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FIBROBLAST AND EPIDERMAL CELL-TYPE I COLLAGEN INTERACTIONS: CELL CULTURE AND HUMAN STUDIES

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Abstract

Fibroblast and epidermal cell-type I collagen sponge interactions were studied in cell culture as well as in humans. In cell culture, fibroblasts were observed to migrate and proliferate throughout a type I collagen sponge containing either hyaluronic acid (HA) or fibronectin (FN). Fibroblasts accumulated in the center of the pores in sponges containing HA and appeared to surround themselves with newly synthesized extracellular matrix. In sponges containing FN, fibroblasts attached to and elongated along the collagen fibers of the sponge. In the absence of FN or HA protein synthesis of fibroblasts appeared to be inhibited by the presence of the type I collagen sponge. Epidermal cells grown on plastic or on type I collagen, formed sheets. Epidermal cells grown on a collagen sponge morphologically appeared different than cells grown on plastic.

The type I collagen matrix studied in cell culture was applied to dermal wounds of patients with pressure ulcers in order to evaluate its effect on dermal wound healing. The areas of ulcers treated for 6 weeks with a type I collagen sponge decreased by about 40% compared with no change in the areas of untreated controls. Preliminary results suggest that a type I collagen sponge is a biocompatible substrate with fibroblasts and epidermal cells and may be effective in enhancing healing of chronic skin ulcers.

KEY WORDS: Wound dressing, porous matrix, collagen, fibroblasts, epidermal cells, skin ulcers.

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Introduction

Chronic mechanical trauma to skin results in skin loss and may be followed by bacterial invasion and can lead to systemic infection in patients with a compromised immunological system. Therefore, it is important that new methods be developed to enhance the rate of healing of chronic wounds.

Many quadraplegics, paraplegics and other patients subject to prolonged bed rest suffer skin loss due to the effects of localized pressure. The resulting pressure sores, also known as decubitus ulcers, exhibit dermal erosion and loss of the epidermis and skin appendages.

Repair of large open wounds involves several events including cellular migration, biosynthesis of connective tissue components and other factors, deposition and remodeling of granulation tissue (Forrester et al., 1969; Peacock, 1984). The remodeling phase has been shown to be accelerated by the presence of a type I collagen porous matrix (Doillon et al., 1984, 1985).

It is well known that components of the extracellular matrix are able to induce cell mobility and attachment. Type I collagen attracts fibroblasts in cell culture (Kleinman et al., 1981) and appears to cause directed migration of cells (Dunn and Ebendal, 1978; Grinnell and Bennett, 1982; Thomaseck et al., 1982). Fibronectin is known to increase chemoattraction and spreading of fibroblasts *in vitro* (Kleinman et al., 1981; Gauss-Muller et al., 1980), and is found in large amounts in dermis during embryonic skin development (Gibson et al., 1983) and in healing wounds (Grinnell et al., 1981). Hyaluronic acid is found in high concentrations during embryonic development, is associated with cell movement and differentiation and is the first connective tissue glycosaminoglycan to appear in the extracellular matrix during wound repair (Alexander and Donoff, 1980).

In addition to high molecular weight components, a variety of low molecular weight cellular products have been shown to stimulate proliferation and enhance cellular biosynthesis of connective tissue components. These factors include epidermal growth factor (Gospodarowicz, 1981), platelet-derived growth factor (see for example Heldin et al., 1981), fibroblast growth factor (Gospodarowicz and Ill, 1980), eye-derived growth factor (Tassin et al., 1983) and cartilage-derived growth factor (Davidson et al., 1985). Heparin has been shown to induce the formation of capillaries (Azizkhan et al., 1980).

Previous work has shown that type I collagen sponges enhance the healing of excised wounds in an animal model (Doillon et al., 1984; Doillon et al., 1985; Doillon and Silver, 1986a; Doillon et al., 1986b). In this paper we extend these studies by analyzing the effects of type I collagen, fibronectin and hyaluronic acid on the biochemical and morphological characteristics of fibroblasts and epidermal cells cultured on type I collagen substrates.

Ultimately, we are interested in enhancing the healing of chronic wounds using connective tissue macromolecules, growth factors and cells to reconstitute synthetic dermal and epidermal replacements. This paper will focus on the interaction of fibroblasts and epidermal cells with a type I collagen sponge in cell culture and the application of collagen sponge technology to healing human skin ulcers.

