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ORIGINAL BASIC SCIENCE STUDIES

The proliferative capacity of neonatal skin fibroblasts is reduced after exposure to venous ulcer wound fluid: A potential mechanism for senescence in venous ulcers

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Purpose: **We have previously shown that fibroblasts cultured from venous ulcers display characteristics of senescence and have reduced growth rates. Susceptibility of young fibroblasts to the microcirculatory changes associated with venous ulcers, such as macrophage trapping and activation, could explain the prevalence of senescent fibroblasts in these wounds.**

Methods: **We tested the in vitro effect of venous ulcer wound fluid (VUWF), as well as pro-inflammatory cytokines known to be present in VUWF (TNF-α, IL-1β, and TGF-β1), on neonatal foreskin fibroblasts (NFFs). NFF growth rates, cellular morphology, and senescence-associated β-galactosidase (SA-β-Gal) activity were determined in the presence or absence of VUWF and the above cytokines. VUWF TNF-α concentration and the effect of anti–TNF-α antibody on VUWF inhibitory activity were determined in samples obtained from four patients with venous ulcers.**

Results: **NFF growth rates were significantly reduced by VUWF (42,727 ± 6301 vs 3902 ± 2191 *P* = .006). TNF-α also significantly reduced NFF growth rates in a dose-dependent manner (*P* = .01). No significant growth-inhibitory activity was seen for IL-1α or TGF-β. Incubation with VUWF significantly increased the percentage of SA-β-Gal–positive fibroblasts in vitro on culture day 12 (*P* = .02). TNF-α and TGF-β1 had similar effects. TNF-α was detected in all VUWF tested, with a mean of 254 ± 19 pg/mL.**

Conclusion: **These data suggest that the venous ulcer microenvironment adversely affects young, rapidly proliferating fibroblasts such as NFFs and induces fibroblast senescence. Pro-inflammatory cytokines such as TNF-α and TGF-β1 might be involved in this process. The role of other unknown inhibitory mediators, as well as pro-inflammatory cytokines, in venous ulcer development and impaired healing must be considered. (J Vasc Surg 1999;30:734-43.)**

The exact pathophysiologic mechanisms leading to ulcer formation and impaired ulcer healing in patients with chronic venous insufficiency has not been completely elucidated. Current theories relate the trapping, migration, and activation of leukocytes—specifically, macrophages, lymphocytes, and mast cells—with the subsequent establishment of a chronic inflammatory microenvironment.[1-3] These theories have been confirmed by studies demonstrating the presence of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and transforming growth factor-β1 (TGF-β1) produced by these cell types in venous ulcer wound fluid (VUWF)[4,5] and in the ulcer microenvironment.[2,6,7]

In previous experiments in our laboratory, we demonstrated that a greater proportion of fibroblasts cultured from venous ulcers were senescent when compared with normal fibroblasts from the ipsilateral limb of the same patient.[8] Similar findings were made

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for patients with venous reflux but no ulcers.[9] Dermal fibroblasts play a critical role during wound healing by producing matrix proteins such as fibronectin, integrins, collagen, and vitronectin to form a basal lamina over which keratinocytes can migrate.

Cellular senescence has been implicated in impaired healing through several potential mechanisms, such as establishment of a proteolitic environment and tissue immunoregulation.[10-12] The mechanism through which a great proportion of dermal fibroblasts from venous ulcers become senescent is not known.

We hypothesized that exposure to the chronic inflammatory microenvironment established by venous hypertension might be involved in the process leading to fibroblast dysfunction and increased senescence within the dermis of venous ulcers. VUWF is considered a reflection of the microenvironment of the venous ulcer, which may contain one or several components with fibroblast-inhibitory activity and from which these components are collected.

To test our hypothesis, we evaluated the in vitro effect of VUWF on growth rates, cellular morphology, and expression of senescence-associated β-galactosidase activity (SA-β-Gal) on neonatal foreskin fibroblasts (NFFs). We also tested the effect of proinflammatory cytokines IL-1β, TNF-α, and TGF-β1, known to be present in VUWF, in growth rate, in morphology and in SA-β-Gal activity of NFFs.

METHODS

Patients

Patients, aged 35 to 75 years, with chronic venous ulcer of greater than 2 months' duration were interviewed and examined. Evaluation of the severity of venous disease in enrolled patients was determined by a trained physician using the guidelines prepared by the Executive Committee of the American Venous Forum ("Clinical Classification and Grading of Chronic Venous Disease").[13] Patients in whom concomitant arterial disease was suspected, as evidenced by absent pulses, patients whose ulcers were caused by malignancy, and patients with infected ulcers were excluded from the study. The Institutional Review Board at Boston Medical Center approved this study, and informed consent was obtained from all enrolled patients.

