast cultures at 30% to 50% confluence were exposed to the staining solution (20 mg/mL X-gal in dimethylformamide; 0.2 mol/L citric acid/Na phosphate buffer, pH = 6.0; 100 mmol/L potassium ferrocyanide; 100 mmol/L potassium ferricyanide; 5 mol/L sodium chloride; 1 mol/L magnesium chloride; and H_2O) after being fixed with 2% formaldehyde/0.2% glutaraldehyde in phosphate buffer saline. Plates were incubated for 12 to 16 hours at 37°C without CO_2. Positive cells characteristically display perinuclear precipitation of blue dye. This allows for clear identification by means of standard light microscopy. Percentage of positive SA-β-GAL cells was determined after counting 300 to 400 cells per plate.

Fibronectin protein analysis (Western blot). Immunoblot analysis (Western blot) for cFN was performed after extraction of matrix proteins from fibroblast culture dishes.

Third passage fibroblasts were allowed to achieve 89% to 90% confluence in 60 mm culture dishes. Urea base extraction of matrix proteins was then performed by lysing cells into 4 mol/L urea buffer and homogenate set for 1 to 2 hours at 4°C. The total amount of protein was quantified by means of the Bradford protein assay technique described elsewhere. Ten to 30 μg of cell lysate protein was separated with a 7.5% sodium dodecyl sulfate-gel electrophoresis. The same amount of cell lysate protein from both wound-fb and normal-fb cultures were loaded. Proteins were then transferred to a nitrocellulose membrane. The membrane was preincubated in 100% Blotto (5 g of nonfat dry milk in 100 mL of PBS) for 1 hour at room temperature. This was followed by incubation with a mouse anti-human monoclonal antibody against cFN (0.2 to 1 μg/mL in 10% Blotto) at 4°C. At the end of incubation, the membrane was washed extensively with PBS containing 0.5% Tween-20, then reacted with the secondary goat anti-mouse antibody labeled with horseradish peroxidase. Chemiluminescent reaction with the ECL Western blotting analysis system was used to detect fibronectin bands by means of autoradiography, exposing membrane to Kodak X-Omat film.

Fibronectin mRNA detection. Total RNA was extracted from third passage fibroblasts at an 80% to 90% confluence in 60 mm culture dishes by lysing the cells with Trizol reagent (1 mL per 60 mm dish). The lysate was equilibrated at room temperature for 5 minutes and extracted with chloroform (0.2 mL per 1 mL of Trizol Reagent). RNA was precipitated by adding 0.5 mL of isopropyl alcohol per 1 mL of Trizol reagent. Seven to 10 μg of total cellular RNA was separated electrophoretically through 1% agarose gel containing 2.2 mol/L formaldehyde, blotted on a Hybond-N nylon membrane, and immobilized by means of short ultraviolet light illumination. The same amount of total RNA was loaded from normal-fb and wound-fb. The blot was prehybridized for 24 hours and incubated with the specific radiolabeled fibronectin insert ($2.0–3.0 \times 10^6$ cpm/mL hybridization solution) for 24 hours. The membranes were washed twice for 30 minutes with 2 X standard saline citrate solution at 65°C and then exposed to Kodak XAR-5 film.

Probe

The complementary DNA (cDNA) specific for fibronectin was purchased from the American Type Cell Culture. cDNA was radiolabeled with [^{32}P]-dCTP (specific activity: 3,000 Ci/mmol) by means of a commercial oligolabeling kit. Ethedium bromide staining of 18S RNA was used to confirm the equal loading of RNA in each lane.

Statistical analyses

Paired Student *t* test was used to compare growth rates and density of fibronectin bands from immunoblots autoradiographs. Nonparametric statistics (Wilcoxon rank sum test) were used to compare SA-β-Gal positivity. Linear regression analysis was used to estimate the growth rate for each cell culture. Image analysis software (Image Tools, The University of Texas Health Science Center, San Antonio) was used to calculate the relative integrated optical densities from scans of autoradiographs of immunoblot and Northern blots.

