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The proliferation of elastic fibres after skin incisions in albino mice and rats: a light and electron microscopic study

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INTRODUCTION

If a wound disrupts the continuity of the skin, usually a predictable sequence of events follows, resulting in the repair of all its components: epithelial, vascular, nervous, and muscular. The healing process has been studied for many years, and excellent references are available on almost every aspect (Arey, 1936; Montagna & Billingham, 1964; Dunphy & Van Winkle, 1969; McMinn, 1969; Peacock & Van Winkle, 1970).

It is apparent from these treatises, however, that although much information is available on the replacement of collagen and the glycoproteins of the dermis, relatively little attention has been paid to elastic fibres. The scattered information that does exist in the literature contributes to a notion that they play no role, or only an insignificant one, in the regeneration process of skin. As a result, most modern textbooks when discussing wound healing do not mention the replacement of elastic fibres.

Although there have been some papers recently in which the regeneration of elastic fibres has been reported, there is a considerable diversity in both the experimental models and the findings; for example, Ordman & Gilman (1966), as part of a much larger work on collagen fibrogenesis, mention that new elastic fibres were seen in the regenerating dermis 15 days after full-thickness incisions were made in piglet skin. In a chronic inflammatory granuloma, Feher, Jennings & Rannie (1971) reported new elastic fibres in the 'muscle layer and subcutaneous tissues' 21 days post-wounding. After removing skin for grafts, Converse & Robb-Smith (1944) in man and later, Hinshaw & Miller (1965) in pigs, found elastic fibres in the new dermis after 28–35 days. Williams (1970) was unable to demonstrate their presence until 56 days after skin burns in rats. There are also authors who deny that elastic fibres are replaced at all. Furthermore, most reports (except for that of Feher *et al.*) deal mainly with the initial appearance of elastic fibres. The older literature, well reviewed by Arey (1936), is equally as varied.

Information about the elastic fibre content of more mature and ageing scars has not been actively pursued. Based on the reports previously cited, and others, one is left with the impression that either these fibres are never replaced, or that they do reappear but not much is known about the matter. In view of all this, and the fact that almost every surgical procedure involves the healing of a skin incision, systematic observations on the replacement of elastic fibres in healing skin wounds are clearly needed.

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MATERIALS AND METHODS

Under ether anaesthesia, four full-thickness skin incisions, 1 cm long, were made in the backs of 50 Swiss-strain albino mice (20 g weight) and 20 Sprague–Dawley rats (200 g weight), In each animal two such wounds were made on each side of, and parallel to, the vertebral column, separated from each other by at least two centimetres. No sutures or other closure devices were used. The animals were returned to cages where they received food and water *ad libitum*, and no special further treatment was given.

Thereafter, at times ranging from 2 to 150 days post-wounding, animals were anaesthetized, and biopsies were taken from all four wound sites at once. Each biopsy included the entire wounded area and some adjacent unwounded tissue. From each animal two of the four wound biopsies were fixed in 10% buffered neutral formol and routinely processed for paraffin sectioning. Sections 6–8 μm thick were cut and stained with either haematoxylin and eosin, Pinkus' orcein, Weigert's resorcin-fuchsin, or Snook's reticulin method (all stains from Luna, 1968). The remaining two wound biopsies were initially fixed at 4 °C in 2% glutaraldehyde in 0·1 M cacodylate buffer, pH 7·2. After 4 hours, each wound biopsy was sliced perpendicular to its long axis into six pieces, and the order of the sections relative to one another was maintained throughout subsequent processing. The six segments from each wound were washed in cacodylate buffer with 10% sucrose (weight/weight), post-fixed in 2% OsO_4 in veronal acetate for 2 hours, embedded in Epon (according to Luft, 1961) and cut on a Porter-Blum MT-1. Sections 2 μm thick were stained with either 1% toluidine blue in 1% borax, 0·02% alcoholic orcein at 60 °C, or 1% aldehyde fuchsin. Thin sections for electron microscopy were stained with either saturated uranyl acetate and 1% lead citrate (20 minutes and 2 minutes respectively), or 0·5% phosphotungstic acid (PTA) in 50% ethanol for 3 minutes.

