

## Collagen Metabolism During Healing of Lacerated Rabbit Corneas

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We previously demonstrated that there are two waves of increased collagen synthesis following corneal laceration in rabbits. In the present study, we have examined whether increases in collagen synthesis and degradation result from increased amounts of mRNAs for collagen and collagenase, respectively. Rabbits were anesthetized by combined administration of ketamine (intramuscular) and pentobarbital (intravenous). A penetrating 8-mm incision was made at the center of each cornea. The lacerated corneas were allowed to heal for 0–49 days. The rabbits were then killed and the corneas excised. The total RNA was extracted from the tissue and subjected to slot-blot hybridization using <sup>32</sup>P-labeled  $\alpha 1(I)$  cDNA. The results indicate that there is a two-phase increase in the amount of  $\alpha 1(I)$  mRNA in injured corneas and that the collagenase mRNA is elevated at most times throughout the healing period. However, the increase in collagenase mRNA may not fully account for the accelerated collagen degradation during corneal wound-healing. Thus, we propose that cells in the wound area may be directly involved in collagen degradation by phagocytosis. To examine our hypothesis, we cultured injured rabbit corneas in the presence or absence of leupeptin, a proteinase inhibitor. The tissues were then examined by electron microscopy. In the presence of leupeptin, lysosomes within fibroblasts or fibroblast-like cells in the wound area of the lacerated corneas healed for 2 and 3 weeks, contain collagen fibrils. In the absence of leupeptin no identifiable collagen was seen in the lysosomes. These observations indicate that fibroblast-like cells in the wound area may directly phagocytose collagen fibrils during tissue remodeling, in addition to secreting collagenase into the extracellular matrix.

*Key words:* collagen metabolism; collagenase; corneal wound-healing mRNA; keratocytes; phagocytosis.

### 1. Introduction

Healing of corneal wounds frequently results in the formation of scar tissue which is less transparent than the original cornea and often impairs vision. The scar tissue is mainly composed of collagen fibrils which are not organized in orthogonal lamella like those found in normal cornea stroma (Matsuda and Smelser, 1973). The reasons for scar tissue formation in injured corneas remain largely unknown.

At least 13 collagen types have been identified in vertebrates (Gordon, Gerecke and Olsen, 1987; Mayne and Burgeson, 1987; Tikka et al., 1988) and most of them have been found in the cornea during early developmental stages and/or in normal adult tissues (Hay et al., 1979; Cintron, Hong and Kublin, 1981; Lee and Davison, 1984; Linsenmayer et al., 1986; Zimmermann et al., 1986; Svoboda et al., 1988). It is known that type I collagen is the major collagenous component in the cornea (Kao, Mai and Lee-Chou, 1982). In addition, many studies have revealed dynamic changes in the metabolism of collagenous components during corneal wound-healing (Cintron et al., 1978; Cionni et al., 1986; Cintron and Hong, 1988; Cintron et al., 1988). For example, Cintron et al. (1978) demonstrated that the pattern of collagen intramolecular crosslinking of the corneal scar tissue

differs from that of normal tissues, even after the corneal wounds have been allowed to heal for up to 50 weeks. Recently, Cintron and Hong (1988) and Cintron et al. (1988) examined the synthesis of different collagens during corneal wound-healing. Their results indicate that the synthesis of different collagen types during wound-healing is similar to that of embryonic development. However, the wound-healing process does not repeat precisely the process of corneal development and thus fails to regenerate a transparent tissue. We previously examined the synthesis and the degradation of collagen in lacerated rabbit corneas (Cionni et al., 1986) and demonstrated that there are two waves of increased collagen synthesis during corneal wound-healing. In addition, the kinetics of the degradation of newly synthesized collagen varies during different periods of corneal wound-healing.

In the present study, we investigated the change in the mRNAs for type I collagen and collagenase to further elucidate the metabolism of collagen in lacerated rabbit corneas. Using light and electron microscopy, we have examined the possible role of keratocytes, the fibroblast-like corneal stroma cell, in collagen metabolism.

## 2. Materials and Methods

### Materials

Adult albino rabbits of either sex weighing 3–4 kg were obtained from Clerco Research Farm (Cincinnati, OH). [<sup>32</sup>P]dCTP was obtained from New England Nuclear-Du Pont Corporation (Boston, MA). Chicken  $\alpha 1(I)$  collagen cDNA was a gift from Dr Ninomiya (Fuller and Boedtker, 1981). The  $\alpha 1(I)$  cDNA used in the present studies is 800 bp long and encodes the C-propeptide of pro  $\alpha 1(I)$  chain. The poly A tail of the cDNA was removed by Hinf I digestion. cDNA of rabbit synovial fibroblast was a gift from Dr Brinckerhoff (Gross et al., 1984; Fini et al., 1987) and mouse 28S ribosomal cDNA was a gift from Dr Arnheim (1979). Leupeptin was purchased from Sigma (St. Louis, MO).