RESULTS

Fibroblasts obtained from 7 patients with ulcers were included in all phases of the study. All ulcers were classified clinically as symptomatic clinical classification 6, according to the American Venous Forum ad hoc committee consensus statement from 1994,[18] with a mean clinical score of 12.7 (range, 11 to 17). Of the 7 patients, 4 were men and 3 were women. The average age of the patients was 51 years

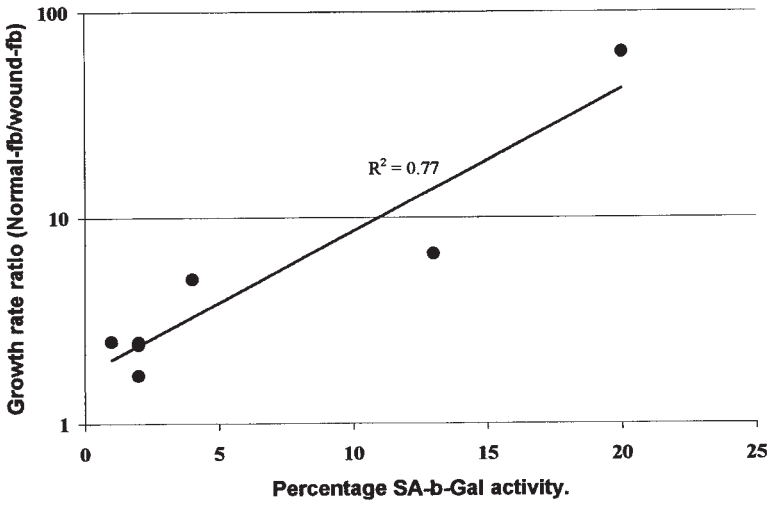


Fig 1. Correlation between percentage of SA-β-Gal positive cells and the ratio between normal fibroblasts (normal-fb) and fibroblasts cultured from venous ulcers (wound-fb) growth rates.

(range, 36 to 67 years). The average duration of the ulcers was 15 months (range, 9 to 36 months). Two patients had documented history of deep venous thrombosis.

Growth rates

Wound-fb growth rates were significantly lower than normal-fb growth rates in all 7 patients (Table I). The average growth rate for normal-fb was 31,190 cells per day vs 11,451 cells per day for wound-fb ($P = .006$).

SA-β-Gal activity

SA-β-Gal activity was not detected in normal-fb cultures, except in the case of 1 patient who had 1.5% of cells staining positive. In the wound-fb populations, SA-β-Gal activity was detected in all patients, and it ranged from 1% to 20%, with a mean of 6.3% and a median of 2% ($P = .016$; Table II).

There was a trend toward a significant correlation between the percentage of SA-β-Gal positive cells and the differences in growth rate between normal-fb and wound-fb, with an R^2 value for the best fit line of 0.74 (Fig 1).

Fibronectin protein and mRNA expression

Six of the 7 patient fibroblast populations were tested. The level of fibronectin protein isolated from all the wound-fb cultures was consistently higher than that isolated from the corresponding normal-fb cultures (Fig 2). This increase ranged from 1.3 to 4.4 fold with densitometry analysis, and the differences were statistically significant ($P = .027$; Table III). In 4 of those patients, the level of fibronectin mRNA was also elevated (Fig 3).

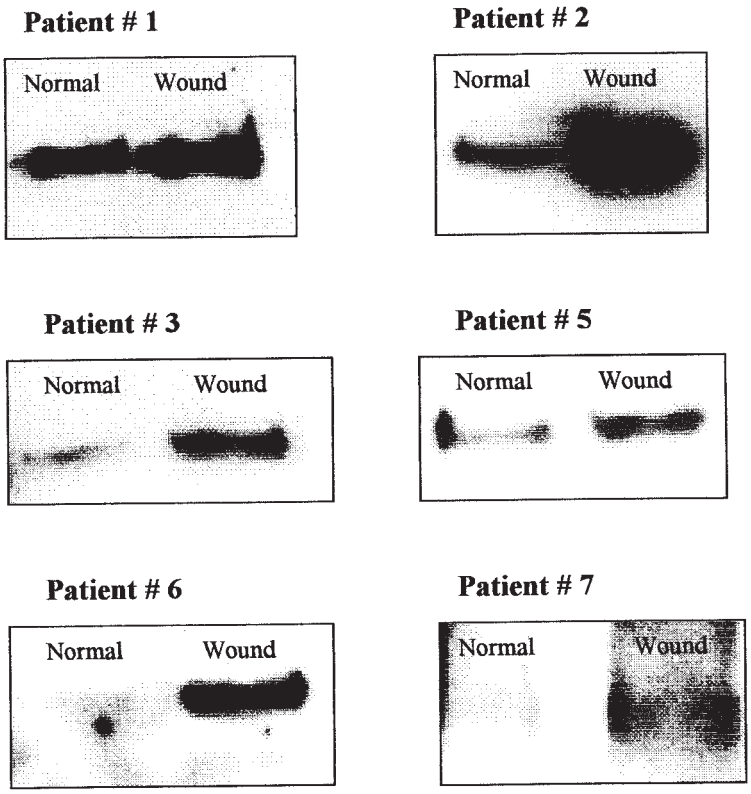
Table I. Growth rates from normal fibroblasts (normal-fb) and fibroblasts cultured from venous ulcers (wound-fb) calculated using the slopes of the best fit lines given by all points of each curve. Points of each curve were determined by counting cells on days 7,10,13, and 16 after plating 1500 cells per plate. The growth rates are given in cells/day.

