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Extension of the Life Span of Pressure Ulcer Fibroblasts with Recombinant Human Interleukin-1β

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Recombinant human interleukin-1β (rhuIL-1β) was investigated in a randomized, blinded placebo-controlled trial to evaluate its effect on the healing of chronic pressure ulcers. The influence of this topically applied cytokine to 26 pressure ulcer patients was correlated with tissue culture and electron microscopic evaluation. Cellular replication studies showed that low (0.01 μg/cm²/day) and medium (0.1 μg/cm²/day) concentrations of rhuIL-1β were not effective in extending replication in pressure ulcer fibroblasts, in vitro. Tissue culture measurements from pressure ulcer biopsies demonstrated that, after 29 days of a high level of rhuIL-1β treatment (1.0 μg/cm²/day), the cytokine was effective in extending the ability of pressure ulcer fibroblasts to replicate. Tissue culture and electron microscopy suggested that, although rhuIL-1β promoted increases in fibroblast numbers, the primary effect appeared to be development of the extracellular matrix. The possible direct and indirect influences of rhuIL-1β therapy on pressure ulcers are discussed. (Am J Pathol 1995, 146:1273–1282)

Typically, pressure ulcers (decubitus) are open skin wounds or sinuses with a loss of both epidermis and dermis. Newly formed ulcers may demonstrate some spontaneous healing via contraction and epithelialization. Often, however, these wound healing processes will slow down and stop, leaving behind an intractable chronic wound. Late stage ulcers appear to undergo an arrest of these natural healing processes, and the ulcer becomes lined with thick fibrotic scar. In this setting, ulcer fibroblasts appear to have a limited proliferative ability or appear senescent because the necessary mitogen (growth) factors may not be available.

Various clinical and basic scientific studies indicate that interleukin-1β (IL-1β) has the ability to affect the wound healing process. Evidence such as IL-1β released by mononuclear cells during chronic inflammation resulting in fibroblast proliferation suggest that IL-1β may be beneficial in treating chronic pressure ulcers.[1] Of further relevance to these wounds is that virtually all nucleated cells have the ability to produce IL-1β for activation of fibroblast division. Additionally, the ability of fibroblasts to respond to this cytokine appears likely as the number of binding sites has been estimated to be 1500 to 5000 sites per cell.[2] Also, in pressure ulcers, which are often bacterially infected, it has been shown that recombinant human (rhu)IL-1β has the ability to reverse the inhibition to contraction.[3]

In view of these potential benefits of IL-1β to pressure ulcer repair, a controlled trial study was conducted to evaluate the safety and efficacy of three different concentrations of IL-1β in chronic pressure ulcers.[4] This study showed that doses of 0.01, 0.10, and 1.0 μg/cm²/day through 29 days were safe to use; however, they produced no significant increase in the rate of repair over placebo-treated wounds. Just before the clinical trial was initiated we discovered that cultured fibroblast populations from the ulcer bed went through significantly fewer replications before becoming senescent when compared with identically cultured fibroblast populations from the ulcer margin and adjacent, normal skin. Additionally we observed that ulcer bed fibroblasts were often larger than fibroblasts from the other populations and vacuolated

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and stained positive with anti-terminin, a cell marker identifying cell senescence.[5] Thus, although the literature indicated that IL-1β stimulated fibroblast proliferation, our clinical study indicated that this cytokine did not significantly improve the rate of ulcer repair over placebo-treated ulcers. In view of our laboratory data, a question that we sought to answer in this study was whether these ulcers did not heal because IL-1β did not stimulate senescent or near senescent ulcer fibroblasts to divide. Such knowledge would be significant toward an understanding of the future of this cytokine in pressure ulcer repair.

Materials and Methods

Biopsies

Pressure sores from 16 patients extended from bone to the subcutaneous tissue (grade III/IV). All patients were denervated in the area of ulceration because of either congenital or acquired spinal cord pathology. Wounds were debrided of all necrotic tissue followed by application of rhuIL-1β or its placebo vehicle. Consecutive patients having a pressure sore between 10 and 100 cm^2 were randomized to receive either placebo or one of the dosage tiers of rhuIL-1β. Patients in the tier 1 group received 0.01 μg/cm^2/day, tier 2 received 0.1 μg/cm^2/day, and the tier 3 group received 1.0 μg/cm^2/day.

Punch biopsies were taken before treatment at day 0 and at day 29 after treatment with low, medium, or high levels of rhuIL-1β or placebo. The biopsies were coded and blinded. After the biopsies were halved, one portion was placed into tissue culture medium and the remaining tissue was minced and placed in fixative before shipment on ice via overnight express to the San Diego Veterans Administration Medical Center.

Tissue Culture

Each biopsy was further divided into smaller portions (0.5 to 1.0 mm^3). Two pieces of tissue were anchored to 60-mm^2 culture dishes and incubated in Eagle's minimal essential medium, pH 7.2, containing 10% fetal bovine serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 4.0 mmol/L glutamine. The method of collagenase digestion of the tissue was tried also; however, the cell yield from this procedure was too small to obtain a viable cell population. Once fibroblast growth started from explant, cells continued to appear and to migrate across the culture dish surface. Cells were then fed every 4 to 5 days and maintained in a humidified atmosphere containing 5% CO_2 at 37 C.

Culture dishes (60 mm^2) were seeded with 5×10^4 ulcer fibroblasts. After 1 week, cells were trypsinized and counted in a Coulter counter. From each preceding passage another population of equal size was seeded into dishes and placed into a cell culture incubator for 1 week. Cell populations were considered senescent when they failed to undergo a 0.5 population doubling within a 1-week period.[6]

Electron Microscopy

Each biopsy was quartered, and two to three tissue samples were randomly selected from each portion. Eight to twelve tissue pieces from the ulcer edge, margin, and normal skin represented each patient.