RESULTS

Unless otherwise noted, the results were demonstrable in both rat and mouse skin with all of the traditional elastic fibre stains used (orcein, resorcein, and aldehyde fuchsin) as well as with toluidine blue and azure B. Although orcein, aldehyde fuchsin and toluidine blue are not usually thought to be useful for the identification of elastic fibres in Epon sections, in fact they provide excellent specificity, and can be applied without prior removal of the Epon. This was constantly confirmed with the electron microscope. Furthermore, the fibres indentified as elastic were not stained by the method for 'reticulin' used.

General wound healing

The full-thickness incisions initially produced a space, hereinafter referred to as the wound space, flanked on all sides by normal skin (Fig. 1). The wound space was first filled with a fibrin clot and inflammatory cells, mainly polymorphonuclear leukocytes. This early stage of acute inflammation lasted only a few days, and then subsided as repair and regeneration progressed. A chronic reaction was never detected in any wound.

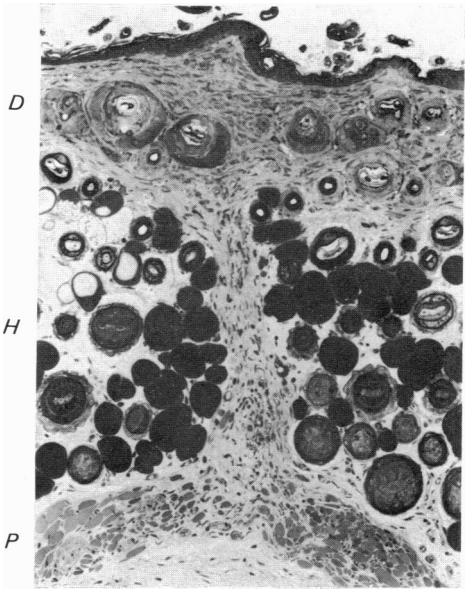

Fig. 1. Mouse skin 2 weeks post-wounding. The wound space is vertically oriented and extends through the dermis (*D*), hypodermis (*H*) and panniculus carnosus (*P*). Toluidine blue-stained, ×100.

Replacement of elastic fibres: light microscopy

The first new elastic fibres appeared at the cut edges of the deepest layer of the integument, below the panniculus carnosus, about 8 days post-wounding. They appeared within the regenerating tissue at locations where, previously, elastic fibres were absent, and they were smaller and more diffusely arranged than those in unwounded regions. During the next few days the number of elastic fibres increased in this region, as other new elastic fibres began to appear throughout the remaining base of the wound. By the eleventh or twelfth day post-wounding, new elastic fibres were very numerous in these deep regions and at the same time were found more superficially in those parts of the wound space bounded laterally by hypodermis. At this time new elastic fibres could not be seen in the regenerating dermis. That is, there was an abrupt termination of elastic fibres at the junction between unwounded and regenerating dermis (Fig. 2A).

By the twentieth day post-wounding new elastic fibres could be found in the regenerating dermis, appearing as darkly staining, fine dots in the light microscope (Fig. 2B). However, they were not distributed uniformly throughout this region. Sections taken from the anterior and posterior extremes of the incision contained new elastic fibres across the entire regenerated dermis. Sections taken from the middle of an incision showed the elastic fibres were more numerous in the deeper and lateral aspects of the new dermis, but were rarely found in its more central or superficial regions.