### Animal Experiment

Rabbits were anesthetized by combined administration of ketamine (intramuscular) and sodium pentobarbital (intravenous). An 8-mm linear penetrating incision was made in the center of the cornea with a microsurgical scalpel as previously described (Katakami et al., 1986). A drop of 0.5% proparacaine was applied. The wounds were allowed to heal for various periods of time, 0–49 days. At the times indicated, the rabbits were killed with an overdose of sodium pentobarbital, and the corneas were excised. The total RNA was extracted from the excised tissues as described below. Some of the corneas were further incubated in Dulbecco's medium in the presence or absence of leupeptin (65  $\mu$ M) for 4 hr prior to fixation for histology (Everts, Beertsen and Tigchelaar-Gulter, 1985).

### Extraction of Total RNA

Five-millimeter strips of the central portions of the corneas, including the linear wounds, were dissected from the injured corneas and quickly frozen in liquid nitrogen. Total RNAs were then extracted from wounded corneal tissues, using an acid-guanidium-thiocyanate-phenol-chloroform procedure as described by Chomczynski and Sacchi (1987).

### Slot-blot Hybridization

The total RNAs were then slot-blotted onto Nytran<sup>®</sup> or nitrocellulose membranes using the procedure recommended by the manufacturer (Schleicher and Schuell, NH). To construct a standard curve, samples of serially diluted cDNA were also blotted on to a Nytran<sup>®</sup> or nitrocellulose membrane. The samples were hybridized with <sup>32</sup>P-labeled cDNA at 41°C overnight. The membranes were then washed twice with 1 × SSC, 0.1% SDS and twice with 0.2 × SSC, 0.1% SDS at 41°C as described by Maniatis, Fritsch and Sambrook (1982). Autoradiograms were then prepared. The amount of mRNA in samples was

estimated with a densitometer (Helena Quick Scan, TX) and the amount of total RNA was estimated by hybridization of <sup>32</sup>P-cDNA of mouse 28S rRNA. The amount of mRNA in each sample was calculated as a ratio of the amount of 28S rRNA in samples.

### Histology

Each cornea was first rinsed in PBS, then immersed in a fixative solution consisting of 2% (w/v) paraformaldehyde, 2.0% (by volume) glutaraldehyde, 0.005% (w/v) calcium chloride in 0.1 M sodium cacodylate buffer, pH 7.3. The tissue was cut lengthwise along the wound into 2-mm wide strips, then sliced into 1-mm slices across the wound to form approximately 1 × 2-mm blocks. The tissue blocks were fixed at room temperature for 2 hr and then at 4°C for 2 more days. After washing in 0.1 M sodium cacodylate buffer for 1 hr the tissue was post-fixed with 1% OsO<sub>4</sub> in the same buffer for 2 hr, and dehydrated through an ethanol series. After propylene xide treatment, the tissue was infiltrated and embedded in Epon 812 (Luft, 1961). Semi-thin sections of 1- $\mu$ m thickness were cut perpendicular to the wound and stained with 0.5% Toluidine Blue. Ultra-thin sections were cut with a diamond knife, stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963), and examined with a Philips-301 electron microscope.

## 3. Results

### Amounts of $\alpha 1(I)$ mRNA in Lacerated Corneas

Using [<sup>14</sup>C]proline incorporation, we have previously demonstrated that there are two waves of increased collagen synthesis during healing of lacerated corneas (Cionni et al., 1986). In order to examine whether the increase in collagen synthesis results from an increase of mRNA, we measured the amount of  $\alpha 1(I)$  mRNA as described in Methods. Figure 1 shows that there are two waves in the increase of  $\alpha 1(I)$  mRNA in the lacerated corneas which have been allowed to heal for 0–49 days. This result parallels our previous observation in which collagen synthesis was measured by quantitating the amount of [<sup>14</sup>C]hydroxyproline synthesized by the lacerated rabbit corneas. The initial decrease in  $\alpha 1(I)$  mRNA 2 days after laceration corresponds to decreased [<sup>14</sup>C]hydroxyproline synthesis by the lacerated tissues at that time. The increased collagen(I) synthesis by lacerated rabbit corneas can be explained in part by the increased amounts of  $\alpha 1(I)$  mRNA (about 25% above control, day 28 after injury). However, the relatively low elevation of  $\alpha 1(I)$  mRNA cannot fully account for the observed 2–3-fold increases in the synthesis of [<sup>14</sup>C]hydroxyproline (Cionni et al., 1986).

