Scanning Electron Microscopy

Volume 1985
Number 2 *Part II*

Article 39

6-24-1985

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SCANNING ELECTRON MICROSCOPY/1985/II (Pages 897-903)
SEM Inc., AMF O'Hare (Chicago), IL 60666

0586-5581/85$1.00+.05

# COLLAGEN DEPOSITION DURING WOUND REPAIR

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(Paper received February 2 1985, Completed manuscript received June 24 1985)

## Abstract

Collagen fiber diameters, amount of birefringent collagen (brightness) and birefringence retardation were measured in implanted collagen-based sponges containing hyaluronic acid (HA) and fibronectin (FN). In the presence of HA and FN, increased number of fibroblasts and brightness were observed 6 days after wounding. Increased brightness in the presence of HA and FN reflected increased deposition of oriented collagen fibers. From days 9 to 12, increased fiber diameters were similar in implanted collagen-based sponges with or without HA and FN. Increased birefringence retardation in sponges containing HA and FN was consistent with increased packing density of collagen fibers observed by scanning electron microscopy.

Our results suggest that HA and FN are effective in promoting fibroblast movement into a collagen sponge and deposition of collagen fibers during the early phases of wound healing. Use of a collagen-based sponge containing HA and FN may enhance collagen deposition in situations where healing is compromised as in the case of dermal ulcers.

KEY WORDS: Scanning Electron Microscopy, Polarized Light Microscopy, Collagen-based sponge, Collagen, Fibronectin, Hyaluronic Acid, Fibroblasts, Wound Healing.

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## Introduction

Repair of large open wounds involves the formation of granulation tissue which is initially characterized by the presence of thin unorganized collagen fibers (Williams, 1970, Ross and Benditt, 1961) associated with non-collagenous interfibrillar material (Kischer, 1974; Kischer and Shetlar, 1974). Later, during the remodeling phase large collagen fibers composed of thicker fibrils are observed. During the remodeling phase of wound closure, the thickness of the collagen fibers is observed to increase but never reaches that observed in normal dermis (Levenson et al., 1965; Gillman, 1968; Knapp et al., 1977; Williams, 1970). The tensile strength of the repair of dermal wounds is related to the amount of collagen (Levenson et al., 1965) and never reaches that of normal skin. It has recently been demonstrated that increased organization of collagen in fibers is related to increased wound tensile strength (Doillon et al., unpublished results).

In the case of immature healing, excessive remodeling results in hypertrophic scar tissue which is characterized by whorls containing randomly deposited collagen that is eventually aligned parallel to flexion lines of the scar (Linares et al., 1972). Transmission electron microscopy shows slightly irregular to ovoid fibrils with diameters less than normal (Kischer, 1974) and an excess of glycosaminoglycans associated with collagen fibrils (Linares et al., 1972, and 1973). By scanning electron microscopy (SEM), collagen fibers are rarely found and appear as a homogeneous mass of material in hypertrophic scar tissue (Kischer, 1974); however, when tension is applied, collagen fibrils can be identified by SEM (Kischer and Shetlar, 1974). The apparently reduced extensibility of hypertrophic scar tissue (Dunn et al., 1985) is a result of partial pre-alignment of the collagen fibers (Kischer and Shetlar, 1974).

Collagen-based materials have been used as a scaffold for tissue ingrowth to improve wound healing. Type I collagen supports fibroblast attachment and growth in vitro (Kleinman et al., 1981) and in vivo (Yannas et al., 1982) and is associated with the spatial deposition of newly formed collagen fibers (Doillon et al., 1984).

Collagen fibers formed in wounds in the presence of collagen-based materials are oriented, uncrimped and have larger diameters than tissue formed in the absence of the sponge, as observed by SEM.

Other components of the extracellular matrix induce cell mobility and attachment in vitro. Fibronectin is known to increase chemoattraction and spreading of fibroblasts in vitro (Gauss-Muller et al., 1980, Kleinman et al., 1981), and is found in large amounts 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 skin development, and is associated with cell movement and differentiation (Fisher and Solursh, 1977; Toole, 1982; Kujawa and Tepperman, 1983). It appears as one of the first extracellular matrix macromolecules synthesized during wound healing (Alexander and Donoff, 1980). Both fibronectin and hyaluronic acid may play a role in collagen fibrillogenesis in vivo.