Table 1. *Fibroblast Growth from Pressure Ulcers Treated with rhuIL-1β*

Trial concentration	Number of patients	Days	% explants displaying cell growth from explant	% explants displaying confluent growth	Number of replications
Controls	3	0	100%	33%	0
0.01 μg/cm^2/day	3	29	67%	33%	0
Controls	4	0	0	0	0
0.1 μg/cm^2/day	4	29	0	0	0
Controls	5	0	20%	20%	4.6
1.0 μg/cm^2/day	5	29	80%	80%	9.5
					13.3
					17.7
					0.5
Placebo controls	3	0	67%	0	0
Placebo	3	29	40%	0	0
Non-ulcer, normal control	1	0	100%	100%	23.5

Response of pressure ulcer fibroblasts to *in vivo* treatment with rhuIL-β. Fibroblast originating from pressure ulcer biopsies before treatment produced only limited cell numbers. These cell populations were of insufficient size to conduct replication studies. Fibroblasts from patient ulcers treated with a high concentration of rhuIL-β (1.0 μg/cm^2/day) for 29 days were able to replicate through additional cell divisions.

Under a dissecting microscope, biopsies were sliced further into small 1- to 2-mm pieces in Karnovsky's buffered fixative. Fixation was continued for an additional hour before washing three times in cold (4 C), 0.1 mol/L sodium cacodylate buffer (pH 7). After three buffer washes, the tissues were post-fixed in sodium cacodylate buffer (pH 7.4, 0.1 mol/L) and 2% osmium tetroxide for 1 hour at room temperature. After three buffer washes of 5 minutes each, the tissues were dehydrated through a graded series of ethanol and propylene oxide and embedded in plastic resin (EM-Bed 812, EM Sciences, Fort Washington, PA). Sections of all tissue blocks (10 to 15 μ thick) for light microscopic examination were stained with 1% toluidine blue, examined, and photographed in a Zeiss photomicroscope. From these sections, specific areas of the tissue blocks were thin sectioned (60 nm), mounted on 200-mesh unsupported mesh grids, and stained in uranyl acetate and bismuth subnitrate. At least four grids, each containing 15 to 20 sections from selected areas, were examined and photographed in a Zeiss EM-10B electron microscope.

Results

Tissue Culture

Low rhuIL-1β Treatment

All tissue explants from control patients before treatment at day 0 were able to produce fibroblasts. However, only explants from one of the three patients produced enough cells to reach confluent growth in a 60-mm^2 dish (Table 1). When these fibroblasts were trypsinized, counted, and plated to determine the remaining number of replications in this population, many cells were incapable of attaching to the culture dish and consequently did not divide.

No benefit from 29 days of low concentration (0.01 μg/cm^2/day) rhuIL-1β treatment to ulcer fibroblasts

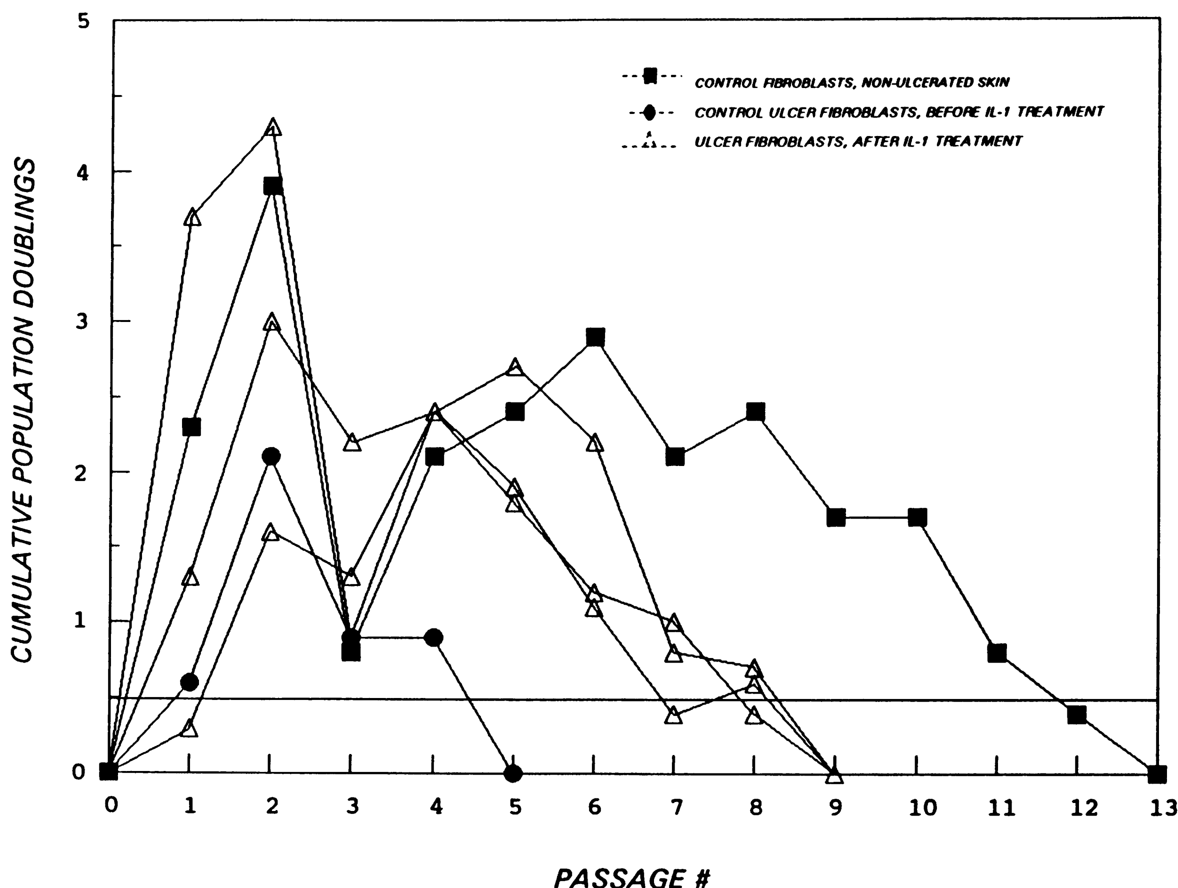

Figure 1. *Graph shows a gradual decline in population doublings through serial passages. The proliferative activity of pressure ulcer fibroblasts before rhuIL-1β treatment became senescent between passages 4 and 5. Fibroblast populations from patient pressure ulcers treated with a high concentration of rhuIL-1β for 29 days showed an extension of proliferative activity before becoming senescent at passage 8. Control fibroblasts from adjacent non-ulcerated skin became senescent after 12 passages. The horizontal bar at 0.5 cumulative population doublings designates the level at which cell lines became senescent. Cell populations were considered senescent when they were unable to undergo one population doubling within a 1-week period.*