Materials and Methods

Collagen Sponge Preparation

Collagen sponges were prepared for cell culture studies and human studies as described previously (Doillon et al., 1984). Type I collagen from cow hide was dispersed at 0.5% (W/V) in a 0.001 N HCl solution (pH 3.0), freeze-dried and crosslinked according to Weadock et al. (1984). Collagen sponges were sterilized by exposure to 2.5 M rads of gamma irradiation.

Fibronectin (FN) was extracted from fresh bovine blood as described by Ruoslahti et al. (1982) and was found by polyacrylamide gel electrophoresis to be composed of two polypeptide chains with a molecular weight of about 220,000 after reduction (Brokaw et al., 1985). FN was dissolved in 0.1 M ammonium acetate.

Hyaluronic Acid (HA) from Sigma Chemical Co. (grade III; potassium salt) was used and dissolved in HCl solution (pH 3.0). FN and HA solutions were mixed with the collagen dispersion in a Waring blender. Mixtures containing weight ratios of 1:99 FN to collagen and 1:19 of HA of collagen were prepared. Tissue ingrowth was maximized with these concentrations of HA and FN (Doillon and Silver, 1986a).

Cell Cultures

Fibroblasts were grown on collagen sponges after the sponge pH was stabilized by immersion in serum-free Dulbecco's Modified Eagle Medium (DMEM) (Gibco Laboratories) for three days. Fibroblasts were derived from embryonic chick tendons as described by Kao et al (1975) and cultured in DMEM supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, ascorbic acid (10 µg/ml added daily) and 5% fetal bovine serum (FBS) (Gibco Laboratories). Fibroblasts were plated as primary cultures at concentrations of 1.5×10^5 to 3.2×10^5 cells/cm^2. To seed the sponges, cells were suspended in 50 to 250 µl of DMEM and the mixture spread on each sponge and allowed to attach for 2 hrs (Doillon et al., 1987). Fibroblasts were grown at 37°C, in a tissue culture incubator in a 10% CO_2 atmosphere. The medium was changed every 2 days and fresh ascorbate added daily.

Epidermal cells were obtained from pieces of guinea pig skin that were excised, defatted and cut into 1 mm^2 pieces (Doillon et al., 1986c). The pieces of skin were washed in culture medium composed of a 1 to 1 mixture of DMEM and Ham's F12 Medium (DMEM/F12; supplied by Sigma Chemical Co.) containing 100 µg/ml of penicillin, 10 µg/ml streptomycin and 3 µg/ml of amphotericin B. Pieces of skin were then placed in a DMEM/F12 solution containing 5 mg/ml of collagenase (Cooper Biomedical) for 90 minutes. The digested skin pieces were then sedimented for 10 minutes under gravity. The sediment rich in epidermal cells was resuspended, counted and used for seeding.

Epidermal cells were cultured in DMEM/F12 supplemented with antibiotics, insulin (1 µg/ml), hydrocortisone (20 µg/ml) and 15% FBS (Doillon et al., 1986c). Cells were plated as primary cultures at a concentration of 2×10^6 cells/cm^2. Cells were seeded on each collagen sponge as described for fibroblasts. After 2 hours, the remaining culture medium was added. Epidermal cells were grown at 37°C, in a tissue culture incubator in a 5% CO_2 atmosphere. The medium was replaced every 2 days. The FBS was decreased from 15% to 10% over a one week period.

Radiolabeling Experiments

Fibroblast cultures were labeled at days 1, 3, 5, 7 and 9 post- seeding using DMEM containing either 2 µCi/ml [^{3}H] thymidine or 2 µCi/ml [^{14}C] proline (New England Nuclear). The labeling medium contained 10 µg/ml ascorbate. Fibroblasts were incubated with [^{3}H] thymidine for four hours. The medium was

removed, a 0.5 N perchloric acid solution added and cells were collected as previously described (Doillon et al., 1987). After sonication, the precipitated DNA was washed two times with 0.5 N perchloric acid and heated at 90°C for 90 minutes. After centrifugation, the supernatant was counted in a liquid scintillation counter (Aquasol-2, New England Nuclear).

Other fibroblast cultures were incubated with 2 uCi/ml [^{14}C] proline for four hours. Eighty percent of [^{14}C] proline incorporated into proteins appeared in collagen (Kao et al., 1975). The medium was then separated from the cells and fresh DMEM was added to the cells. Cell and medium fractions containing radiolabeled [^{14}C] proline were treated with protease inhibitors (Kao et al., 1977) and sonicated. The samples were brought to a final concentration of 2% sodium dodecyl sulfate (SDS), dialyzed against SDS sample buffer containing 2% (W/V) SDS, 10% (V/V) glycerol, 0.005% bromophenol blue in 0.124 M Tris-HCl pH 6.8, and an aliquot was counted in a liquid scintillation counter.