Using SEM and specific staining techniques, the present study involves the analysis of collagen deposition and remodeling that occurs in a collagen-based sponge in the presence of fibronectin and hyaluronic acid. The goal of these studies is to determine if these factors enhance wound healing.

## Materials and Methods

### Materials

Collagen-based sponges are made from reconstituted type I collagen from bovine hide after dispersion in HCl solution at 1% w/v. After freeze drying the dispersion, a sponge is obtained with pores that interconnect to form channels as previously described (Doillon et al., 1984). Collagen sponge biodegradation is controlled by crosslinking; the result of cross-linking is peptide bond formation as previously described (Weadock et al., 1984).

Collagen-based sponges can be used as a carrier of other connective tissue macromolecules. Fibronectin (FN) extracted from fresh bovine blood as described by Ruoslahti et al. (1982) and hyaluronic acid (HA) (grade III, potassium salt, from Sigma Chemical Co.) extracted from human umbilical cord were incorporated into collagen sponges in this study. A HCl solution (pH 3.0) containing FN or HA was progressively mixed with the collagen during dispersion. Ratios of 1:99 and 1:19 of FN or HA to collagen (w/w) were tested. Other collagen sponges were made in the same way with both 1% FN and 1% HA; 1% FN and 5% HA; or 5% FN and 5% HA. The top surface of all specimens was coated with a thin layer of silicone and sterilized by gamma irradiation at 2.5 M rads for in vivo studies.

### Animal Studies

A square of 2 by 2 centimeters was excised down to the panniculus carnosus on the back skin of guinea pigs (one wound on each guinea pig). A collagen-based sponge cut to the same dimension as the wound, was sutured into place as previously described (Doillon et al., 1984). Animals were sacrificed at days 6, 9 and 12 post implantation. For each period of time and each collagen sponge tested, 6 animals were used.

### Histology and Scanning Electron Microscopy Preparations

At sacrifice, two pieces of wound tissue containing collagen sponges were excised including pieces of normal skin at both edges. For histology, one specimen was fixed in Carson's buffered formalin, and processed using the routine paraffin embedding. Tissue sections were stained with Hematoxylin and Eosin (H & E), and picro-sirius red (Junqueira et al., 1979).

For SEM, the other piece was fixed in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 3 days, and washed in cacodylate buffer for 5 days. Specimens were dehydrated through a series of ethanol baths and acetone, and critically point dried using $CO_2$. After critical point drying, specimens were fractured through the wound area using forceps, then coated with gold in a sputter coater for 3 minutes at -30°C, and examined in an Amray SEM model 1400 (Bedford, Mass.) at 30 kV.

### Qualitative Data Analysis

H & E stained tissue sections were observed using light microscopy and the frequency and infiltration of tissue ingrowth into the collagen sponge were qualitatively determined. Picro-sirius stained tissue sections were observed under polarized light. In the presence of this stain the birefringence of the newly formed collagen was increased as described by Junqueira et al. (1979). SEM observations were used to follow the deposition of extracellular collagen as well as collagen fibril assembly into fibers.

### Quantitative Data Analysis

Numbers of fibroblasts were determined on H & E stained tissue sections, using micrographs representing approximatively 0.5 $mm^2$ of each tissue section from each animal. Cells were considered as fibroblasts based on elongated shape with an elongated nucleus. Cells of at least 3 fields of view were counted on a section through the middle of the wound for each animal.

Brightness of picro-sirius stained tissue sections was used to follow the deposition of collagen in fibers. The degree of brightness was quantitatively determined using a computerized image analysis system. Briefly, a video camera (Venus TV-2M) mounted on a Leitz polarizing light microscope (12 Pol), was controlled by a Hamamatsu Video Frame Memory (C1440-02, Hamamatsu Systems Inc., Waltham Mass.). The frame memory was interfaced with a Digital Equipment Corporation PDP-11/03 minicomputer via a RS-232 serial interface. Intensity calibration of the system was done for each histologic slide (to correct for differences in section thickness) by using normal dermis as the "exposure setting". The area for analysis was digitized and integrated using a window. The sum of the intensities of pixels was then divided by the total number of pixels in the window to give the average pixel intensity. The average pixel intensity was divided by the maximum pixel intensity and multiplied by 100 to give what was

termed the % brightness.