Collection of VUWF

Recruited patients were treated with foam wafer occlusive dressing (Allevyn), which was placed over the ulcer and covered by a paste bandage (Unna's boot) and a compression wrap. Dressings were changed weekly, and VUWF was extracted from the foam wafer with a sterile syringe with a 20-gauge needle. The volume recovered from the dressings varied among patients (0.3-3 mL). The collected VUWF was then immediately diluted (1:10) with Dulbecco modified Eagle medium (DMEM), which contained the protease inhibitor aprotinin (2 μg/mL) and phenylmethylsufonyl fluoride (10 μg/mL). The diluted VUWF was filtered through a 0.2-μm tissue culture filter unit to remove large debris, including bacteria. Subsequently, the protein concentration (expressed as μg/μL of VUWF) was determined and distributed into 1-mL vials and kept frozen at –70°C until further testing. VUWF from four different donors was tested. The average age of the patients was 50 years (range, 45-57 years); the only significant comorbidity reported was coronary artery disease (one patient). All four patients had a history of recurrent venous ulcers, and the duration of the ulcer from which the VUWF was collected was an average of 6 months.

Fibroblast isolation and culture

Cultured fibroblasts used in these experiments were isolated from foreskins obtained from two healthy neonates. NFFs have the highest growth capacity among fibroblasts cultured from all age groups.[14,15] NFFs from the same donor were used in each experiment to exclude the possibility of donor variability. Neonatal foreskin was obtained within 2 to 6 hours of elective circumcision, and primary cultures of dermal fibroblasts were prepared with techniques described in detail by Stanley et al.[16] In brief, biopsy specimens were treated with a 5-minute povidone-iodine bath followed by a 5-minute bath in 70% ethanol. Overnight treatment with trypsin at 4°C (trypsin solution = 1 μg/mL) was performed, which caused 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. Complete medium (CM) that consisted of DMEM with 10% calf serum and antibiotic solution (penicillin 100 U/mL, methicillin, fungizone 0.25 μg/mL, and streptomycin 100 μg/mL) was added to support cell growth. Cells at the second passage were frozen (1×10^6 cells per vial) by using dimethyl sulfoxide solution and were stored in liquid nitrogen at –140°C. A vial of frozen second-passage cells was thawed and replated for each experiment. For all growth-rate and morphology experiments, an equal number of cells per plate (1500 cells/plate) were seeded into 30-mm tissue culture dishes. For assessment of SA-β-Gal activity, 1500 cells were seeded into 60-mm tissue culture dishes.

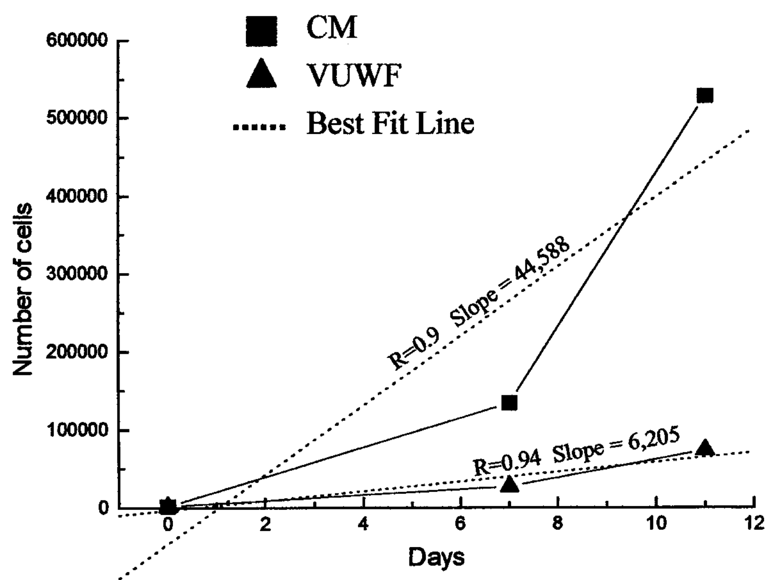

Fig 1. Representative sample of growth curves of neonatal foreskin fibroblasts cultured in the presence of complete media (*squares*) and in the presence of venous ulcer wound fluid (*triangles*). The best-fit line for each curve was calculated by regression analysis and is shown for each curve. The slope of this line *(dotted line)* was then calculated and used as an estimate of the growth rate.

Growth curves

Growth curves were assessed by the plating of fibroblasts at 1500 cells per plate and the determination of total cell number per plate with the use of a Coulter counter, on days 7 and 11. A combination of trypsin and ethylenediamine tetraacetic acid solution was used to detach cells from tissue culture dishes, and detachment was monitored by means of light microscopy. Trypsin and ethylenediamine tetraacetic acid action was neutralized with CM containing 10% calf serum. Cells were then harvested and transferred to plastic vials containing Isoton (Coulter Balanced Electrolyte Solution, Coulter, Miami, Fla). Complete harvesting of cells was reassessed by examining plates under light microscopy. A Coulter counter was then used for cell counting. Growth curves were generated by the plotting of total cell number against harvesting days. For each growth curve, the best-fit lines were calculated using regression analysis, and the slopes of those lines were used as estimates of the growth rates of each cell population in cells per day (Fig 1).