### Amounts of Collagenase mRNA in Lacerated Corneas

Our previous studies suggest that active remodeling of the collagen matrix takes place during the second

## COLLAGEN METABOLISM IN LACERATED CORNEAS

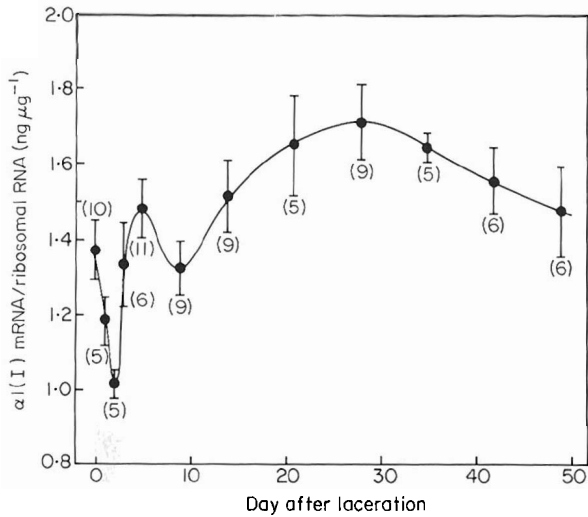


FIG. 1. Amounts of  $\alpha 1(I)$  mRNA in lacerated corneas. An 8-mm penetrating incision was made in the center of rabbit corneas. The wounds were allowed to heal for various periods of time. At the times indicated, the animals were killed and corneas were excised. The central portion (2.5 mm from either side of the wound) was cut off from the cornea button. Total RNA was extracted (see Method). The RNA samples were serially diluted and slot-blotted to nitrocellulose or Nitran® membranes and hybridized with  $^{32}\text{P}$ -labeled cDNA of chicken  $\alpha 1(I)$  chain. Autoradiograms were prepared. The membranes were then hybridized to  $^{32}\text{P}$ -labeled cDNA of mouse 28S rRNA and autoradiograms were prepared. The amounts of  $\alpha 1(I)$  mRNA and rRNA were then estimated by scanning the autoradiograms with a densitometer (Helena). The values shown are the average of triplicate assays of each of a number of corneas (number in parentheses). The bars indicate the standard deviation from the average value.

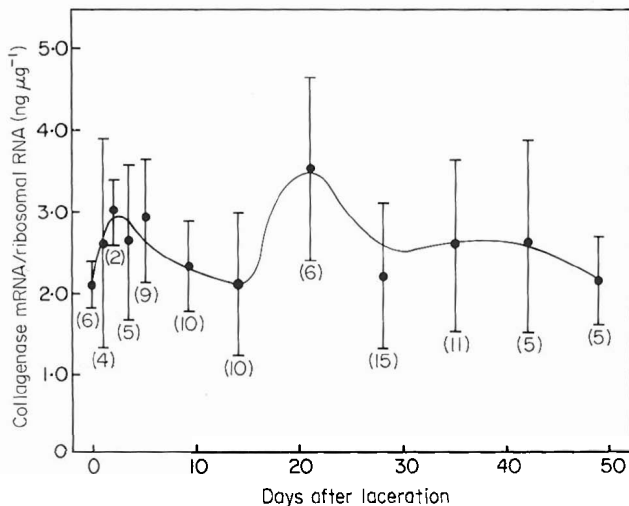


FIG. 2. Amounts of collagenase mRNA in lacerated corneas. Samples were prepared as described in Fig. 1, except  $^{32}\text{P}$ -labeled cDNA of rabbit synovial fibroblast collagenase was used to quantitate the amount of mRNA for collagenase present during various stages of wound healing.

wave of increased collagen synthesis (Cionni et al., 1986). In the present study, we have examined whether there is a parallel increase in collagenase mRNA. Therefore, slot-blot hybridization using  $^{32}\text{P}$ -labeled cDNA of synovial fibroblast collagenase was

performed with the total RNA prepared from lacerated corneas. Figure 2 shows the variations in collagenase mRNA in lacerated corneas which were allowed to heal for 0–49 days. This result indicates that normal corneas contain about 2.2 ng collagenase mRNA  $\mu\text{g}$  rRNA $^{-1}$  (day 0 in Fig. 2). It also indicates that mRNA of collagenase increases in two waves. There is a 36% increase in lacerated corneas which have been allowed to heal for 2 days. Thereafter, the amount of collagenase mRNA remains at a higher than control level at most time points during healing. There is a 60% increase of collagenase mRNA in injured tissues healed for 21 days. This is significantly higher than the levels of collagenase mRNA found in normal corneas ( $P < 0.025$ ) and injured corneas healed for 35 days ( $P < 0.05$ ). Following this, the increase of collagenase mRNA levels is marginal and within the variations of the measuring procedures used. The increase of collagenase mRNA can partially account for the rapid degradation of newly synthesized collagen during the healing period. (Cionni et al., 1986).