Using polarized light, and a Brace-Kohler compensator ($\lambda$/10 at 546 nm), birefringence retardation was measured on picro-sirius red stained tissue sections by determination of the compensator extinction angle using a video-camera mounted on the microscope. After zeroing the compensator, the fiber to be measured was rotated to a position of maximum extinction, and then oriented diagonally by turning the stage 45°. The determined angle of rotation ($\omega o$) was measured by turning the compensator drum until maximum extinction of the fiber was observed. The phase difference ($\Gamma$) which is related to the birefringence retardation, was approximated with the following formula.

$$\Gamma = \Gamma_o \sin(2\omega o) \qquad (1)$$

where $\Gamma_o$ is the polychromatic light calibration value of 71.84 nm.

Collagen fiber diameters were measured from 1) light micrographs taken at 600x on picro-sirius red stained tissue sections and 2) from scanning electron micrographs taken at magnifications between 700x and 10,000x.

Statistical Analysis

Each data point obtained represented the mean of samples ± standard error of the mean; a two-way analysis of variance (Anova) was used to statistically test differences between treatments for each animal.

## Results

Microscopic Observations

Histological sections showed new tissue ingrowth into the collagen-based sponges, but uniform ingrowth into the sponge was not found in all the samples. Good tissue ingrowth was defined as a tissue rich in fibroblasts (see Table 1) and dense in newly synthesized collagen. Good ingrowth was found when the collagen sponge was associated with 1% FN and 1% HA and with a collagen sponge in the presence of 5% HA at days 6, 9, and 12 and with a collagen sponge in the presence of 1% FN at days 9 and 12.

Other studies (Doillon and Silver, unpublished results) showed that large amounts of FN alone (5%), small and large amounts of HA (1% and 10%), and increased amounts of both HA and FN resulted in less than optimal tissue ingrowth within the collagen sponge. In this present study, the tissue ingrowth was analyzed in a control collagen sponge and in a collagen sponge in the presence of FN and HA at 1% ("HAFN collagen sponge").

Picro-sirius red staining showed orientation of the newly formed uncrimped collagen fibers parallel to the collagen bundles of the sponge as previously described (Doillon et al., 1984). This orientation of densely stained fibers was more frequently observed within the collagen sponge containing HA and FN and the brightness was greater than in the control group (see Figure 1). The same level of brightness was observed in the presence of 1% FN or 5% HA particularly at day 9.

SEM observations showed different forms of collagen deposition within the collagen sponge. Basically there were 3 types of morphologic observations.

1) "Spider-like" morphology where collagen fibrils or thin fibers were spread in all directions; collagen fibers were composed of 2 or 3 collagen fibrils (see Figure 2a) and bridged the inner surface of the pores and channels of the sponge. This morphology was frequently seen at day 6 post implantation either in the control group or in the HAFN collagen sponge.

2) Oriented fibrils aggregated into elongated fibers (see Figure 2b) were observed at days 9 and 12 in control collagen sponges and at days 6, 9, and 12 in the presence of FN and HA.

3) Collagen fibrils densely packed into fibers were observed at days 9 and 12 in the presence of 5% HA and with the HAFN collagen sponge (see Figure 2c). This behavior was particularly apparent at day 12 (see Figure 2c). Measurements of collagen fiber diameter were made on compact fibers as seen in Figure 2c.