was observed by tissue culture. Although 67% of the tissue explants produced cells, only explants from one of the three patients produced confluent fibroblast growth (Table 1). A replication study was not possible for the same reasons as found with cultured patient fibroblasts before rhuIL-1β treatment at day 0.

Medium rhuIL-1β Treatment

Explants from patients in the medium treatment group did not produce fibroblasts in culture (Table 1). Fibroblasts in explants from patients treated with a medium concentration of rhuIL-1β (0.1 μg/cm²/day) also were not stimulated to replicate after 29 days of treatment.

High rhuIL-1β Treatment

Replication studies were possible with mass populations of fibroblasts from all but one of the patient ulcers after 29 days of high treatment levels of rhuIL-1β (1.0 μg/cm²/day). Table 1 showed that four of five (80%) ulcer fibroblast populations from two separate patients were extended an additional 9.5 and 17.7 population doublings after eight passages.

Three 60-mm² culture dishes containing four randomly selected tissue samples from each biopsy represented the origin for each fibroblast population. Before treatment at day 0, control explants from one of five patients produced fibroblasts in culture to reach confluent growth. This cell population was able to replicate 4.6 times before becoming senescent. Ulcer fibroblasts from the same patient after treatment went through 13.3 replications or an additional 8.7 population doublings. All three cell populations became senescent after approximately eight passages (Figure 1). Thus, it appeared that a high concentration of rhuIL-1β therapy stimulated cell replication and thereby extended the life span of pressure ulcer fibroblasts. A fibroblast population was considered senescent when it did not complete a 0.5 population doubling within a 1-week period.[6]

Placebo Treatment

Explants from two of three patients (67%) in the control placebo group at day 0 and two of five (40%) ulcer explants after 29 days of placebo treatment pro-

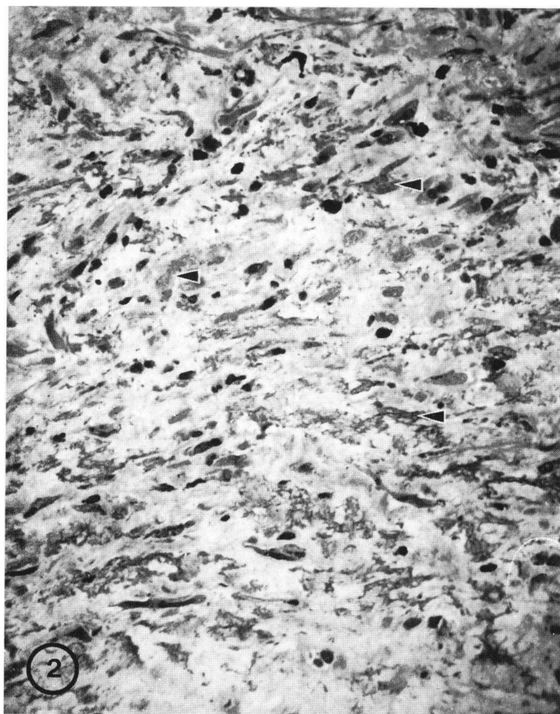

Figure 2. *Light micrograph of pressure ulcer tissue before rhuIL-1β treatment. Random fibroblasts (arrows) and inflammatory cells were generally found in an extracellular matrix composed of collagen remnants. Fibroblasts could be cultured from these tissues, but only one of three explants produced a cell population of 1000 cells or less. These populations became senescent before reaching confluency. Magnification, ×100.*

Figure 3. *Light micrograph of pressure ulcer biopsy from patient in Figure 2 after a low concentration (0.01 μg/cm²/day) treatment of rhuIL-1β for 29 days. Fibrin (FI) was commonly found associated with skeletal muscle (S) from the ulcer bed to the ulcer surface. Red blood cells (RBC) and other inflammatory cells were observed in areas of the ulcer tissue that showed little evidence of wound repair. Cultured fibroblast populations from these tissues became senescent and were too small to conduct replication studies. Magnification, ×100.*