Epidermal cell cultures were labeled between days 15 and 22 using the methods described for the fibroblast cell cultures (see above).

Light Microscopy

Primary cell cultures were observed 9 days post-seeding. Collagen-based sponges and plastic dishes seeded with cells were washed briefly with phosphate buffered saline solution then fixed with modified Karnovsky's fixative. Specimens were embedded in glycol methacrylate as described previously (Doillon et al., 1984). Control plastic dishes containing cells were treated in a similar manner for light microscopy. Light micrographs were taken with a Laborlux 12 Pol light microscope equipped with a 35 mm camera at a magnification of 128X.

Human Studies

Sterilized collagen sponges were applied to non-infected dermal ulcers ranging from about 1 to 10 cm^2 in surface area. Only patients with ulcers in which loss of dermis without exposure of muscle, tendon or bone were treated. A patient consent form was signed by all subjects. The degree of subcutaneous erosion varied from patient to patient. The patients ranged in age from about 35 to 70 years of age. Six patients were studied in the control and collagen treated groups, respectively.

Both control and collagen treated wounds were cared for using the protocol that follows. All wounds were washed with a 1% (W/V) solution of hydrogen peroxide followed by normal saline. The collagen sponge was then packed into the wound (except for controls). All wounds were then covered with saline wetted cotton gauze. A layer of dry cotton gauze was then applied and taped to surrounding healthy skin. All wounds were washed with hydrogen peroxide and saline and rebandaged daily. Wounds treated with the collagen sponge were repacked daily after wounds were washed.

The rate of dermal wound healing was estimated by photographing the wound once a week and tracing the wound perimeter using a plastic transparency directly laid over the wound. Wounds were

Table 1. 9-Day Fibroblast Cell Culture Experiments-Morphological Results

Substrate	Observation
plastic	1. superficial layers of confluent cells 2. formation of multiple cell layers of rounded or triangular shaped cells 3. formation of some ECM*
collagen sponge	1. superficial layers of confluent cells 2. elongated superficial cell layers 3. presence of ECM* throughout top 1/4 of sponge
collagen sponge + 5% hyaluronic acid	1. superficial layers of confluent cells 2. a high degree of cell infiltration throughout the sponge 3. rounded and triangular shaped cells surrounded by ECM* seen in pores
collagen sponge + 1% fibronectin	1. superficial layers of cells 2. moderate cell infiltration throughout sponge 3. fibroblasts appear to attach to and elongate along fibers of sponge

* ECM = Extracellular Matrix

photographed from a constant distance with a ruler placed next to the wound.

The wound area was calculated using a digitizing pad interfaced with an IBM PC. Wound areas were normalized by dividing by the original wound area (time=0) to yield a value of % area change.

Results And Discussion

Previous studies (Doillon et al., 1984; Doillon et al., 1985 and Doillon and Silver, 1986a) indicate that type I collagen sponges enhance repair of animal dermal wounds by organizing the spatial deposition of newly synthesized collagen and accelerating remodeling. In addition, incorporation of hyaluronic acid and fibronectin into a type I collagen sponge results in increased numbers of fibroblasts that migrate into the collagen-based sponge and consequently an increased deposition of newly synthesized collagen is observed (Doillon and Silver 1986a).

The purpose of this study is to examine the interaction of fibroblasts and epidermal cells with collagen sponges in a simple cell culture model as well as in a more complicated human dermal ulcer.

Fibroblasts grown on plastic adopt a flattened shape and synthesize some extracellular matrix (ECM)(see table 1 and figure 1). In comparison, cells grown on a collagen sponge form several confluent layers of elongated cells and deposit a large amount of ECM. However, by day 9 only about 25% of the collagen