Quantitative Results

The number of fibroblasts inside the sponge at day 6 was similar for all the sponges, except

Table 1: Results of tissue ingrowth within different collagen sponges. Means and standard errors of numbers of fibroblasts (No fibrob.), % of brightness, fiber diameter ($\varnothing$), birefringence retardation (bir. ret) are represented; fiber diameters are determined by scanning electron microscopy (SEM) and by light microscopy (LM). The birefringence retardation per unit fiber thickness is also represented.

| | No fibrob. / 0.5 mm$^2$ | brightness % | fiber $\varnothing$ (SEM) µm | fiber $\varnothing$ (LM) µm | bir. ret. nm | bir.ret.x10$^3$ / fiber $\varnothing$ |
|---|---|---|---|---|---|---|
| Control SP. | | | | | | |
| day 6 | 69.2±6.9 | 6.9 ± 0.8 | -- | -- | 9.0 ± 1.8 | -- |
| day 9 | 70.4±5.4 | 7.7 ± 0.6 | 2.5 ± 0.5 | 2.7 ± 0.2 | 21.9 ± 1.3 | 8.4 |
| day 12 | 68.6±6.4 | 7.6 ± 0.7 | 3.0 ± 0.6 | 3.2 ± 0.2 | 26.4 ± 1.8 | 8.5 |
| HAFN Coll. SP. | | | | | | |
| day 6 | 97.1±6.9 | 7.9 ± 0.8 | -- | -- | 17.9 ± 1.4 | -- |
| day 9 | 89.0±6.0 | 8.8 ± 0.7 | 2.4 ± 0.5 | 2.1 ± 0.2 | 22.7 ± 1.3 | 10.0 |
| day 12 | 97.1±5.4 | 10.8 ± 0.6 | 3.7 ± 0.6 | 3.5 ± 0.2 | 28.2 ± 1.2 | 7.8 |

in the HAFN collagen sponge; at days 6, 9 and 12 it was significantly ($p< 0.0001$) higher than in the control group. The number of fibroblasts was unchanged from days 6 to 12 (Table 1).

The brightness inside the sponge was similar at day 6 for all the sponges. However, the brightness was found significantly ($p< 0.005$) to be higher in the HAFN collagen sponge. A significantly increased amount of birefringent collagen ($p< 0.01$) was observed in the control group as well as in the HAFN collagen sponge between days 6 and 12 (Table 1).

Using SEM, observation of fused collagen fibrils into compact fibers was distinct by day 9. However, in a few cases, in the presence of HA and/or FN, collagen fibrils were assembled into thin fibers at day 6. From days 9 to 12, fiber thickness increased without any significant difference between the control group and the HAFN collagen sponge. These results were also observed in the presence of 1% FN or 5% HA alone, but the number of good specimens for SEM observations was not sufficient for statistical analysis.

Using micrographs of picro-sirius stained tissue sections, collagen fiber diameters increased significantly ($p< 0.005$) from days 9 to 12, but the sponges containing HA and FN did not modify fiber diameters (Table 1).

Birefringence retardation was higher in the collagen sponge containing HA and FN than in the control group ($p> 0.006$), and increased as a function of time ($p< 0.0001$). From days 6 to 9, the birefringence retardation showed a sharp increase. In the presence of 5% HA, a high birefringence retardation was noted, similar to that of the collagen sponge associated with HA and FN, particularly at day 9 (data not shown). Birefringence retardation per unit thickness decreased from days 9 to 12 in the presence of HAFN collagen sponge (Table 1).

## Discussion

These studies were conducted in order to determine whether factors that are known to be associated with fibroblast movement and attachment can enhance wound healing in a collagen-based sponge. We have recently shown (Doillon et al., unpublished results) that the tensile strength of wounds that are sutured closed is directly related to collagen fiber diameter. In addition, in the presence of HA and FN the morphology of a collagen-based sponge is modified such that interchannel connections are more numerous (Doillon and Silver, unpublished results) and is associated with increased tissue ingrowth. Therefore it is important to determine if HA and FN can enhance wound healing by increasing collagen fiber diameters.