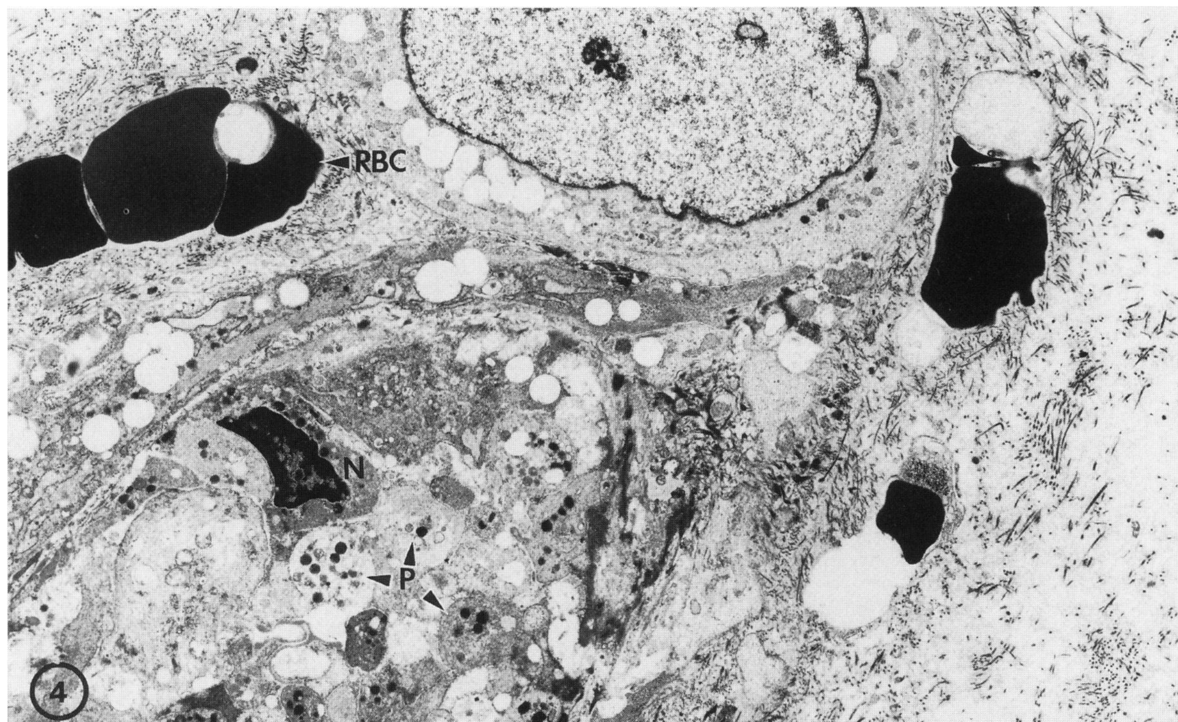

Figure 4. *In certain areas of ulcers receiving low rhuIL-1β treatment, blood vascular areas were occluded with inflammatory cells (N) and platelets (P). Single and/or small groups of red blood cells (RBC) were often observed in the extracellular matrix. Magnification, ×7500.*

duced fibroblasts in culture. However, less than 200 cells were observed in each culture dish (Table 1).

Non-Ulcerated Normal Tissue

From normal, non-ulcerated skin explants near the ulcer site, a confluent population of fibroblasts was easily cultured. This fibroblast population replicated through 23.5 population doublings before becoming senescent after 12 passages (Table 1).

Light and Electron Microscopy

From ulcer biopsies before and after low, medium, and high levels of rhuIL-1β therapy, remaining random pieces of tissues that were not used for cell culture were used for light and electron microscopic examination. These data specifically compared a patient before treatment (control) with the effect of rhuIL-1β treatment on that same patient after 29 days of treatment. Figures were selected that were representative of the histology of each treatment group.

Low rhuIL-1β Treatment

The appearance of pressure ulcer before treatment at day 0 showed that this tissue was in a general state of dissolution (Figure 2). Numerous inflammatory and some fibroblast-like cells were noted among fragmented collagen fibers.

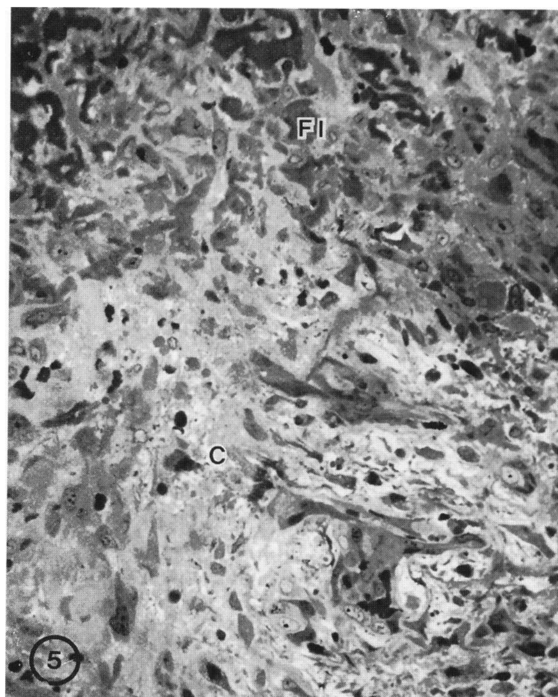

Figure 5. *Pressure ulcer tissue before treatment in the medium level concentration (0.1 μg/cm²/day) rhuIL-1β group. Characteristically, these tissues were composed of fibroblasts in a matrix of amorphous collagen fibers (C) and fibrin (FI). Fibroblasts could not be cultured from these tissues. Magnification, ×100.*