### Light and Electron Microscopy of Lacerated Corneas

In order to examine the possible role of keratocytes in tissue remodeling, we carried out a series of experiments to examine the morphology of lacerated corneas which have been allowed to heal for 1, 2, 3 and 4 weeks. As shown by the cellularity and filling in of the wound area (data not shown), the lacerated corneas progressively synthesize extracellular matrix to repair the wounds. One week following laceration, the wounded cornea is swollen, the epithelial surface has closed, and internally the wound is still filled with a fibrin plug; meanwhile, increased numbers of keratocytes (or fibroblast-like cells) begin to appear in the wounds (data not shown). Many fibroblast-like cells appear along the edge of the same wound. After 3 weeks of injury, there are many fibroblast-like cells in the wound, and the wound is filled with extracellular matrix. The swelling is reduced after the injured corneas healed for 4 weeks and the wound is filled with fibroblast-like cells and extracellular matrix components (data not shown).

Electron microscopic examination reveals that laceration interrupted the organized collagen network typical of normal cornea [Fig. 3(A)]. Figures 3(B), (C) and (D) show that the highly organized collagen was replaced by disoriented collagen fibrils in wounds which had been allowed to heal for 1, 3 and 4 weeks respectively. It is apparent that cells within the wounds are fibroblast-like and have well-developed endoplasmic reticulum. These fibroblast-like cells are probably responsible for the synthesis of components of extracellular matrix. As the wounds healed, progressively more collagen fibrils were deposited. However, in the time periods studied the collagen fibrils were not organized in orthogonal lamellae; instead they were irregular, with most of the collagen fibrils perpendicular to the plane of section. This irregularity of