The results of this study indicate that fibroblast numbers and collagen deposition during tissue ingrowth can be increased in the presence of HA and FN and is associated with the organization of collagen fibrils into fibers. The presence of HA and FN, factors that are known to be chemotactic to fibroblasts, increased the number of fibroblasts observed within composite collagen-based sponges. Delpech and Delpech (1984) described a hyaluronic acid-binding glycoprotein during embryonic development which can be bound to HA, FN and collagen (Turley and Moore, 1984). This glycoprotein appeared in embryonic extracellular matrix and may play an important role in migration and differentiation of mesenchymal cells, and may provide the basis of the HA-FN complex associated with collagen type I used in our studies. Associated with this increase in fibroblast number was an increase in the % brightness and birefringence retardation at day 6. The increased brightness is probably a result of increased amounts of collagen synthesized while increased birefringence retardation may in part reflect the increased organization observed particularly by SEM. By day 9 little difference was observed in these parameters in the presence or absence of HA and FN suggesting that the effect of these macromolecules is primarily during the proliferative and early synthetic phases of wound healing. In the presence of HAFN, birefringence retardation increased while fiber diameters were unchanged, this observation suggests that collagen fibrils may have had a higher packing density in the presence of HA and FN. Indeed, in the presence of HAFN sponge, the birefringence retardation per unit thickness which is related to the packing density was observed to decrease between days 9 and 12. Since type I collagen fibers have been observed to bind less sirius red than type III containing fibers (Junqueira et al., 1979) this result indicates that the increase in packing density of newly synthesized collagen fibers with increased diameters is associated with decreased type III collagen content.

The increased number and size of collagen fibers that appear to be deposited at day 6 in the presence of HA and FN could have been a result of changes in the morphology of the pores and channels in the collagen sponge. Although increased numbers of interchannel connections were observed in the presence of HA and FN (Doillon and Silver, unpublished results) the size of each pore or channel was observed to be relatively constant (between 50 and 300 μm) and independent of the sponge composition. Increased collagen fiber diameters observed with time post-wounding in all the sponges is consistent with other observations made during the remodeling phase of wound healing (Williams, 1970) and during tendon development (McBride et al., 1985).

Increased birefringence at day 6 in the presence of FN and HA may be associated with changes in the types of glycosaminoglycans synthesized by the fibroblasts within the sponge. Alexander and Donoff (1980) have shown that increased levels of hyaluronate and chondroitin sulfate proteoglycan appeared during the early

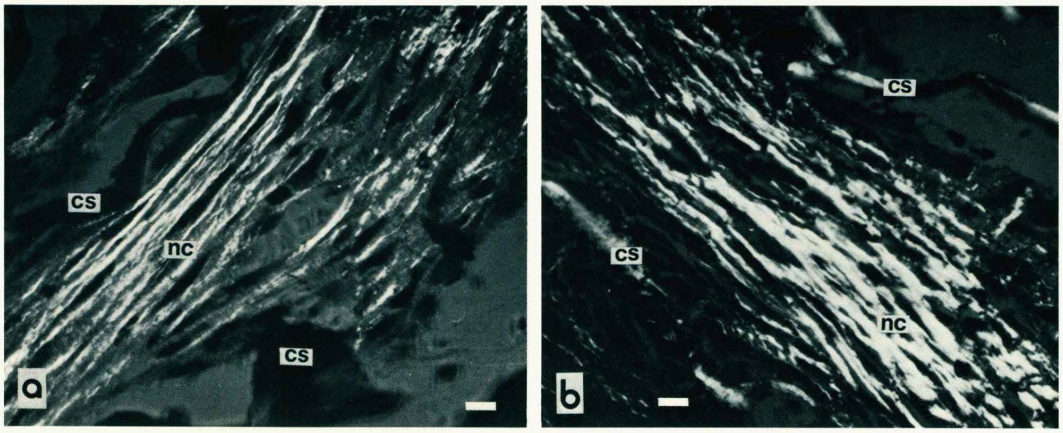


Figure 1: Micrographs of picro-sirius red stained tissue sections observed under polarized light. The birefringence of the newly formed collagen (NC) is oriented following the bundles of the collagen sponge (CS). At day 9 post implantation, the birefringence is less in the control collagen sponge (a) than in the collagen sponge associated with hyaluronic acid and fibronectin (b). Bar = 10 µm.