Patient	*Normal*	*Ulcer*
1	40,466	8,100
2	47,033	19,600
3	17,033	6,753
4	17,600	276
5	28,066	16,033
6	60,866	24,586
7	7,270	4,812
Average	31,190*	11,451

*$P = .006$ (comparing normal-fb vs wound-fb, using paired Student *t* test).

DISCUSSION

Fibroblasts isolated from venous ulcers showed reduced growth rates, an increase in SA-β-Gal activity, and increased production of fibronectin when compared with normal-fb isolated from uninvolved skin of the same patient. Increase in wound-fb size in our laboratory has been described previously.[6] These characteristics are consistent with senescence



Integrated optical densities

Patient	Normal	Ulcer	Ratio
1	384	634	1.6
2	493	1,780	4.4
3	181	576	3.2
5	228	304	1.3
6	175	763	4.3
7	335	1,254	3.7
Average	285	885*	3.7

(*p=0.02, comparing normal-fb vs. wound-fb using paired Student T-test)

Fig 2. Cellular fibronectin protein extracted from 80% to 90% confluent third passage fibroblasts isolated from venous ulcers (wound-fb) and uninvolved skin (normal-fb) of the ipsilateral thigh of the same patients. Densitometry was performed on scans from immunoblot autoradiograph using image analysis software. Results are presented in the table.

phenotype. These findings support the theory that accumulation of senescent fibroblast within the ulcer microenvironment is associated with impaired healing of these lesions.

Human fibroblasts upon senescence acquire resistance to apoptotic programmed cell death[25] for a long period, rendering them quite stable and allowing for accumulation in tissues upon aging.[16] Senescent fibroblasts have been shown to overexpress proteolytic activity by means of an increase in the production of enzymes such as matrix metalloproteinases and to underexpress protease inhibitors. This may contribute to dermal thinning and venous ulcer matrix breakdown, facilitating ulcer formation and impairing the capacity for re-epithelization and healing.