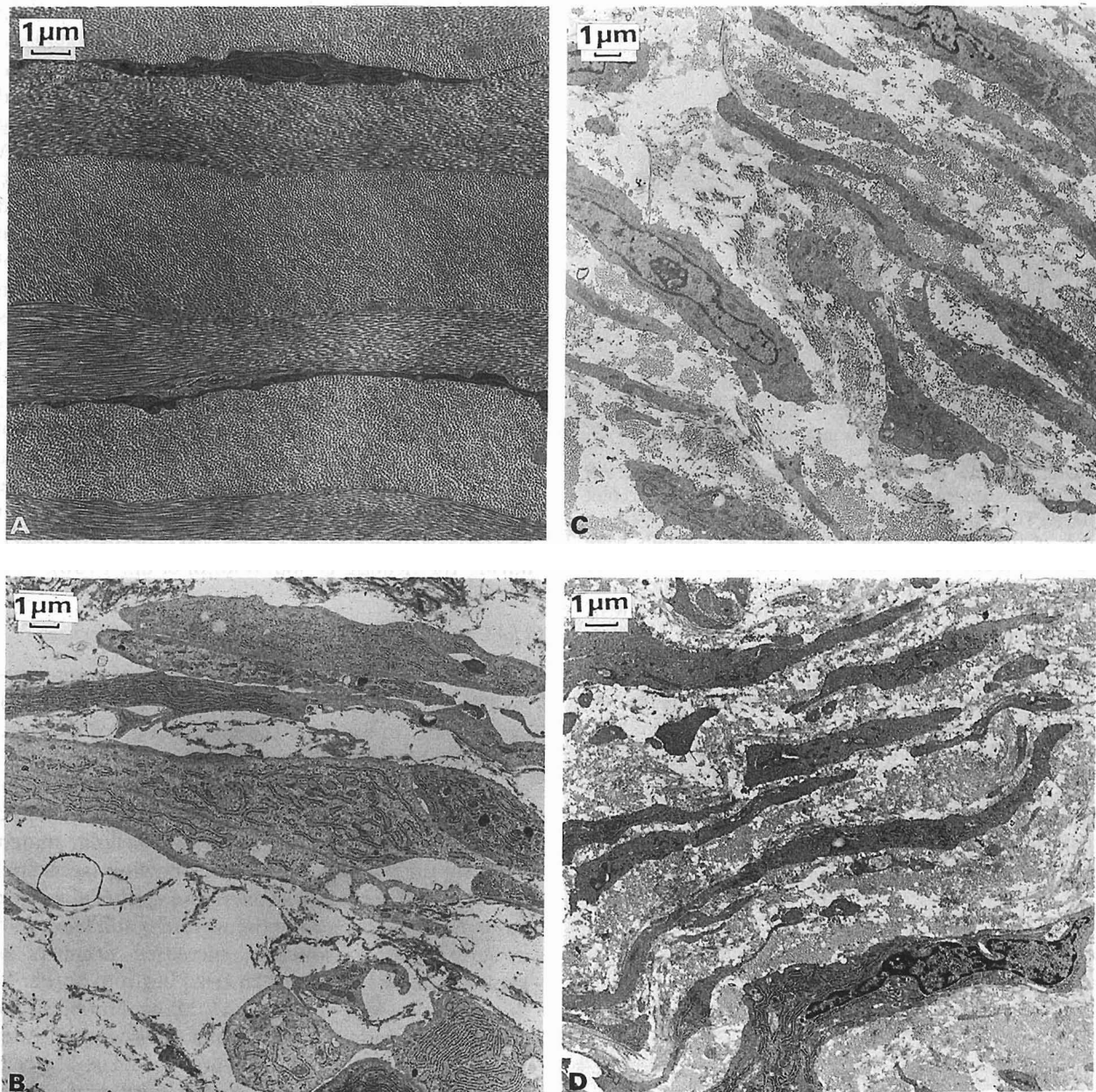


Fig. 3. Electron microscopy of lacerated rabbit corneas. The lacerated corneas were allowed to heal for 1, 3 and 4 weeks. A, Micrograph of a normal stroma; B, lacerated stroma healed for 1 week; C, lacerated stroma healed for 3 weeks; D, lacerated stroma healed for 4 weeks.

collagen fibrils can in part account for the opacity of scar tissue.

#### *Effect of Leupeptin on Lysosomes of Fibroblast-like Cells*

In our previous studies, we demonstrated that corneal wounds undergo active remodeling 3 weeks after laceration (Cionni et al., 1986). Our data from the present study indicates that production of collagenase mRNA in lacerated corneas increases at most time points during healing, with peak levels at 2 and 21 days, (Fig. 2). Because high levels of collagenase mRNA are not sustained throughout periods at rapid collagen turnover, we suggest that the cells in the wounds may play an active role in tissue remodeling during corneal wound-healing by lysosomal degra-

dation of collagen as well as secretion of collagenase. To examine this hypothesis, lacerated corneas were allowed to heal for 1, 2 and 3 weeks. The excised corneas were then incubated for 4 hr in Dulbecco's Minimum Essential medium with or without  $65 \mu\text{M}$  leupeptin, a proteinase inhibitor. The tissue was prepared for electron microscopic examination as described in Methods. Figure 4(A) is an electron micrograph of fibroblast-like cells in a wounded cornea which has been allowed to heal for 1 week. The cells have well-developed endoplasmic reticulum, and few lysosomes can be seen in the field. When the 1-week-healed corneas were treated with leupeptin prior to fixation for electron microscopy, many enlarged lysosomes filled with amorphous components were seen within the cells [Fig. 4(D)]. The nature of this