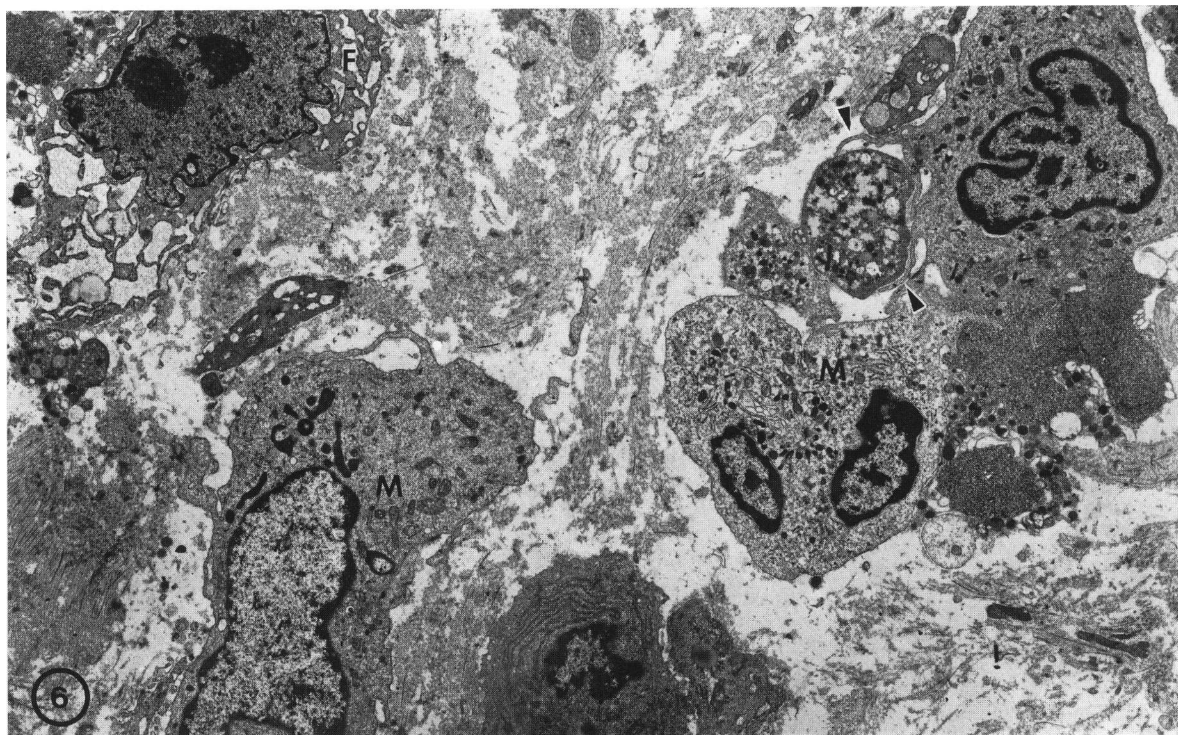

Figure 6. *Higher magnification of tissue from same patient pressure ulcer as in Figure 5 showed the details of the amorphous nature of the collagen matrix (C). Typically, these tissues displayed numerous macrophages (M) in various stages of removing cell fragments (arrows) and debris (*) by phagocytosis. Magnification, ×5000.*

After 29 days of low concentration rhuIL-1β therapy to the same patient there was no noticeable difference in pressure ulcer histology. Figure 3, a longitudinal section, shows skeletal muscle at the ulcer bed from which issued fibrin and irregular collagen fibers. The ulcer surface typically consisted of large concentrations of inflammatory cells within a fibrin matrix. Red blood cells were often observed outside the blood vasculature within the connective tissue matrix. In addition, electron microscopy showed that some blood vessels were occluded with inflammatory cells and platelets (Figure 4).

Medium rhuIL-1β Treatment

Pressure ulcer histology at day 0 showed evidence of an increased cellularity compared with that noted at the same time interval in the group of patients receiving the low concentration (Figure 5). The ulcer surface was characteristically composed of a fibrin mesh. The extracellular matrix was composed of amorphous remnants of collagen fibers (Figure 6). Within this area, macrophages were commonly observed in removing cell fragments and debris. The fibroblast cytoplasm was often vacuolated and without cellular organelles.

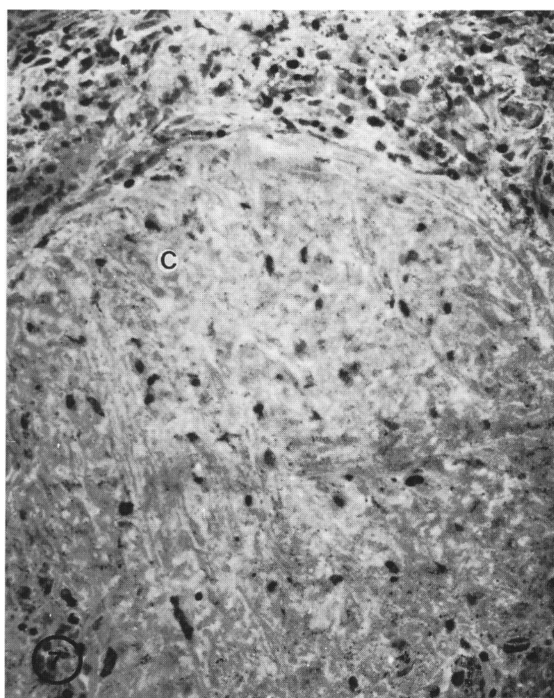

Figure 7. *After 29 days of rhuIL-1β (0.1 μg/cm²/day) treatment, no evidence of matrix remodeling in pressure ulcer tissue was noted. Fibroblasts could not be cultured from these tissues. Magnification, ×100.*

After 29 days of treating this pressure ulcer with 0.1 μg/cm²/day, no noticeable change in pressure ulcer histology from day 0 was noted. Numerous inflammatory cells occupied a matrix of unwoven and amorphous collagen fibers (Figure 7).

High rhuIL-1β Treatment

The histopathology of this pressure ulcer was similar to those control biopsies at day 0 before low and medium therapy (Figure 8). Many inflammatory cells, some fibroblasts, and occasional blood vessels could be identified in these tissues. The extracellular matrix was composed of loose and amorphous collagen fibers. The pressure ulcer surface was composed of primarily inflammatory cells within a fibrin mesh.

After 29 days of 1.0 μg/cm²/day of rhuIL-1β therapy, noticeable improvement was observed in the overall appearance of the pressure ulcer tissue (Figure 9). Blood vessel development was significant within a developing extracellular matrix. Although inflammatory cells were still evident, there were increased numbers of fibroblasts. Electron microscopy showed that both fibroblasts and myofibroblasts contained functional appearing mitochondria and endoplasmic reticulum (Figure 10). Within a developing extracellular matrix of new collagen fibers, cell fragments were being actively removed by macrophages (Figure 11). Additional evidence of matrix remodeling was evident by the apparent synthesis of collagen fibers at the cell membrane (Figure 12).