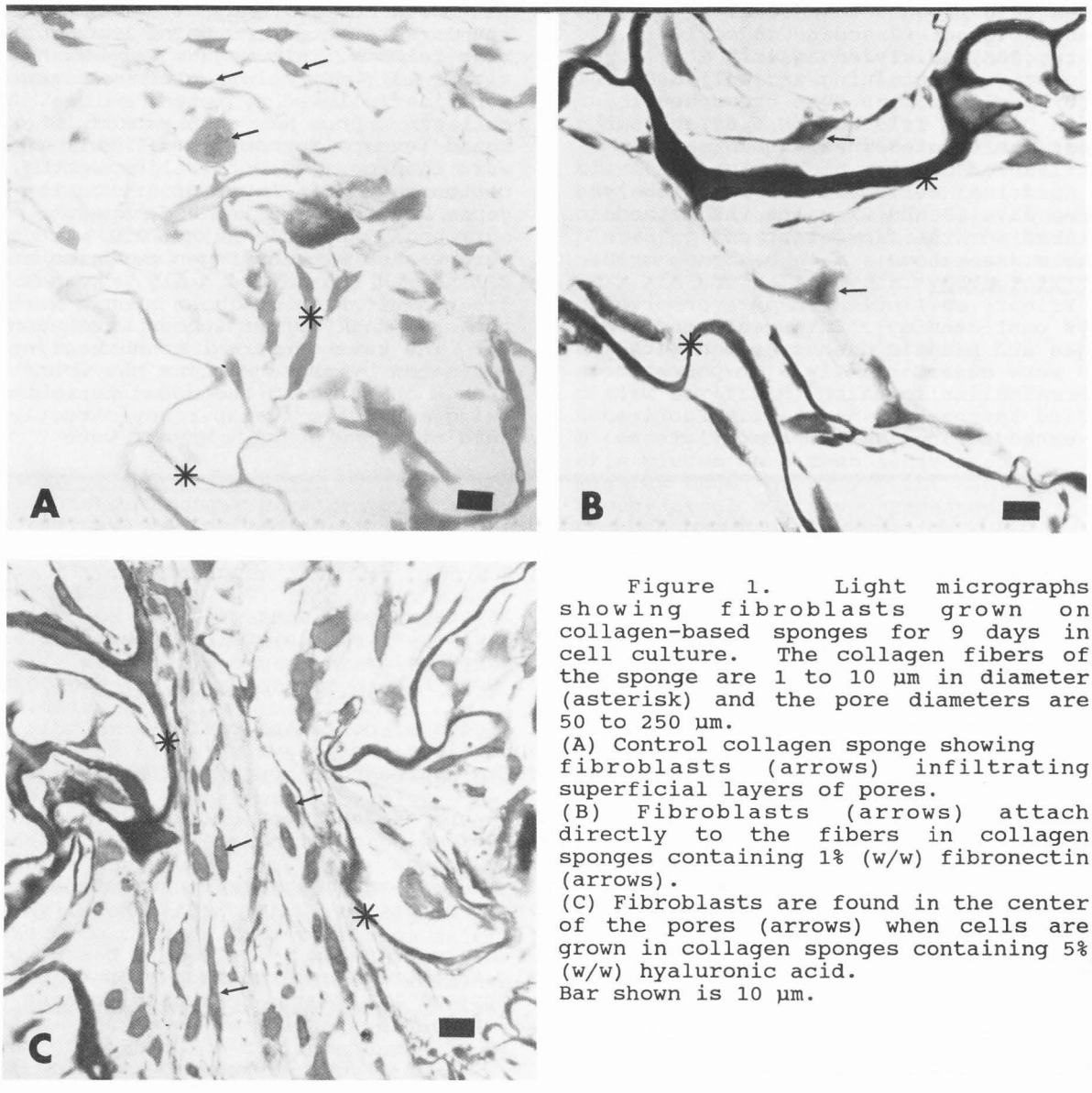

Figure 1. Light micrographs showing fibroblasts grown on collagen-based sponges for 9 days in cell culture. The collagen fibers of the sponge are 1 to 10 µm in diameter (asterisk) and the pore diameters are 50 to 250 µm.
(A) Control collagen sponge showing fibroblasts (arrows) infiltrating superficial layers of pores.
(B) Fibroblasts (arrows) attach directly to the fibers in collagen sponges containing 1% (w/w) fibronectin (arrows).
(C) Fibroblasts are found in the center of the pores (arrows) when cells are grown in collagen sponges containing 5% (w/w) hyaluronic acid.
Bar shown is 10 µm.

Table 2. [^{14}C]-Proline and [^{3}H]-Thymidine incorporation into fibroblasts in cell culture after 9 days (protein and DNA synthesis results). Cells were labeled for 4 hours and the values shown in counts per minute are means and standard errors of mean of six samples. Letters in parentheses represent groupings for statistical analysis. Significant differences at a p value <0.0001 are not observed if the means are followed by the same letter or letters (i.e. A, B, C or AB) in each column.