VUWF. NFFs were cultured in the presence of VUWF from four different donors, and growth rates were obtained as explained above. The amount of VUWF from each sample added to each culture dish was standardized to a total protein concentration of 300 μg/mL, which meant the addition of 5 to 30 μL, depending on the sample, to the total volume of CM (1.5 mL).

Pro-inflammatory cytokines. NFF growth rates were also calculated in the presence of the following: (1) TNF-α at three different final culture concentrations (0.2, 2.0, 20 ng/mL), (2) IL-1β at three different final culture concentrations (0.025, 0.25, and 2.5 ng/mL), (3) TGF-β1 at three different final culture concentrations (0.1, 1.0, and 10 ng/mL), and (4) combinations of the above cytokines with the highest concentration of each one tested.

Cytokines were reconstituted as recommended by the provider and added to CM to achieve the highest concentration tested (5-10 μL). Further dilutions were done by using CM. In addition, NFF growth rates were calculated for TNF-α at a concentration of 20 ng/mL in four separate experiments and for TGF-β1 at a concentration of 10 ng/mL in three separate experiments. Further testing was not performed for IL-1β because no inhibitory effect was observed in the preliminary dose-response curves.

Assessment of senescence in vitro

For determination of SA-β-Gal activity, NFFs were cultured in the presence of (1) complete media alone, (2) VUWF from four different donors, (3)

TNF-α at a final culture concentration of 20 ng/mL, (4) TGF-β1 at a concentration of 10 ng/mL, and (5) IL-1β at a concentration of 2.5 ng/mL. The effects of CM alone, VUWF, and TNF-α were additionally tested in three separate experiments to confirm the preliminary findings.

SA-β-Gal activity determination with the use of X-gal–based staining is a well-established method of detecting senescent fibroblasts in vitro.[17] SA-β-Gal staining was performed according to a protocol described by Dimri et al.[17] Briefly, NFF cultures exposed to the conditions described above were allowed to achieve a 30% to 50% confluence at 37°C and 5% CO_2. At this time, CM was removed, plates were washed with sterile phosphate-buffered saline, and fibroblasts were exposed to the staining solution (20 mg/mL X-gal in dimethylformamide; 40 mmol/L citric acid/Na phosphate buffer, pH = 6.0; 5 mmol/L potassium ferrocyanide; 5 mmol/L potasium ferricyanide; 150 mmol/L sodium chloride; 2 mmol/L magnesium chloride; and H_2O) after they were fixed with 2% formaldehyde/0.2% glutaraldehyde in phosphate-buffered saline. Plates were incubated for 12 to 16 hours (37°C without CO_2), and the plates were then examined under light microscopy. Positive cells characteristically display perinuclear precipitation of blue dye, which allows for clear identification in standard light microscopy. The percentage of positive SA-β-Gal cells was determined after a total of 300 to 400 cells per plate were counted.

Cell morphology

Morphologic light microscopic characteristics were evaluated for NFFs in the presence of the above culture conditions at ×10 and ×25 magnifications.

Determination of TNF-α concentration in VUWF

Immunoreactive TNF-α was determined in five different samples of VUWF, including those used in NFF growth and senescence experiments. Enzyme-linked immunosorbant assay (ELISA) (Cytoscreen TNF-α US Ultrasensitive ELISA kit; Biosource International, Inc, Camarillo, Calif) for human TNF-α was used for this determination, with the protocol provided by the manufacturer.

Effect of anti-human TNF-α antibody on the in vitro inhibitory activity of VUWF

NFFs were plated in 35-mm tissue culture dishes and exposed to CM, CM containing TNF-α at a final concentration of 20 ng/mL (TNF-α media), and anti-human TNF-α antibody–treated TNF-α media. Anti-human TNF-α antibody–treated TNF media was obtained by incubating TNF-α media with 0.04 μg/mL of anti-human TNF-α antibody for every 0.25 ng/mL of TNF-α (this is equivalent to a neutralizing dose of 50%) for 60 minutes at room temperature. Total cell count was then determined on day 11 of culture with a Coulter counter.

NFFs were then cultured in the presence of four VUWF samples from four different ulcer patients who were treated with and without anti-human TNF-α antibody. The amount of anti-human TNF-α antibody used was calculated by the amount of TNF-α determined with ELISA for each VUWF sample (0.04 μg/mL of anti-human TNF-α antibody for every 0.25 ng/mL of TNF-α). Bovine serum albumin (250 μg) was added to CM for control purposes. A total of 250 g of VUWF protein was used from each sample.

Materials

DMEM and trypsin were purchased from Gibco BRL Life Technologies, Inc. (Gaithersburg, Md). Bovine calf serum was obtained from Hyclone Laboratories, Inc (Logan, Utah). X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) was purchased from Sigma (St. Louis, Mo). Alevyn was obtained from Smith & Nephew (Largo, Fla). Cytokines (TNF-α, IL-1β, and TGF-β1) were purchased from R&D Systems, Inc (Minneapolis, Minn). Cytoscreen TNF-α US Ultrasensitive ELISA kit was purchased from Biosource International, Inc (Camarillo, Calif). Recombinant goat anti-human TNF-α antibody was purchased from R & D Systems, Inc (Minneapolis, Minn).