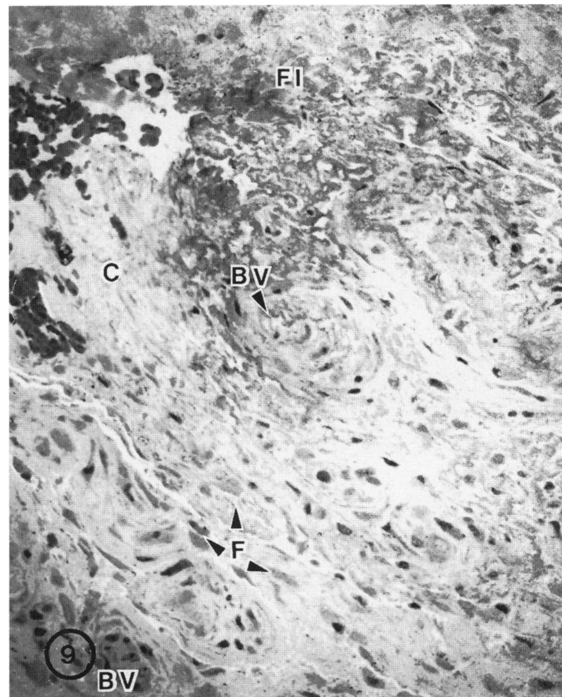

Figure 9. *After 29 days of high rhuIL-1β treatment, the same patient as shown in Figure 8 displayed a more organized extracellular matrix with collagen bundles (C), blood vasculature (BV), and fibroblasts. Fibrin (FI) was still noted at the pressure ulcer surface. Culture fibroblasts for replication studies were grown from 67% of the pressure ulcer biopsies. Magnification, ×100.*

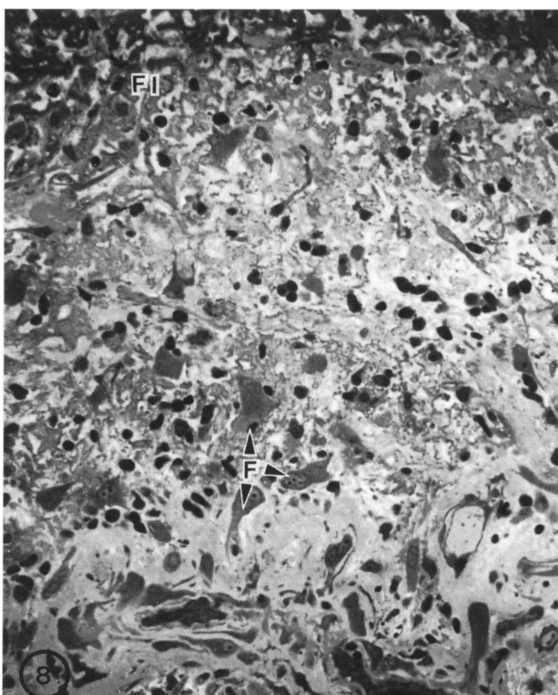

Figure 8. *Pressure ulcer tissue before high (1.0 μg/cm²/day) rhuIL-1β treatment displayed some fibroblasts (F) and inflammatory cells in a matrix of amorphous collagen and fibrin (FI). No fibroblasts could be cultured from this tissue. Magnification, ×100.*

Placebo Treatment

The histopathology of pressure ulcer biopsies at day 0 and after 29 days of placebo treatment appeared similar. These tissues were also similar to day 0 treatment from patient groups treated with low, medium, and high levels of rhuIL-1β therapy. After 29 days of placebo, tissues demonstrated large numbers of inflammatory cells. In some specimens, there were occasional observations of fibroblasts as well as open blood vasculature in an amorphous collagen matrix. A large portion of the stromal matrix appeared to be composed of inflammatory cells and fibrin (Figure 13).

Discussion

The influence of IL-1β has been evaluated in several types of wounds. Split thickness wounds created on 36 healthy volunteers and treated with 0.5 g/day

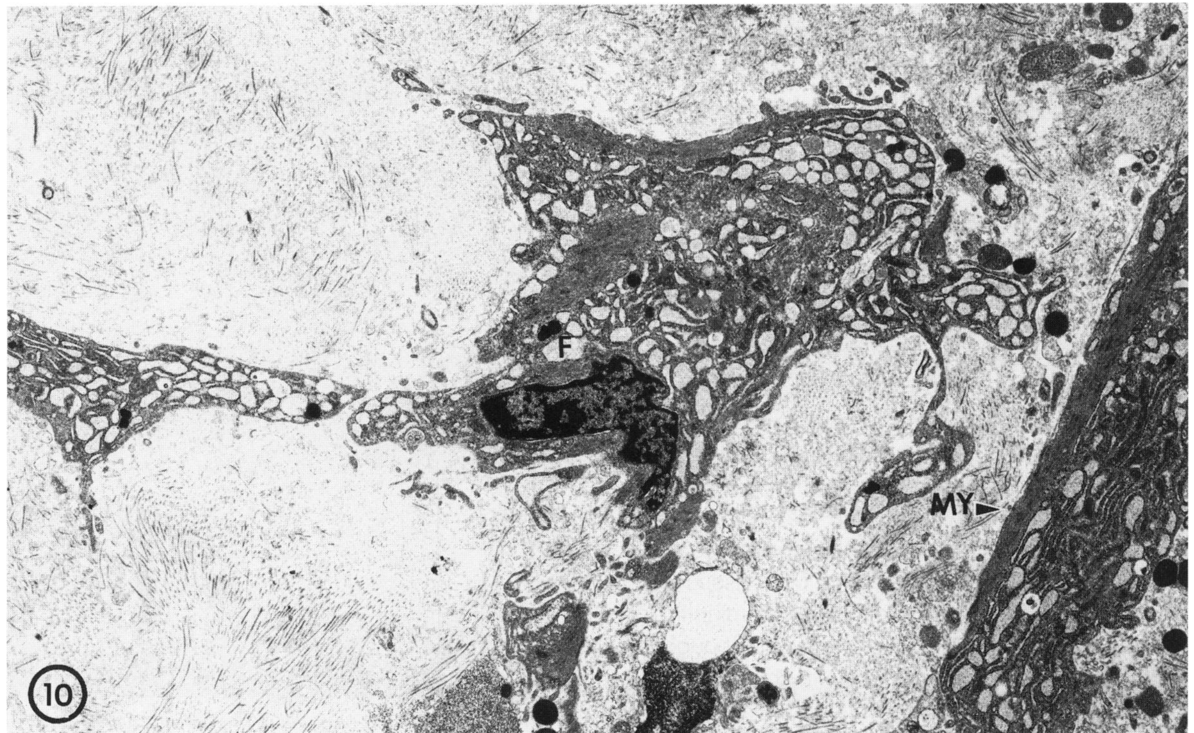

Figure 10. *Electron micrograph of high level rhuIL-1β treatment showed that fibroblasts (F) and myofibroblasts (MY) were prominent in a matrix of distinguishable collagen fibers. Although cells in these tissues appeared active, many still displayed large intracellular vacuoles suggesting a limited ability to contribute to ulcer repair. Magnification, ×5000.*