	[^{14}C] proline			[^{3}H] Thymidine	Proteins Synthesized
	on substrate	in medium	total		
Plastic	511±26(AB)	787±48(A)	1298	500±34(A)	type I procollagen + collagen + procollagen intermediates
collagen sponge	279±38(B)	430±37(B)	709	395±48(B)	
collagen sponge + 5% Hyaluronic Acid	638±21(A)	788±22(A)	1426	595±21(C)	
collagen sponge + 1% Fibronectin	638±21(A)	731±48(A)	1369	600±6(C)	

sponge is infiltrated with fibroblasts based on histological observation of cell density as a function of depth within the sponge. Fibroblasts grown on collagen sponges containing 5% (W/W) hyaluronic acid are found throughout the collagen sponge primarily in pores that are formed by the collagen fibers of the sponge (see figure 1C). Fibroblasts grown on collagen sponges containing 1% (W/W) fibronectin attached and elongated along the collagen fibers and infiltrated throughout the sponge (see figure 1B). In the presence of hyaluronic acid or fibronectin several superficial layers of confluent cells are seen on the surface of the collagen sponge.

Fibroblasts grown on plastic for 3 days in cell culture incorporated about five times more of [^{14}C] proline counts (collagen synthesis) than were incorporated into cells grown on a type I collagen sponge (Doillon et al., 1987). This result suggests that the presence of a collagen matrix inhibits collagen synthesis by fibroblasts. [^{3}H] thymidine incorporation (cell replication) into fibroblasts after three days in cell culture was similar for cells grown on plastic and on a collagen sponge; however, [^{3}H] thymidine incorporation was increased by the addition of 5% HA or 1% FN (Doillon et al., 1987). By day 9, these trends were still present (see table 2). Fibroblasts grown on all substrates synthesized type I procollagen, type I collagen and several processing intermediates based on [^{14}C] proline incorporation and fluorography (data not shown). The results suggest that the interaction between a porous type I collagen substrate and fibroblasts leads to inhibition of collagen production. Cell replication and collagen synthesis can be increased by addition of small amounts of HA or FN.

Collagen synthesis and cell replication were evaluated for epidermal cells grown on a type I collagen sponge (see table 3). Epidermal cells formed more layers of stratified cells when grown on collagen than when grown on plastic. Tight packing of cells on the sponge surface was observed which was not observed when these cells were grown on plastic. In the presence of a collagen

Table 3. Morphological Results of Epidermal Cell Cultures.

Substrate	Observation
plastic	1. cells stratified into a few loose layers 2. no evidence of basement membrane formation
collagen sponge	1. cells grow into several stratified layers on sponge surface 2. some cultures show evidence of a basement membrane-like structure 3. in interior, cells form clusters of cuboidal cells that contain intracellular accumulations of a lipid-like substance

Table 4. [^{14}C] Proline and [^{3}H] Thymidine incorporation into epidermal cells. Cells were labeled for 4 hours and the values shown in counts per minute are means and standard errors of mean of six samples obtained for cells grown in culture for 15 to 22 days. Letters in parentheses represent groupings for statistical analysis. Significant differences at a p value <0.0001 are not observed if the means are followed by the same letter or letters (i.e. A, B) in each column.

	[^{14}C] Proline substrate	medium	[^{3}H] Thymidine
plastic	245±42(A)	422±86(A)	880±25(A)
collagen sponge	247±58(A)	529±64(A)	525±21(B)

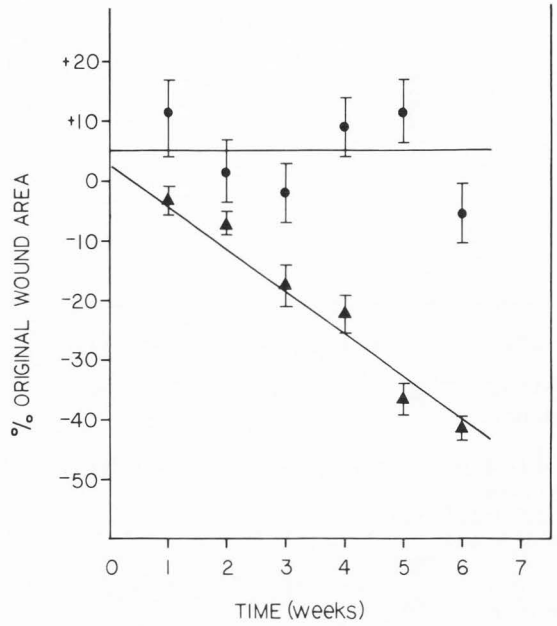

Figure 2. Plot of percent of original wound area for controls (●) and ulcers treated with a collagen-based material (▲). Both controls and collagen treated ulcers were treated daily as described in methods. Error bars show standard deviations.

sponge some cultures showed evidence of a basement membrane-like structure between the collagen sponge and a layer of basal cells. In addition, in the interior of the collagen sponge epidermal cells formed clusters that resembled primitive glands. However, as indicated by the results listed in table 4 the collagen sponge did not inhibit incorporation of [^{14}C] labeled proline into collagen. These results suggest that a type I collagen sponge seems to encourage basement membrane deposition (type IV collagen and laminin) by epidermal cells.