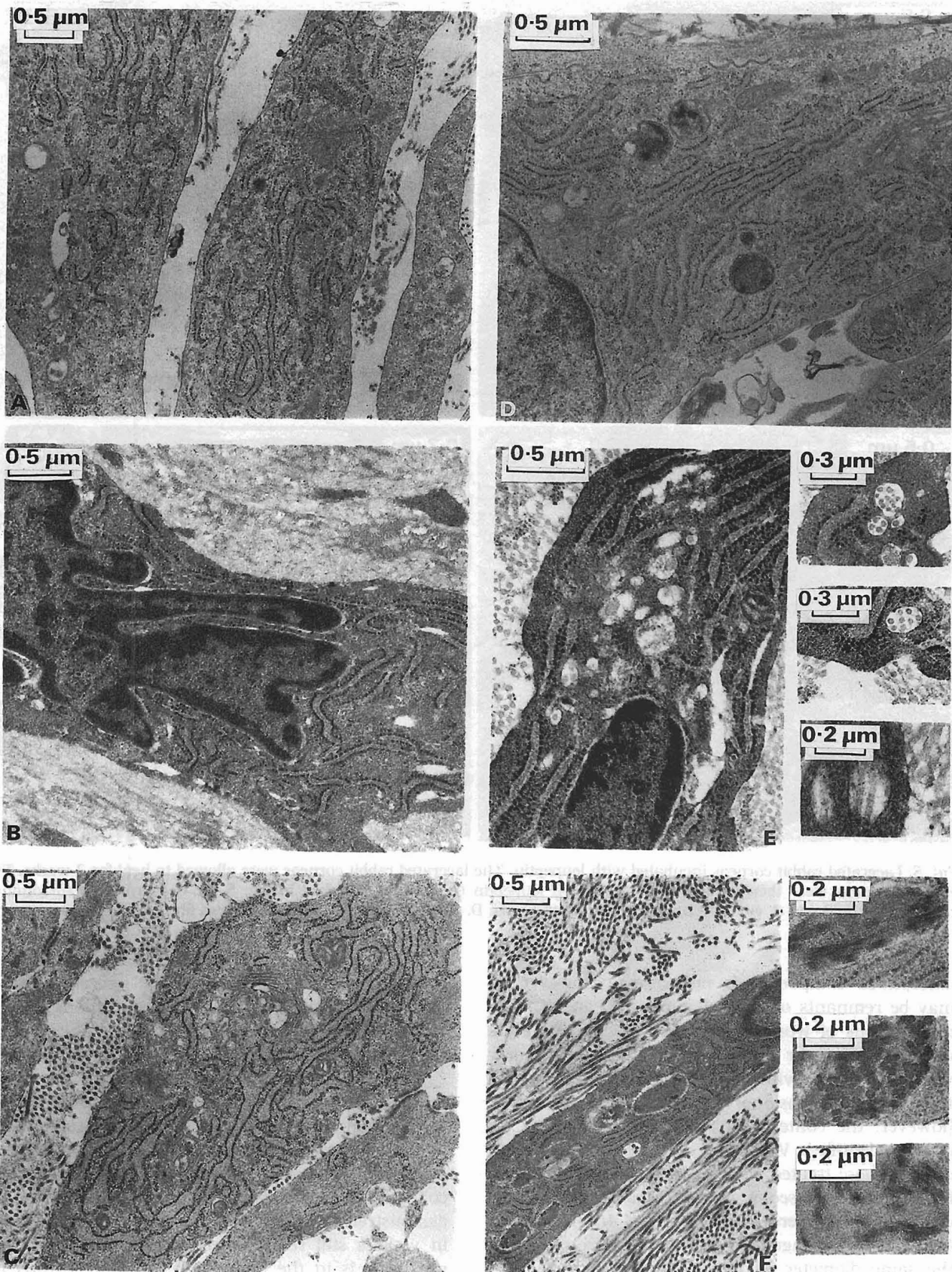


FIG. 4. Lacerated rabbit corneas incubated with and without leupeptin. The lacerated rabbit corneas were allowed to heal for 1, 2 and 3 weeks. The corneas were incubated in DME with or without leupeptin ( $65 \mu\text{M}$ ) at  $37^\circ\text{C}$  for 4 hr. The corneas were then prepared for electron microscopy. A, B and C, Corneas incubated without leupeptin; D, E and F, corneas incubated with leupeptin; A and D, lacerated stroma healed for 1 week; B and E, lacerated stroma healed for 2 weeks; C and F, lacerated stroma healed for 3 weeks.