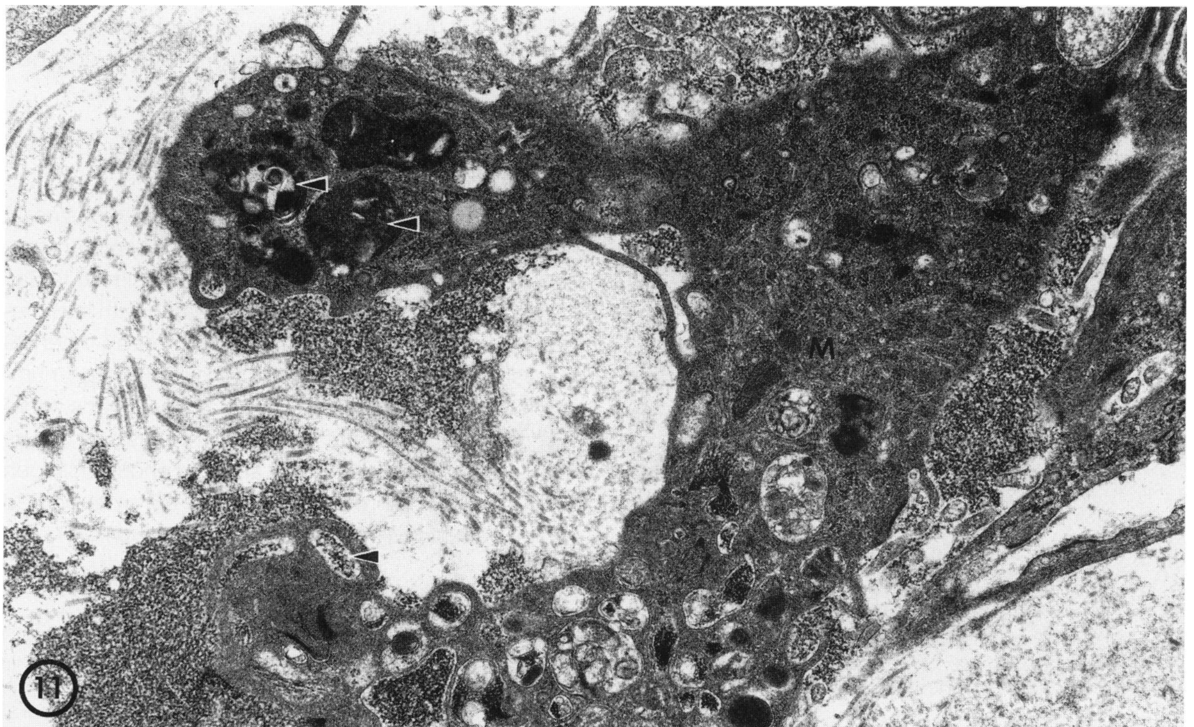

Figure 11. *In these tissues, macrophages (M) were very active as evidenced by the incorporation of cell fragments and debris into intracellular lysosomes (arrows). Magnification, ×15,500.*

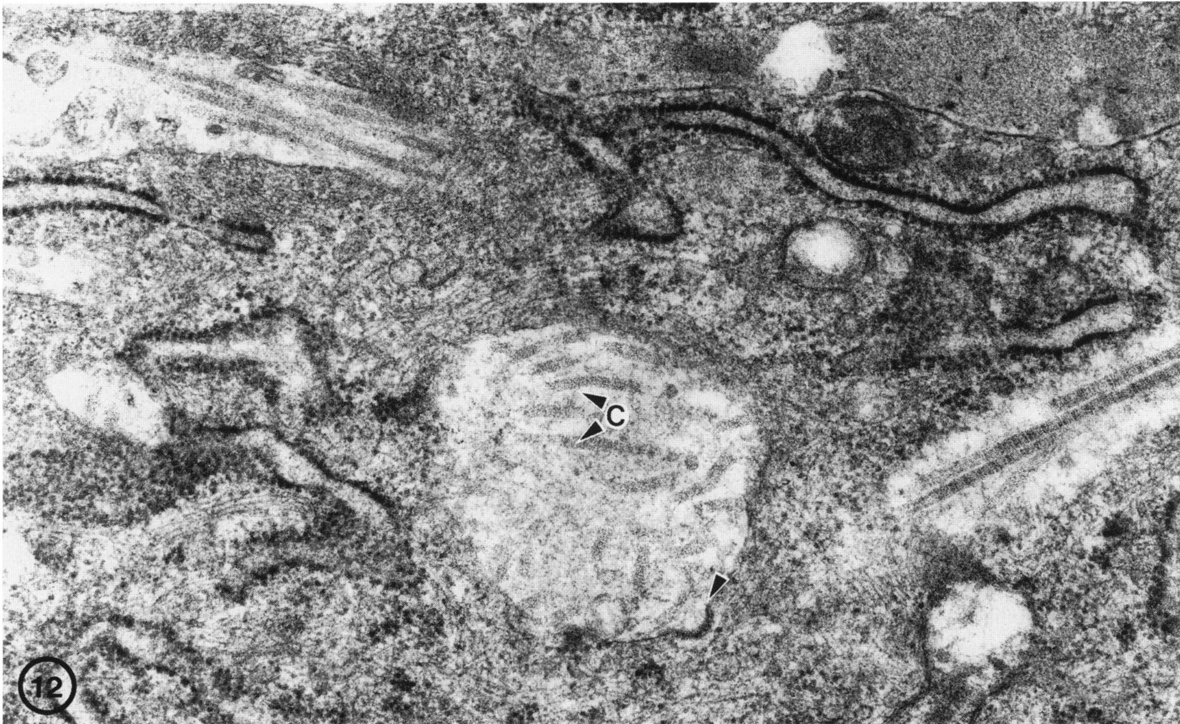

Figure 12. *Evidence of constructive remodeling of the extracellular matrix was shown by the development of collagen fibers (C) near coated vesicles (arrow) in the cell membrane of a fibroblast. Magnification, ×50,000.*