Statistical analyses

Linear regression analysis was used to estimate the best-fit line for each growth rate obtained. The slope of the best-fit line was used as an estimate of the growth rate in cells per day ± SEM. Comparisons of growth rates were done with two-tailed paired t-test analysis, with significance represented by a P value of less than .05. Paired t-test analysis was also used to compare SA-β-Gal data.

RESULTS

Growth rates

VUWF. The average growth rate of NFFs in CM was 42,727 ± 6301 cells/d with minimal variability between the two foreskin donors. The average NFF growth rate in the presence of VUWF was 3902 ± 2191 (P = .006). The average percent reduction of growth rates was 96% (86%-100%) (Fig 2).

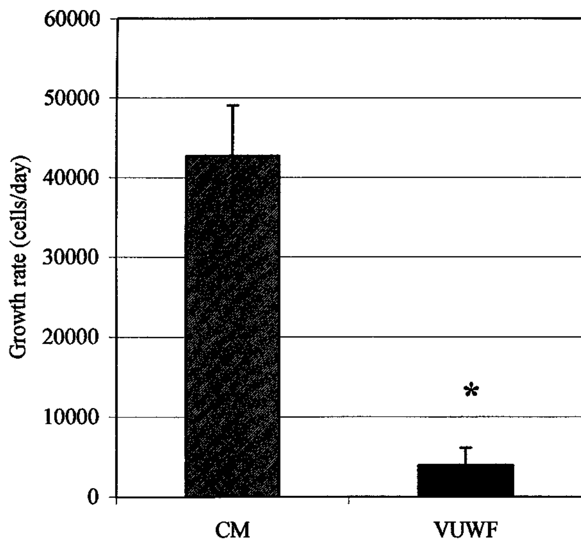

Fig 2. Neonatal foreskin fibroblast growth rates in the presence or absence of venous ulcer wound fluid (VUWF). The VUWF bar represents the mean ± SEM of four different samples tested.
CM, Complete media; asterisk, $P = .006$

Cytokines. NFF growth rates were reduced in a dose-dependent manner by TNF-α and by TGF-β1, whereas IL-1β did not modify NFF growth rates at the concentration tested (Fig 3).

Four separate experiments were performed with the final TNF-α concentration of 20 ng/mL, with an average NFF growth rate of 25,729 ± 5555 cells/d in the presence of TNF-α versus 46,099 ± 3453 cells/d in the presence of CM, a reduction of 56% ($P = .01$). NFF growth rates were inhibited by TGF-β1 at a concentration of 10 ng/mL, but this inhibitory effect did not reach statistical significance (44,476 ± 4312 vs 24,500 ± 8422, $P = .4$).

No synergistic effects were noted when combinations of the above cytokines were used, and the inhibition observed was equivalent to that of the TNF-α concentration used alone (data not shown).

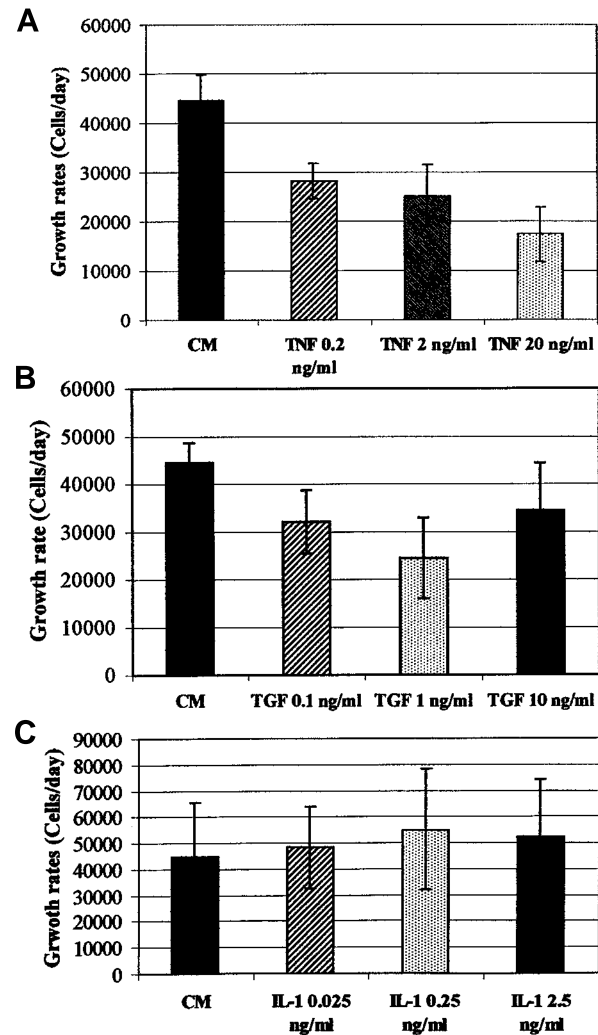

Fig 3. Neonatal foreskin fibroblast growth rates (± SEM) when culture in the presence of tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and interleukin (IL)-1β. Dose-response curves were performed for each cytokine. Each data point represents the growth rate derived by calculating the slope of a 3-point growth curve. Data points in each growth curve derive from duplicate samples.