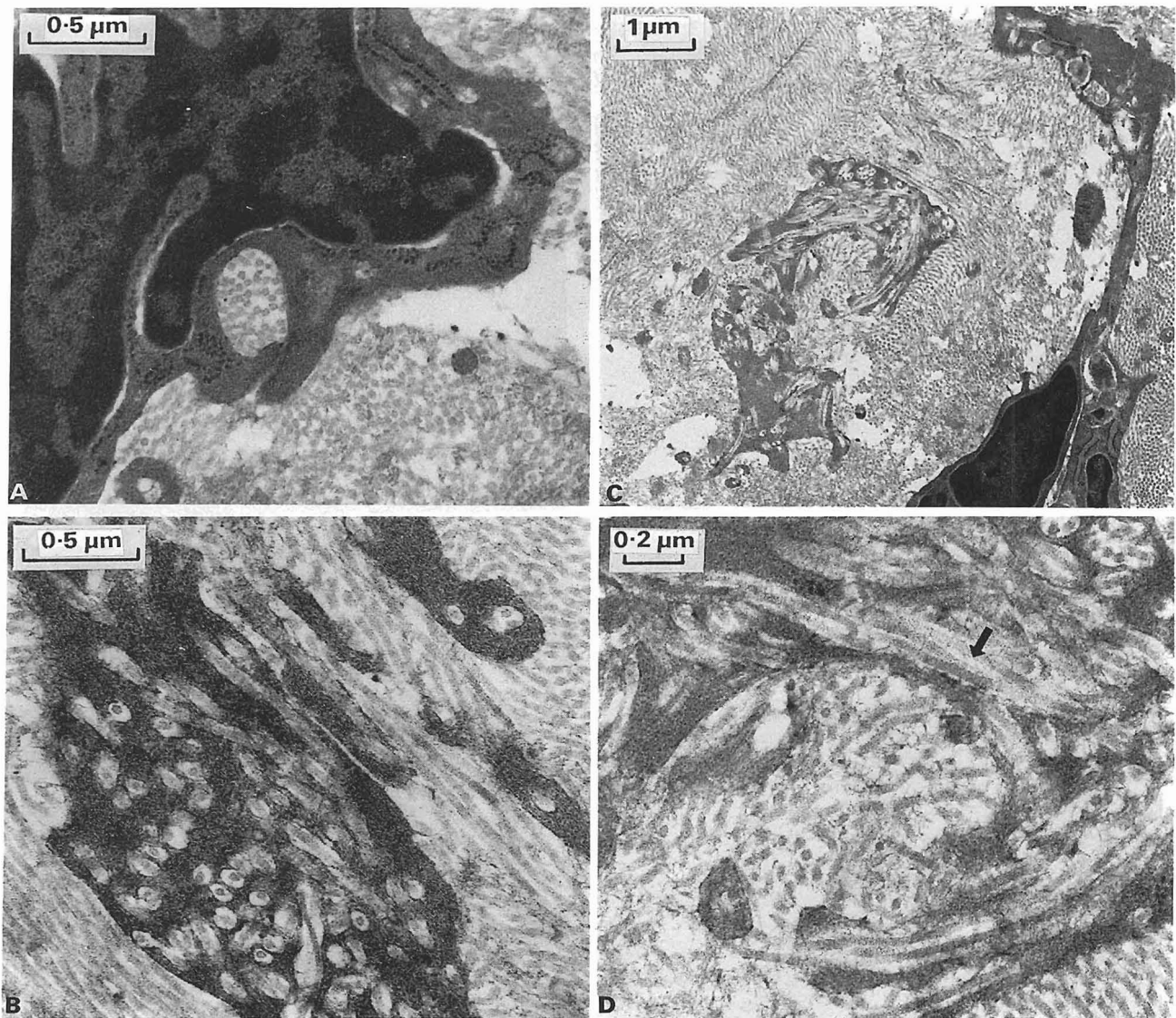


FIG. 5. Lacerated rabbit corneas incubated with leupeptin. The lacerated rabbit corneas were allowed to heal for 2 weeks. The excised corneas were then incubated for 4 hr with leupeptin ( $65 \mu\text{M}$ ) as described in Fig. 5. A, Cell processes surrounded collagen fibrils; B and C, vesicles containing collagen fibrils; D, enlarged vesicles. Arrow indicates fibrils having characteristic collagen striations.

amorphous component is not known, however, it may be remnants of fibrin. Figures 4(B) and (C) are micrographs of wounded corneas which have been allowed to heal for 2 and 3 weeks respectively, and were not treated with leupeptin prior to fixation. In these corneas, occasional lysosomes can be identified; however, the contents of the lysosomes cannot be readily identified. When the 2- and 3-week-healed corneas were treated with leupeptin prior to fixation for electron microscopy, many lysosomes containing collagen fibrils were identified [Figs 4(E) and (F)]. Many of the collagen fibrils within lysosomes have the same diameter as those found in extracellular matrixes. Occasionally, the characteristic striations of collagen fibrils can also be seen in the collagen fibrils located within the lysosomes [Figs 4(E) and (F)]. However, striated collagen fibrils within the lysosomal vesicles are more difficult to find. This can be explained in part by the possibility that leupeptin may not

completely inhibit all the lysosomal proteases under our experimental conditions. Our observations support the hypothesis that fibroblasts may phagocytose collagen fibrils during the healing of lacerated corneas.

To further examine this hypothesis, we have repeated our electron microscopy experiments. Figure 5(A) demonstrates that collagen fibrils are surrounded by cell processes which are probably at the initial stage of phagocytosis. Figures 5(B) and (C) show many collagen fibrils are included in vesicles. Figure 5(D) demonstrates that some of the collagen fibrils included in vesicles still retain characteristic striations, suggesting it is in the early stages of phagocytosis by fibroblasts.

Electron micrographs of normal cornea or corneal tissues taken some distance away from the injury indicate that no lysosomes containing collagen fibrils can be found in the stroma cells, whether the corneas were treated with leupeptin or not (data not shown).

This observation suggests that cells within the normal corneal stroma distant from the laceration do not actively engage in tissue remodeling during wound-healing.