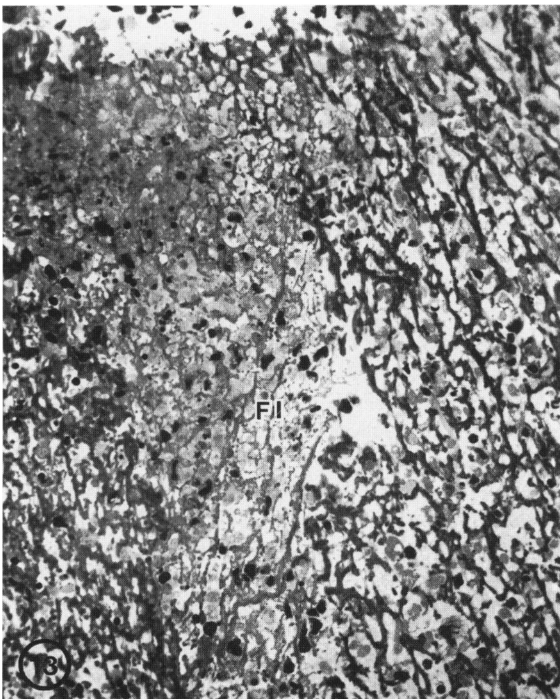

Figure 13. *Light micrograph of pressure ulcer treated with placebo for 29 days. Some ulcer tissues contained inflammatory cells and fibroblasts in an amorphous collagen matrix; however, many biopsied tissues showed primarily inflammatory type cells in fibrin (FI) and little evidence of matrix development. Magnification, ×100.*

rhuIL-1β showed a significantly faster rate of complete repair.[7] However, lower and higher doses of this cytokine demonstrated no significant increase in the rate of healing compared with wounds treated with placebo. Similarly, Kucukcelebi and associates[3] showed an increase in the rate of wound repair when rhuIL-1β was applied to an acute rat wound model.

In a controlled clinical trial that paralleled this study, doses of 0.01, 0.10, and 1.0 μg/cm^2 through 29 days produced no significant increase in the rate of repair over placebo-treated wounds.[4] However, the changes in histopathology before and after treatment suggested that rhuIL-1β did have an effect on the extracellular matrix and the cellular composition. Most pressure ulcers, before treatment and those treated with low concentrations of rhuIL-1β or with placebo, displayed an extracellular matrix that was amorphous and/or composed of loose collagen fibrils and fibrin. In general, most fibroblasts from these biopsies appeared in various stages of dissolution. However, pressure ulcers treated with 1.0 μg/cm^2/day of rhuIL-1β for 29 days exhibited fewer inflammatory cells, more viable appearing fibroblasts, and evidence of wound remodeling.

With these changes in stromal morphology, changes in viability were noted by the ability of fibroblasts from the high treatment group to proliferate. In

80% of the cell lines, the influence of rhuIL-1β was sufficient to extend the life span through several additional population doublings. It was interesting that, although variations in the number of population doublings occurred at each passage, senescence was observed after eight passages. Although fibroblasts from some control ulcer explants and ulcers treated with a low level of rhuIL-1β exhibited some ability to divide, populations greater than 1000 cells were never realized. The validity of these observations was based on the fact that, although pressure ulcer fibroblasts were treated by *in vivo* applications of rhuIL-1β, all cell lines were grown with identical culture conditions.

The precise mechanism by which rhuIL-1β was able to extend the proliferative capacity of pressure ulcer fibroblasts and promote matrix development is very complex. The reasons may be that IL-1 is made by nearly every cell type[2] and that it is capable of producing a multitude of responses *in vitro*.[8] Raines and associates[9] suggested that this cytokine may stimulate fibroblast division indirectly by inducing the release of the mitogen, platelet-derived growth factor (PDGF) by endothelial and smooth muscle cells. These researchers demonstrated that, in cultured human dermal fibroblasts, IL-1 induced expression of the PDGF A chain gene, followed by release of PDGF into the culture medium. Antibodies to PDGF were found to block the mitogenic response to IL-1. Evidence for the indirect effect of IL-1 on matrix development was shown by the ability of PDGF to stimulate fibroblasts to synthesize transforming growth factor-β as well as other stromal constituents.[10–12]

Light and electron microscopic examination of pressure ulcer biopsies showed that a high concentration of rhuIL-1β was capable of promoting remodeling of the extracellular matrix in addition to fibroblast proliferation. Unlike the study by Raines et al[9] involving human dermal fibroblasts, the mitogenic effects of a high concentration of rhuIL-1β on senescent ulcer fibroblasts may occur by another mitogen or pathway. Evidence for this assumption is based on a separate study in which our preliminary data show that PDGF-AA or PDGF-BB (40 ng/ml) in a defined culture medium only slightly stimulated ulcer fibroblast to divide. These factors may also explain why there was no correlation in the rate of repair in our parallel clinical study.

Although our sample size was limited, we conclude that rhuIL-1β may stimulate pressure ulcer repair through extension of fibroblast viability and the development of the extracellular matrix. However, with respect to our associated clinical trial,[4] our data indicate that rhuIL-1β alone does not stimulate fibroblast viability to promote a significant increase in the rate of repair in pressure ulcers. This study underlies the importance that future studies, involving the treatment of pressure ulcers with growth factors, should be conducted with senescent fibroblasts.

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