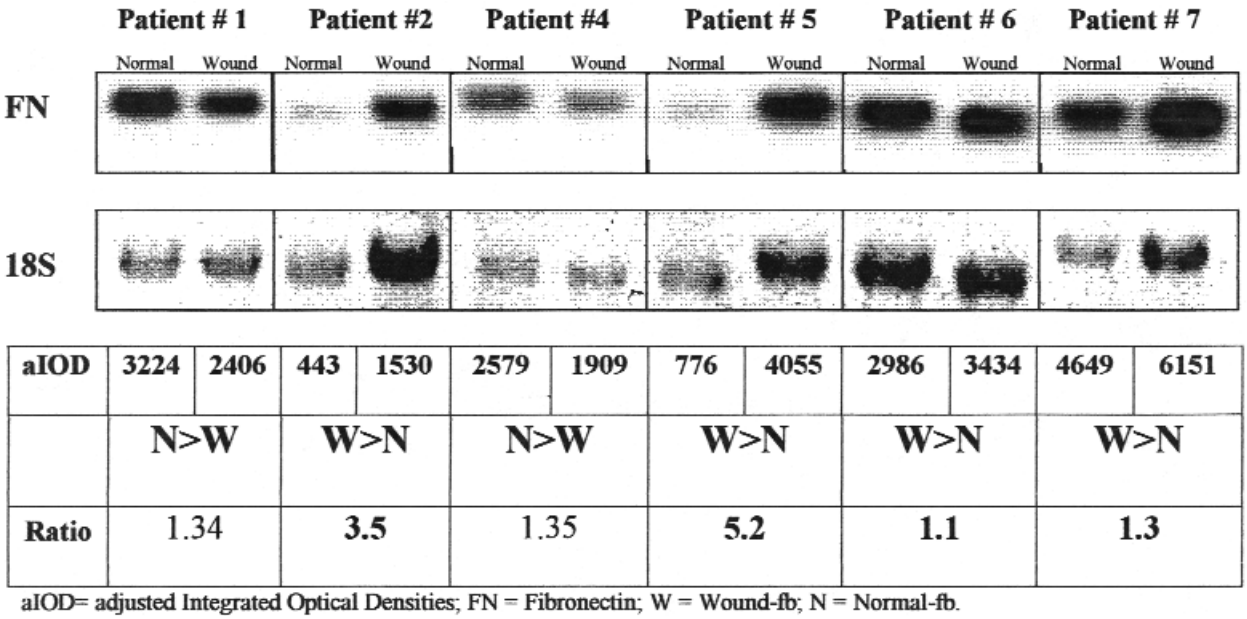


Fig 3. Cellular fibronectin messenger RNA (mRNA) extracted from 80% to 90% confluent third passage fibroblasts isolated from venous ulcers (wound-fb) and uninvolved skin (normal-fb) of the ipsilateral thigh of the same patients. Ethidium bromide labeled 18S subunit of total RNA loaded into each lane was used to calculate the ratio between cellular fibronectin mRNA and total RNA for purposes of comparison. Densitometry was performed on scans from Northern blot autoradiograph and picture of ethidium bromide labeled gel using image analysis software. Results are presented in table.

Table II. Percentage of cell positively stained for SA-β-Gal activity in cultures of normal fibroblasts (normal-fb) and fibroblasts cultured from venous ulcers (wound-fb).

Patient	*Normal*	*Ulcer*
1	0	4
2	0	2
3	0	1
4	0	20
5	0	2
6	0	2
7	1.5	13
Average	0.2	6.3
Median	0	2.0*

*P = .015 (comparing normal-fb vs. wound-fb using Wilcoxon signed rank test).

Table III. Demographic characteristics and ulcer details of the patient population.

Variables	
Number	7
Age	51 years (range, 36 to 67 years)
Sex	4 men, 3 women
Greater diameter (mean)	5.8 cm (range, 2 to 10 cm)
Clinical score (mean)	12.7 (range, 11 to 17)
Duration of ulcer	15 months (range, 9 to 36 months)

Senescent fibroblasts also have a significant immunoregulatory role that may affect immune cellular interactions during the process of wound healing. Senescent fibroblasts overexpress interleukin 1 (IL-1),[8] have a diminished proliferative response to tumor necrosis factor (TNF)-α, and underexpress interleukin 6 and 8 (IL-6, IL-8) after TNF-α challenge,[20] all of which may alter the immunologic mechanisms associated with tissue repair.

Tissue fibronectin increases with age,[21] and its gene sequence has been shown to be overexpressed in senescent and Werner's's syndrome human fibroblasts.[17] This glycoprotein encourages cell-to-cell and cell-to-substrate interactions, playing an important role in wound healing. However, fibronectin has been found to be an important component of pericapillary fibrous caps associated with venous hypertension.[22] The products of fibronectin degradations are strong chemoattractants for monocytes and macrophages and probably act to stimulate differentiation of the mono-

cyte to the activated macrophage.[23] Also, age-related increases in macrophage adhesion to fibronectin have been found in vitro.[24] Taken together, overexpression of fibronectin by senescent fibroblasts from venous ulcers may: (1) result in pericapillary matrix deposition and act as a diffusion barrier; (2) promote monocyte trapping, differentiation, and activation with proinflammatory consequences; and (3) provide the substrate for macrophage homing within the ulcer environment, facilitating a chronic inflammatory process. The increase in cFN could be caused by either a more stable or efficient glycoprotein production by senescent cells or an increase in the fibronectin message at a transcriptional level.