SA-β-Gal activity

The percentage of SA-β-Gal–positive NFFs after 12 days in culture was a mean of 2.56% ± 1.41% in the presence of CM, versus 24.3% ± 6.2% in the presence of VUWF ($P = .009$) and 34.8% ± 20.6% in the presence of TNF-α ($P = .03$) (Fig 4). In a single experiment, TGF-β1 induced NFF SA-β-Gal activity to a similar degree (23%), but no SA-β-Gal activity was detected in NFFs cultured in the presence of IL-1β.

Cellular morphology

Morphologic changes were noted in an increased proportion of fibroblasts cultured in the presence of VUWF and TNF-α. These changes included increase in size, polygonal and irregular shapes, and presence of intracytoplasmatic stress fibers. These changes are consistent with those previously reported for senescent fibroblasts (Fig 5).

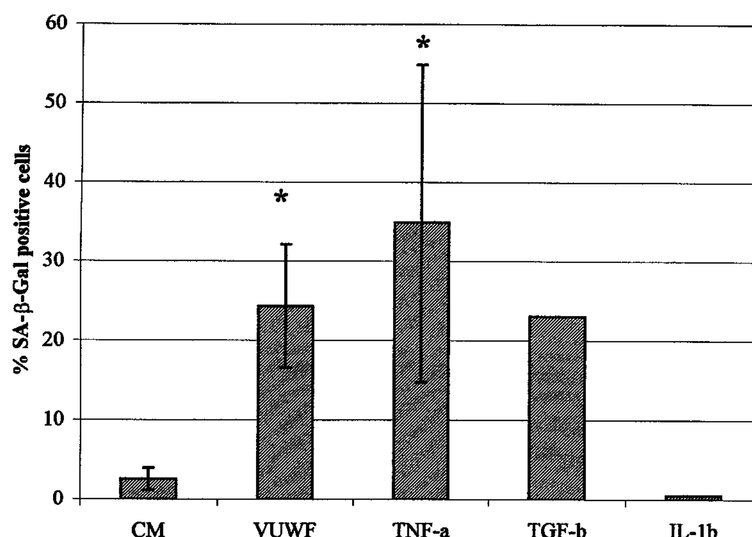

Fig 4. Neonatal foreskin fibroblast senescence associated β-galactosidase activity (SA-β-Gal) activity after 12 days in culture in the presence of venous ulcer wound fluid (VUWF) and pro-inflamatory cytokines. The VUWF bar represents the mean ± SEM of four different VUWF samples tested. The tumor necrosis factor (TNF)-α bar represents the mean ± SEM of four independent observations.
CM, Complete media; *TGF-β,* tranforming growth factor-β; *IL-1β,* interleukin-1β; asterisk, $P < .03$ when compared to control (CM).

Determination of TNF-α in VUWF

TNF-α was detected in all VUWF tested, with a mean of 0.254 ± 19 ng/mL (range, 0.188-0.309 ng/mL).

Effect of anti-human TNF-α antibody on the in vitro inhibitory activity of VUWF

Anti-human TNF-α antibody successfully blocked the inhibitory effect of TNF-α in NFF cultures exposed to a TNF-α concentration of 20 ng/mL (Table I). All VUWF samples tested in this experiment inhibited NFF growth. Incubation of VUWF samples in the presence of anti-human TNF-α antibody did not significantly modify the in vitro inhibitory effect of VUWF (Table II).

Table I. Average number of neonatal foreskin fibroblasts (NFFs) ± SEM harvested from two separate tissue culture dishes counted after 11 days in culture in the presence of complete media (CM), CM + TNF-α (TNF-α), and CM + TNF-α + anti-human TNF-α antibody (anti–TNF-α)

	CM	*TNF-α*	*Anti–TNF-α*
NFFs	31,820 ± 1460	9805 ± 1965	21,760 ± 2860

The initial number of NFFs seeded was 500 cells.

DISCUSSION

In this study, we have demonstrated that healthy young fibroblasts are susceptible to VUWF. If we accept that VUWF is a reflection of the venous ulcer microenvironment, these findings demonstrate that the dysfunction found on fibroblasts isolated from patients with venous ulcers and venous reflux[8,9] is a direct consequence of the microenvironment established by venous hypertension. In addition to the reduction of NFF in vitro growth rates, VUWF also increased the NFF expression of SA-β-Gal activity and induced senescent-like morphologic characteristics. This is consistent with the findings in our previous experiment, in which a greater proportion of senescent fibroblasts were found on isolates from the dermis of biopsy specimens taken from venous ulcers.[8] This suggests that the venous ulcer microenvironment affects fibroblast function, at least in part, by inducing fibroblast senescence.