#### 4. Discussion

We previously demonstrated that there are two waves of 2–3-fold increases in collagen synthesis during healing of lacerated corneas as measured by the synthesis of [<sup>14</sup>C]hydroxyproline (Cionni et al., 1986). In the present studies, we measured the amount of  $\alpha 1(I)$  mRNA in injured corneas and the data indicate that the change in collagen synthesis can be explained in part by the changes of  $\alpha 1(I)$  mRNA. It should be noted, however, that the increase of  $\alpha 1(I)$  mRNA in lacerated corneas is only 25% higher than the control and cannot fully account for the 2–3-fold increases of [<sup>14</sup>C]hydroxyproline synthesized by lacerated rabbit corneas. This can be explained by the hypothesis that in lacerated corneas, the translation of  $\alpha 1(I)$  mRNA is probably much more efficient than that in normal corneas. Alternatively, synthesis of many collagen types may account for the higher increase in the synthesis of [<sup>14</sup>C]hydroxyproline. For example, it is known that injured corneas synthesize many different collagen types, i.e. collagen types III, IV, V, VI, etc. (Cintron and Hong, 1988; Cintron et al., 1988). Although type I collagen has been considered to be the major collagenous component synthesized by lacerated corneas, measurement of  $\alpha 1(I)$  mRNA may not fully account for the increased synthesis of [<sup>14</sup>C]hydroxyproline. It should be noted that cDNA of  $\alpha 1(I)$  used in present studies shares homology with C-propeptides of type II and type III collagen (Ninomiya et al., 1984). However, our previous studies demonstrated that no type II or type III collagen synthesis could be detected in lacerated rabbit corneas (Cionni et al., 1986). Thus, the mRNA levels observed in Fig. 1 should represent mostly  $\alpha 1(I)$  chains. Furthermore, type VI collagen was considered to be a minor collagenous component in corneas but recent data suggest that type VI collagen may represent more than 20% of the total collagenous component in corneas (Zimmerman et al., 1986). It is not known, however, whether type VI collagen is the major collagen type synthesized by lacerated corneas and further investigation is needed to elucidate this possibility.

Our previous studies also suggested that active remodeling of collagen takes place during corneal wound-healing (Cionni et al., 1986). We reported that the half life of [<sup>14</sup>C]collagen synthesized by injured corneas healed for 1 week, progressively decreases from 21 days to 4 days during healing periods between day 7 and day 15, and day 17 and day 24 after laceration. The [<sup>14</sup>C]collagen synthesized by lacerated corneas healed for 21 days has a half life of 14 days (Cionni et al., 1986). The accelerated degradation of [<sup>14</sup>C]collagen synthesized by lacerated corneas healed

for 1 week precedes the increase of [<sup>14</sup>C]hydroxyproline synthesis by lacerated corneas (Cionni et al., 1986). It is of particular interest to note that the accelerated degradation of [<sup>14</sup>C]collagen is concurrent with the second wave of increase of collagenase mRNA in the injured tissues (Fig. 2). The immediate increase of collagenase mRNA after laceration (day 2) coincides with the decrease in [<sup>14</sup>C]hydroxyproline synthesis and  $\alpha 1(I)$  mRNA. The nature of this coincidence is not clear. It is known, however, that numerous PMN infiltrate lacerated corneas within 24–48 hr. The PMN collagenase is immunologically distinct from fibroblast collagenase (Kao et al., 1986). It was recently demonstrated that human neutrophil collagenase possesses 57% identity with the deduced sequence for human fibroblast collagenase (Hasty et al., 1990). It is possible that the immediate increase of collagenase mRNA may be due to the infiltration of PMN into the lacerated corneas. Further experiments are needed to examine this hypothesis.

Nevertheless, the increased amount of collagenase mRNA may not fully account for active collagen metabolism during healing of lacerated rabbit corneas. We should be cautious about this interpretation, however, because the process of collagenolytic activity includes a complex cascade of reactions of activating latent collagenase (Harris, Welgus and Krane, 1984). Thus, a low level in increase of collagenase mRNA in lacerated corneas does not exclude the possibility of increased collagenolytic activity via activation of latent collagenase. It has been shown by immunofluorescent staining that latent collagenase exists in normal corneas as well as in many other tissues (Montfort and Perez-Tamayo, 1975; Gordon, Bauer and Eisen, 1980). It is likely that the latent collagenase is activated during corneal wound-healing (Brown and Weller, 1970).

Our study demonstrates that lysosomes containing collagen fibrils are found in fibroblast-like cells in the wound after the tissue was treated with leupeptin. A similar observation has been reported by Everts, Beertsen and Tigchelaar-Gulter (1985) who demonstrated that collagen fibrils are phagocytosed by periosteal fibroblasts of cultured bone. In addition, Fauer et al. (1970) suggested that fibroblasts resulting from keratocytes differentiation are active in phagocytosis during inflammatory reactions. It should be noted that in their experiment no inhibitor was included to inhibit the lysosomal proteases. We were not able to identify lysosomal particles which contained collagen fibrils when leupeptin was not included in the culture medium (Fig. 4). Our results, as shown in Figs 4 and 5, are consistent with the hypothesis that fibroblast-like cells in injured corneal stroma actively participate in tissue remodeling by phagocytosis of extracellular collagen.

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