Results of cell culture experiments suggest that both fibroblast and epidermal cells replicate and synthesize collagen when grown on a type I collagen matrix. The collagen matrix inhibits collagen synthesis by fibroblasts but appears to be highly biocompatible with both fibroblasts and epidermal cells. The biocompatibility of this collagen matrix and the chemotactic properties of collagen-derived peptides suggests that a collagen matrix may be useful in enhancing healing of chronic wounds such decubitus ulcers.

Patients treated with a standard protocol that included daily irrigation of the wound and gauze bandaging exhibited no decrease in wound area over a 6 week period (see figures 2 and 3). In many cases the wound area remained constant for three or more months. When the protocol for treatment of these patients was modified to include packing the wound daily with a type I collagen sponge, the wound area decreased by an average of 20% in three weeks and 40% in six weeks. After three weeks of collagen treatment the wounds appeared to have increased blood supply (see figure 3b) based on their color and by six weeks significant epidermal migration is observed (see figure 3c).

Preliminary results suggest that the rate of wound healing of decubitus ulcers can be enhanced by daily treatment with a type I collagen sponge. The mechanism of the enhancement probably involves attraction of dermal and inflammatory cells into the wound area. Further clinical work is necessary to completely evaluate the effects of incorporation of HA, FN and growth factors into the collagen sponge.

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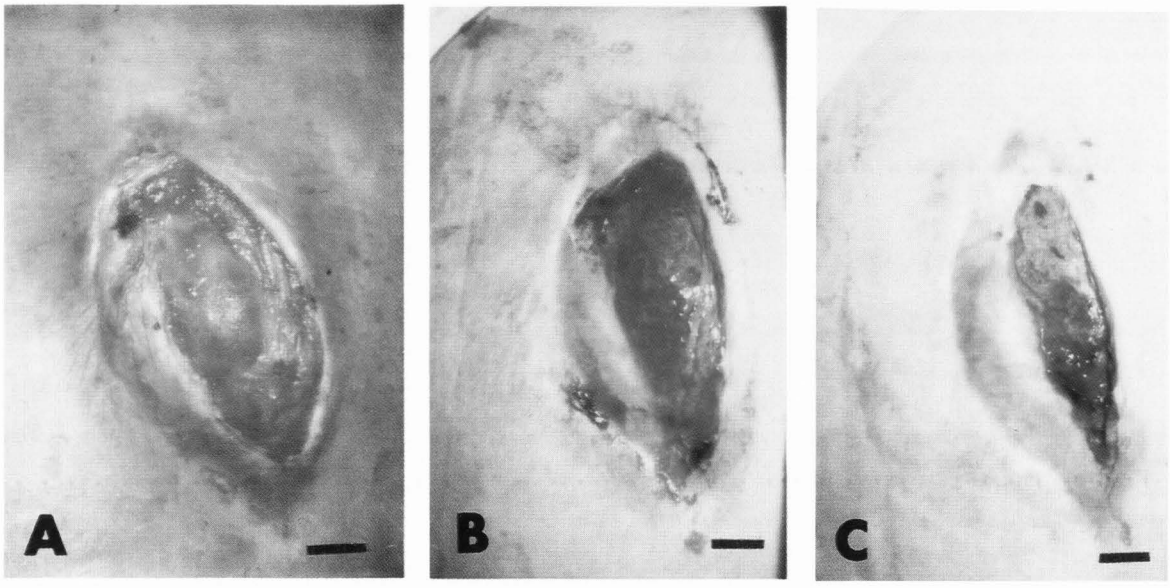
Figure 3. Photographs showing dermal ulcers at time zero (A), 3 weeks (B) and 6 weeks (C) after treatment with collagen sponges. Control wounds did not change in their appearance at 3 and 6 weeks. Both control (N=6) and collagen treated ulcers (N=6) were irrigated and rebandaged daily. Bar shown is 1 cm.


A
B
C

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