The inhibitory components of VUWF on fibroblast growth and function are not known. Potential candidates include endogenously produced cytokines involved in modulating chronic inflammation[4]; extensively degraded vitronectin or fibronectin, which has

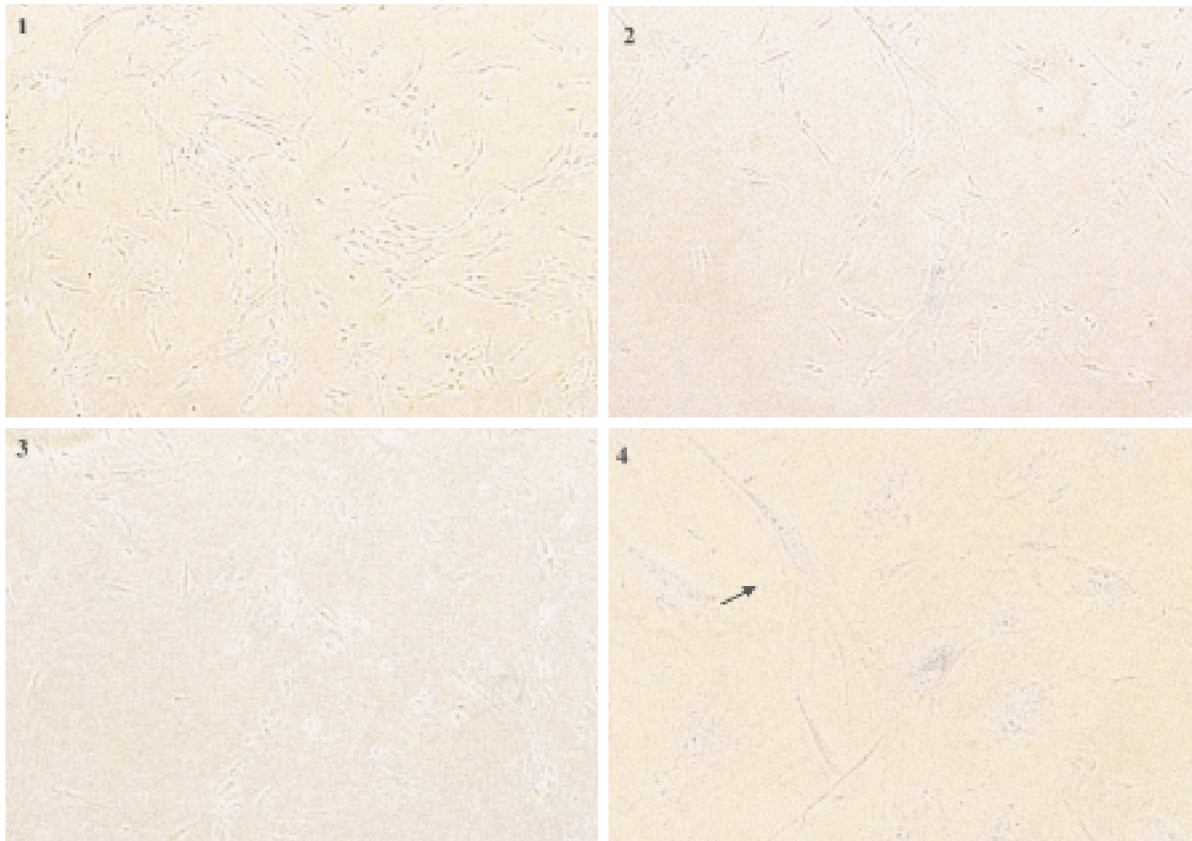

Fig 5. Representative photomicrographs of neonatal foreskin fibroblasts (NFFs) under light microscopy at ×10 **(1,2,3)** and ×25 **(4)** magnification. All photomicrographs were obtained from tissue culture plates subjected to the same culture conditions and on day 7 after initial seeding (500 cells/plate). **1,** NFFs cultured in the presence of complete media (CM) alone. **2,** NFFs in the presence of CM and venous ulcer wound fluid (VUWF). **3** and **4,** NFFs in the presence of TNF-α. The *arrows* mark fibroblasts with the typical morphologic features of senescence.

Table II. Average number of neonatal foreskin fibroblasts ± SEM harvested after 11 days in culture from two separate dishes for each condition

	CM-BSA	*VUWF 1*	*VUWF 2*	*VUWF 3*	*VUWF 4*
CM-BSA	82,585 ± 5685	750 ± 20	56,550 ± 4515	205 ± 75	35,975 ± 905
Anti-TNF	91,190 ± 12,040	760 ± 140	22,470 ± 590	455 ± 135	32,440 ± 320

Venous ulcer wound fluid (VUWF; 250 μg of protein) from four different donors was tested after incubation with or without anti-human TNF-α antibody (Anti-TNF). Bovine serum albumin (BSA, 250 μg) was added to complete media (CM) for control.

been shown to affect cellular adhesion[18,19]; or increased levels of metalloproteases, which could lead to an increased matrix breakdown.[20] Bacterial toxins or by-products have also been proposed.