SA-β-Gal activity determination using X-gal based staining is a well-established method of assessing senescent fibroblasts in vitro.[16] Cells from all 3 embryonic layers express SA-β-Gal upon senescence. Still, some cells, such as adult melanocytes and sebaceous and eccrine gland cells, may express this activity independent of senescence or age, but overall it is considered a good marker of senescence in human cells such as dermal fibroblasts.[16] It is unlikely that SA-β-Gal activity is directly involved in the process of proliferative arrest of senescent cells, and most likely it reflects a change in cell function that invariably accompanies senescence.[16]

Fibroblasts isolated by explant technique are by definition cells that have the ability to replicate, and thus they have not reached senescence. In the in-vitro environment, as these cells proliferate they exhaust their replicative life span, ultimately reaching senescence. At any given time, the proportion of cells that reached senescence within a particular cell population is dependent on the replicative "age" of those cells at the time of biopsy. The greater the number of fibroblasts closer to replicative senescence in vivo, the greater the number of cells that will reach senescence upon culture in vitro at any given time. In our experience, we determined the percentage of SA-β-Gal positive cells when third passage fibroblasts reached approximately 50% confluency in 60 mm tissue culture dishes (approximately 3 to 4 weeks after the biopsy specimen was taken). At this endpoint, a mean of 6.3% (median, 2%) of wound-fb had reached senescence vs. a mean of 0.2% (median, 0%) of normal-fb. The logical conclusion is that a greater proportion of dermal fibroblasts from the ulcer biopsy specimens was closer to replicative senescence than fibroblasts from normal skin biopsy specimens.

As previously stated, replicative senescence is dependent upon cumulative cell divisions and not chronologic or metabolic time, indicating that proliferation is limited by a "mitotic clock."[26,27] This mitotic clock has been recently characterized as the length of chromosomal telomeres and the extension of life span achieved through elongation of telomeres after introduction of telomerase into normal human fibroblasts.[28]

We can then speculate that inflammation, infection, or injury may locally promote accumulation of senescent fibroblasts by:

1. Stimulating proliferation and cell turn over with subsequent exhaustion of replicative life span. In each population doubling, fibroblast telomeres shorten approximately 50 to 100 base pairs (bp) in culture and 15 bp per year of donor age,[28] approaching a threshold telomere length with each cell division. Several cytokines and growth factors with stimulatory effects on fibroblast proliferation, such as transforming growth factor β-1 (TGF-β-1)[30] and IL-1, have been detected to be increased in venous ulcer tissues.
2. The possibility that cytokines and/or oxygen-free radicals delivered by leukocytes trapped and activated within the microvasculature of a venous ulcer[32-34] may induce cell death in young, actively replicating fibroblasts selecting out for more stable and resistant senescent cells.

Fibroblasts isolated from venous ulcers are at a more progressed stage of senescence than fibroblasts isolated from healthy skin. Whether this phenomena is induced by extrinsic ulcer mechanisms, such as injury or infection, or by intrinsic mechanisms, such as chronic venous hypertension, is the question addressed by an ongoing project in our laboratory that is evaluating fibroblast senescence in patients with venous reflux but no ulcers.

There was only a trend toward a correlation between the percentage of SA-β-Gal positive cells and the reduction of wound fibroblasts growth rates. Also, it is difficult to explain a 2-fold to 20-fold reduction in growth by the presence of an average of 6.3% SA-β-Gal positive cells. A possible explanation originates from the concept that as cells approach senescence, cell growth rate diminishes.[7,16] It is possible that a significant proportion of wound fibroblasts isolated from venous ulcers are approaching senescence (near senescent), explaining the reduced growth rate found in this study, but at the same time SA-β-Gal might not be expressed in sufficient amounts by near senescent fibroblasts to be detected by the bioassay.

CONCLUSIONS

Fibroblasts cultured from venous ulcers exhibited more characteristics associated with senescent cells

when compared with fibroblasts isolated from unaffected skin of the same patient. This implies that the local accumulation of senescent fibroblasts within the venous ulcer may be related to impaired healing.

We introduce a novel pathophysiologic concept to explain the cellular mechanisms of disease in venous ulcers. Potential different therapeutic approaches can derive from these findings. Bypass of replicative senescence has been achieved in the laboratory through different mechanisms,[28,29] and this technology could have therapeutic implications in the management of venous ulcers in the future.

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