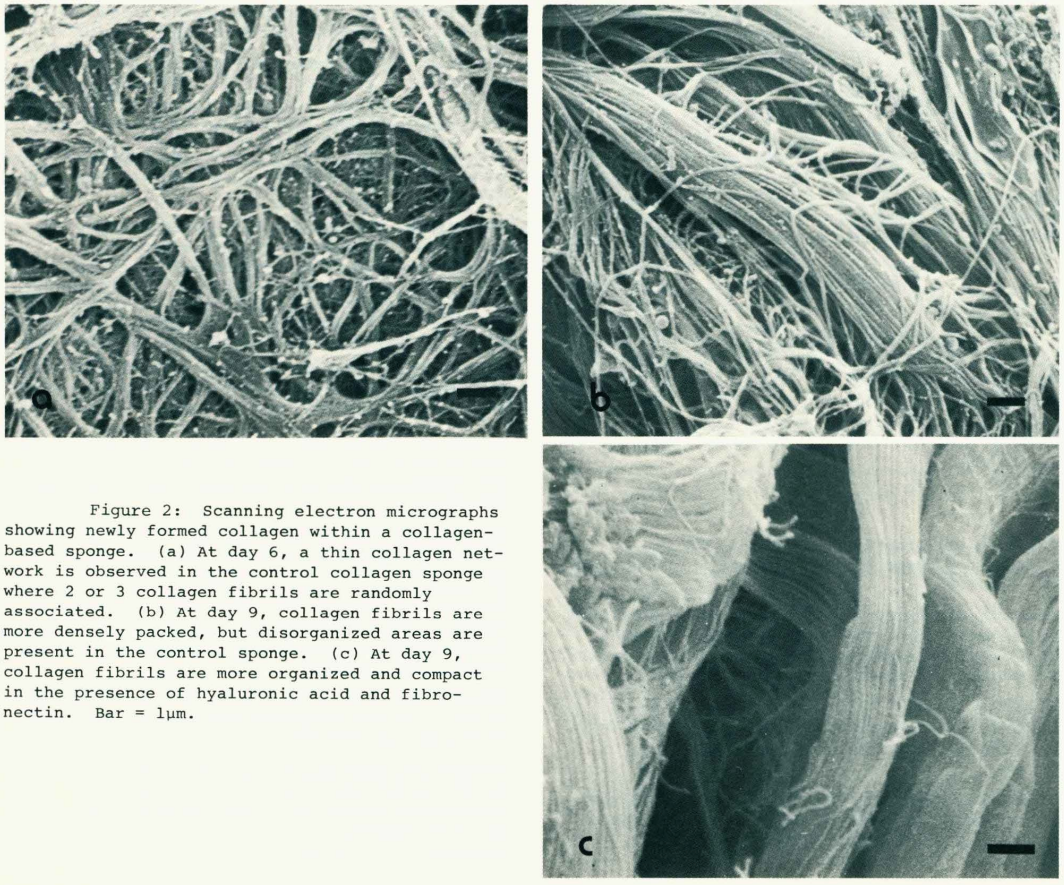


Figure 2: Scanning electron micrographs showing newly formed collagen within a collagen-based sponge. (a) At day 6, a thin collagen network is observed in the control collagen sponge where 2 or 3 collagen fibrils are randomly associated. (b) At day 9, collagen fibrils are more densely packed, but disorganized areas are present in the control sponge. (c) At day 9, collagen fibrils are more organized and compact in the presence of hyaluronic acid and fibronectin. Bar = 1µm.

phase of wound healing. Scott (1980; 1984) has demonstrated an increase and then a decrease in these glycosaminoglycans and increased dermatan sulfate proteoglycan levels during the period in which collagen fiber diameters increased during tendon development. The presence of hyaluronate in the collagen sponge may depress HA synthesis and promote dermatan sulfate synthesis thereby accelerating the fusion of collagen fibrils into bundles that make up collagen fibers (Birk and Trelstad, 1984; McBride et al., 1985).