Previous studies have demonstrated that chronic wound fluid decreases the proliferation of fibroblasts, keratinocytes, and endothelial cells in vitro,[21] in contrast to acute wound fluid, which has been found to stimulate proliferation of fibroblasts and endothelial cells.[20,22] In previous experiments in our laboratory, we have found that chronic wound fluid is not cytotoxic to fibroblasts, has a reversible inhibitory effect on NFF growth rates, has inhibitory activity that is heat sensitive,

and selectively inhibits other fast-growing cells such as melanoma cell lines.[29]

Of the endogenously produced cytokines, pro-inflammatory cytokines, such as IL-1β, TNF-α, and TGF-β1, have previously been isolated from VUWF.[4] These pro-inflammatory cytokines play an important beneficial role in the early stages of wound healing and tissue repair, but their role in chronic inflammatory processes like venous ulcers is not known.

In our study, TNF-α and, in part, TGF-β1 had an in vitro inhibitory effect on NFF growth rates. The concentrations at which these inhibitory effects were noted were significantly higher than those expected physiologically within the venous ulcer microenvironment but did reproduce, in part, the effects of VUWF. Also, the inhibitory effects of TNF-α and TGF-β1 may be dependent not only on concentration but also on time of exposure. TNF-α, a cytokine produced by macrophage upon activation, is a modulator of chronic inflammatory responses, as well as TGF-β1, which is also largely produced by activated macrophages and was detected in all VUWF tested in our experiments. IL-1β, another pro-inflammatory cytokine, did not share the same direct inhibitory activity on NFFs. In vivo IL-1β could still play an indirect role in fibroblast inhibition, modulating chronic inflammation. Other pro-inflammatory cytokines not tested in our experiments that could also be involved in this process are interferon-γ and IL-12.[23,24]

The VUWF inhibitory effect on NFF was not significantly modified by the blocking of VUWF TNF-α activity, suggesting that, at least in vitro, TNF-α plays a secondary role. This also suggests the presence of unknown strong inhibitors of fibroblast growth within VUWF.

Clinical improvement of venous ulcers has been associated with decreased levels of VUWF TNF-α concentration,[5] and, in an experimental model, topical application of TNF-α has been shown to be detrimental to wound healing.[27] Furthermore, a prospective multicenter study of pentoxifylline (a well-known down-regulator of TNF-α production) in patients with venous ulcers demonstrated a significant reduction in ulcer size in treated patients compared with untreated patients.[28]

VUWF, TNF-α, and TGF-β1, but not IL-1β, induced NFF senescence in vitro. To our knowledge, this effect has not yet been reported. Senescent fibroblasts are unable to replicate but remain metabolically active with altered cell functions[25]; are less motile[25]; can accumulate in tissue due to their resistance to apoptosis[26]; and produce a different array of proteins, including elevated levels of matrix metalloproteases[11] and pro-inflammatory cytokines[10]—all of which can affect tissue integrity and normal healing.

CONCLUSIONS

We conclude that VUWF, through one or more of its components, is able to inhibit NFF growth and induce senescent characteristics. This suggests that the venous hypertension microenvironment leads to subsequent development of venous ulcers by inducing severe fibroblast dysfunction. Accumulation of TNF-α and TGF-β1 in this microenvironment may contribute significantly to this process. Further studies are needed to isolate other fibroblast inhibitory components of VUWF and to elucidate the role of pro-inflammatory cytokines in chronic venous insufficiency.

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DISCUSSION

Dr John Mannick (Boston, Mass). Manuel, I'd like to congratulate you and Dr. Menzoian and your associates for pursuing this very important clinical problem at the molecular and the cellular levels.

I have a couple of problems with the data, which perhaps you can straighten me out on.

The first has to do with your control cultures, which contain 10% fetal calf serum. Now, what did you actually do with the wound fluid? Did you just filter the wound fluid, determine what its protein concentration was, and then put the cultures in that fluid, at a protein concentration of 300 μg/mL? Is that what you did?

Dr Manuel V. Mendez. Once the wound fluid was obtained, we filtered and diluted it in culture media without calf serum.

Dr Mannick. Is it not possible that the entire difference we're looking at here is just the absence of fetal calf serum in the culture?

Dr Mendez. We cultured the cells in 1.5 mL of CM including 10% calf serum. When we measured our concentration of protein in the wound fluid, it was an average of 6 μg/μL. This meant that we usually added anywhere between 25 and 50 μL per plate, so we think that even though this wound fluid does not have the protein concentration of calf serum, there was enough calf serum to support growth.

Dr Mannick. So, if I understand you correctly, then you have these cells still in medium with 10% calf serum, but you've added some of the wound fluid in there, in addition to the regular medium containing the calf serum.

Dr Mendez. Exactly. That's the way we did it.

Dr Mannick. Do you think that it might be a good idea to do a control that had some human serum, from the same patient or a different patient, that was treated the same way as your wound fluid? And then add it just to make sure that diluting out the fetal calf serum doesn't explain the results?

Dr Mendez. Yes, I think that's a good point. We should probably, to get the ideal control, use the same amount of volume used for the VUWF media.

Dr Mannick. Yes, I would think so.

The second problem I have is with the TNF experiments. The TNF concentration in your wound fluid is about 200 pg/mL, which is very believable in an area of inflammation. But, the only concentration of TNF that really did any statistically significant damage to your cultures was, if I recall your data correctly, 20 ng.

That's 200 times the physiologic concentration of TNF. So I can't believe that the TNF concentration in that wound fluid and the concentration that caused the damage could really be said to be similar and that TNF is the explanation.

I think you're going to have to look elsewhere, besides TNF, to explain your findings.

Dr Mendez. Yes.

I think that there are definitely other inhibitors, components of VUWF, which we have not identified.

I didn't mean to make a connection between the level of TNF and VUWF as a direct cause of the inhibitor activity in vitro. The issue that we find inhibitor activity of TNF in vitro just points out the possibility that TNF in vivo may have an effect in terms of fibroblast function and in terms of fibroblast senescence.

Dr Mannick. Well, I guess, just in defense of what I said, 20,000 pg/mL of TNF should be pretty toxic to any cell I can think of. So I think probably the 200 pg dose, which didn't do much to your cultures, cannot really explain the findings. I certainly would agree with you there.

Dr B. Sumpio (New Haven, Conn). I'd like to follow up with three questions.

One is a follow up on Dr. Mannick's point, regarding the causality issue of TNF, as you stated in your abstract—venous fluid. And he's made a point already, concerning the doses.

I guess the killer experiment, if you will, is to really add some kind of neutralizing antibody. If you were, perhaps, to add a neutralizing antibody to TNF, or to IL-1, or whatever you'd like to pick, and you found that you could attenuate that response, then that might give more evidence that TNF is involved.

So, the first question is, have you done any neutralizing antibody studies to support the contention of TNF-α or other cytokines in this role?

Dr Mendez. No. We are in the process of planning out those experiments, but we have not done those studies yet.

I want to mention one thing, that there are clinical data in the literature to support that anti-TNF therapy may have an effect in venous ulcer healing. We know about the beneficial effects of using pentoxifylline in a randomized trial for venous ulcer healing. And we know that pentoxifylline has regulating activity of TNF, among other effects. This is indirect evidence that maybe by blocking TNF we can induce healing on venous ulcers.

Also Salomon et al showed that topical application of TNF decreases wound healing strength and increases the level of collagenoses and diminishes RNA for procologen type one and two. Again, we have not done the studies neutralizing TNF within the wound fluid, and we show that actually blunts the inhibitory effect on neonatal fibroblasts.

Dr Sumpio. And just to be quick, the next question really is, have you looked at the effects of this fluid in other cell types, besides fibroblasts? For instance, on the endothelium cell, or to see if this has an effect, and would you tell us more about the senescence effect? Is it truly senescence? Are you killing cells? Is it lytic to the cells, or is it causing apoptosis?

Dr Mendez. In our lab, we've done studies with VUWF showing, first of all, that it's not cytotoxic. We've also tried VUWF in other cell types, and it appears to be inhibitory for fast growing cells, such as melanoma cell lines, but not for slow growing cells, like melanocytes.

We think that this inhibitory effect has to do with the growth rate. And we see here, that there is significant inhibitory activity on NFFs. It's probably related to the growth rate of the cells.

Dr Allan Callow (Boston, Mass). Manuel, I think it's time to rally around the flag. And therefore I'm getting up to say what a splendid study I think this is.

It's not a Nobel Prize winner, but I think it's a very good piece of work.

I, too, have some questions. I wonder if you would see the same effect with endothelial cells or muscle cells.

I also wonder if, because β-galactosidase is such a ubiquitous substance and associated with trauma, whether or not you might demonstrate some of this effect if you had just a simple acute wound, a dermabrasion, or an open wound and took the serum.

But overall, I want to say that I think it's a step forward. And of course it's one small step but, nevertheless, a very important one.

Dr Mendez. Thank you, Dr. Callow.

We have not tested VUWF, nor have we looked at senescence in other cell types, yet.

There is one comment I want to make about acute wounds and chronic wounds. There are studies out in the literature showing that fluid that came from acute wounds has the opposite effect. It stimulates fibroblast proliferation as well as collagen activity. So, I think there is a distinction to make between chronic wounds, like venous ulcers, and acute wounds.

I think it would be interesting to look at the acute setting, whether fibroblasts obtained from those wounds do actually behave like senescent cells. But we have not done that type of study yet.

Dr Robert Hopkins (Providence, RI). Perhaps I missed it, but I didn't hear you mention the possible microbiology of the ulcer fluid. The wound is open and, to some extent, subject to contamination for a long time. I assume you cultured it and that it was negative. On the other hand, there could be other microorganisms there that did not grow out. Could they have contributed to your findings?

Dr Mendez. The inhibitory effect of VUWF could be caused by components of exogenous nature such as bacterial toxins or could be of endogenous origin.

We filtered this fluid through filters that remove all bacteria, but some toxins produced by bacteria may actually go through.

The issue that we don't see bacteria overgrowth in our cultures makes us very confident that there's no contamination of bacteria, but we cannot rule out the possibility of bacterial toxins within the VUWF as potential inhibitors for fibroblast function, as well as inducers of senescence.