In the presence of a collagen-based material, deposition and orientation of collagen is similar to that observed in sutured wounds, except that collagen fiber diameters are thicker in the presence of the collagen-based material at earlier times than in sutured wounds. SEM observations of sutured wounds on rats (Levenson et al., 1965) and guinea pigs (Doillon et al., unpublished results) showed average collagen fiber diameters of 3 μm ± 1 at day 14 post wounding. Wound healing within a collagen-based sponge is similar to that within wounds that are sutured closed except the time lag for collagen fiber deposition observed in the absence of a collagen-based sponge is greater.

Our results indicate that in the presence of HA and FN the time lag for deposition of newly assembled collagen fibers within the collagen sponge is decreased. Increased birefringence in the presence of HA and FN reflects increased collagen deposition. We conclude that HA and FN enhance collagen deposition during the early phase of wound repair by attraction of increased numbers of fibroblasts. Use of a collagen-based sponge containing HA and FN may enhance collagen deposition in situations where healing is compromised as in the case of dermal ulcers.

## Acknowledgements

The authors would like to acknowledge the technical assistance of Miss Rita Hahn and Miss Joy Caspers, and thank Mr. Edward Bender for help in image analyses and Dr. Ronald Cody for help in the statistical analyses. We would like to thank Mrs. Maria Ayash for typing the manuscript.

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## Discussion with Reviewers

R.C. Hallowes: Why were the specimens not cryo-fractured whilst in absolute ethanol so that the collagen fibres are fully supported rather than totally unsupported as after CPD?
Authors: We have tried both of these techniques and it appears that distinct collagen fibrils and fibers were observed after the CPD. We believe that non-collagenous proteins are discarded during SEM preparations and this indirectly improves observation of collagen.

M. Chvapil: Description of collagen sponge preparation is incomplete. How was it cross-linked, which agent, how did it change sponge characteristics? This is a critical step and information, as cross-linking with aldehydes is cytotoxic. Thin layer of silicone - how permeable to vapors?
Authors: The crosslinking procedure and modifications of sponge characteristics has been already described in: Weadock et al., 1984 by formation of synthetic peptides bonds using carbodiimide and dehydration. Crosslinking agents are washed extensively with deionized water and in vitro and in vivo studies show no cytotoxicity.

The thickness of the silicone layer varies between 0.6 and 0.8 mm, the water vapor transmission is about 500 g of water vapor/$m^2$ per 24 hours.

M. Chvapil: The determination of the strength in wounds relates to breaking strength, not tensile strength, which is a highly defined parameter involving the cross-section area. From the pictures enclosed it is not clear how fibroblasts were identified in light microscopy. If only by shape, it needs to be stressed. Do the authors have data on how long HA or FN stayed within the sponge, diffusion out..
Authors; Although, it is difficult to measure the exact cross sectional area at the failure which is required to determine the tensile strength, the tensile strength is an engineering parameter. Calculation of the tensile strength allows comparison between repair tissue and normal skin which have different cross sectional areas.

Fibroblasts were identified by their elongated shape and counted on H & E stained tissue sections. No experiments were performed to determine rates of loss of HA or FN from sponges.

C.W. Kischer: Fiber diameter probably refers to large aggregates - not individual collagen fibrils. Since the authors admit that there were not enough good specimens for statistical analysis one wonders what value they are at all. What really would reflect "age" of healing would be fibril diameter as measured by TEM as per Kischer (1974 text reference) and Hendrix et al. (Invest. Opthalmol. 22:359, 1982).
Authors; Fiber diameters were measured when at least 2 collagen fibrils were aggregated together to form a fiber.

There were not enough good specimens for statistical analysis when either HA or FN were separately associated with the collagen sponge. However, in the presence of both HA and FN together at low concentration, the number of specimens were sufficient for statistical analysis.

It is true that fibril diameters increase with time, but we have found that fiber diameters are increased as a function of time and the tensile strength reflects the presence of fibers as observed within the network of collagen fibers in normal dermis.

C.W. Kischer: If a 2 x 2 cm piece of skin was excised, was the sponge not then covered? If so, was it kept moist? And how?
Authors; Before sterilization and implantation, the sponge is covered by a thin layer of silicone which controls fluid flux as described by Yannas et al. (1982). A 2 x 2 cm piece of sponge is sutured in place and the silicone is on the exterior surface.