Between the twentieth and thirtieth days post-wounding, there began a period of rapid proliferation of elastic fibres to levels far above those found in the adjacent unwounded tissue. This increase was seen throughout the entire wound space, but was especially noticeable in the new dermis (Fig. 2C). The one exception to this was the relative sparsity of elastic fibres in the most superficial regions of the dermis,

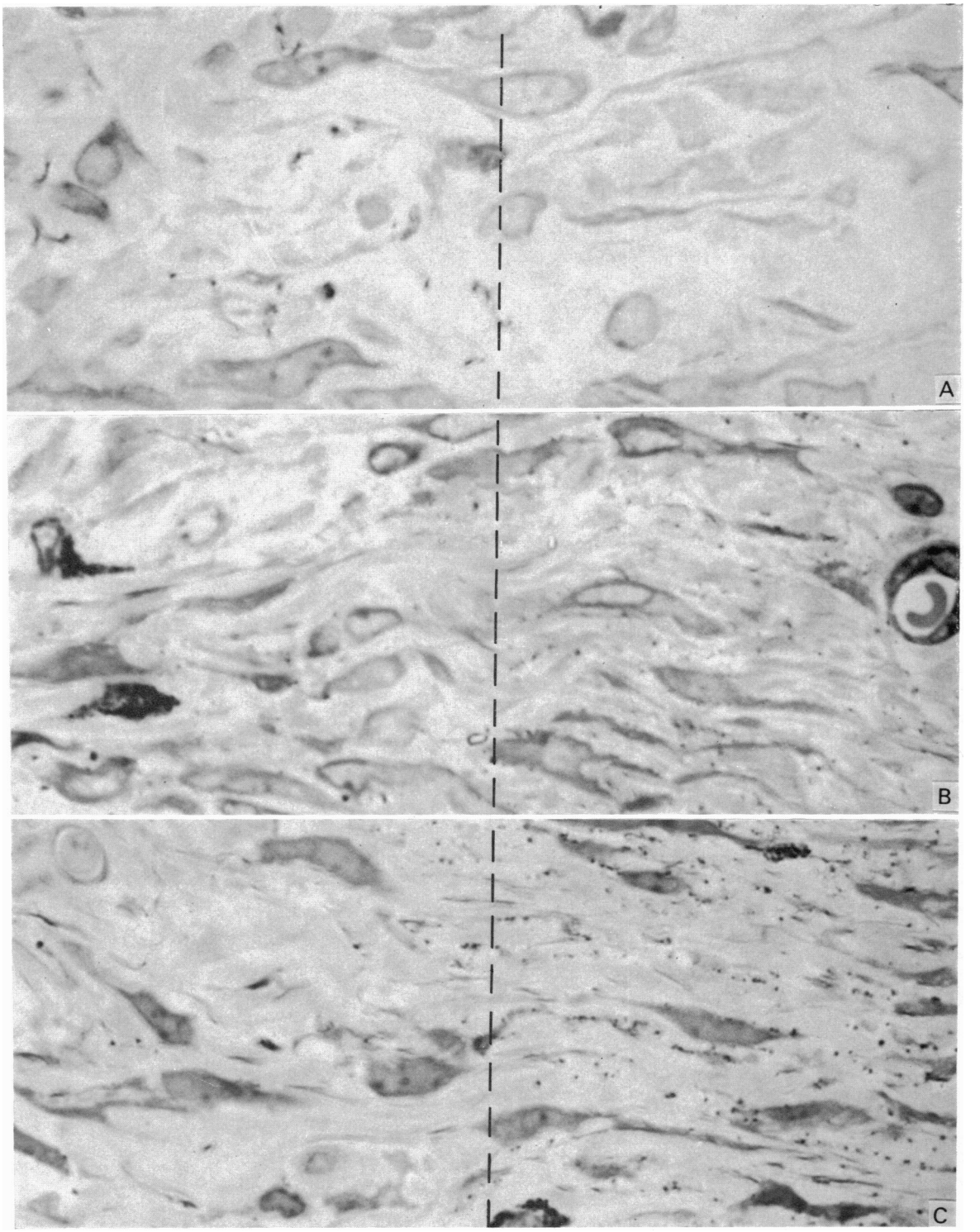

Fig. 2. Mouse dermis at various times after wounding. The wound space is to the right of the broken line. (A) 10 days post-wounding, no elastic fibres in the wound space. (B) 20 days post-wounding, numerous small, darkly staining elastic fibres are just barely visible. (C) 30 days post-wounding demonstrates the extent of proliferation of the new elastic fibres. Toluidine blue-stained. ×1250.

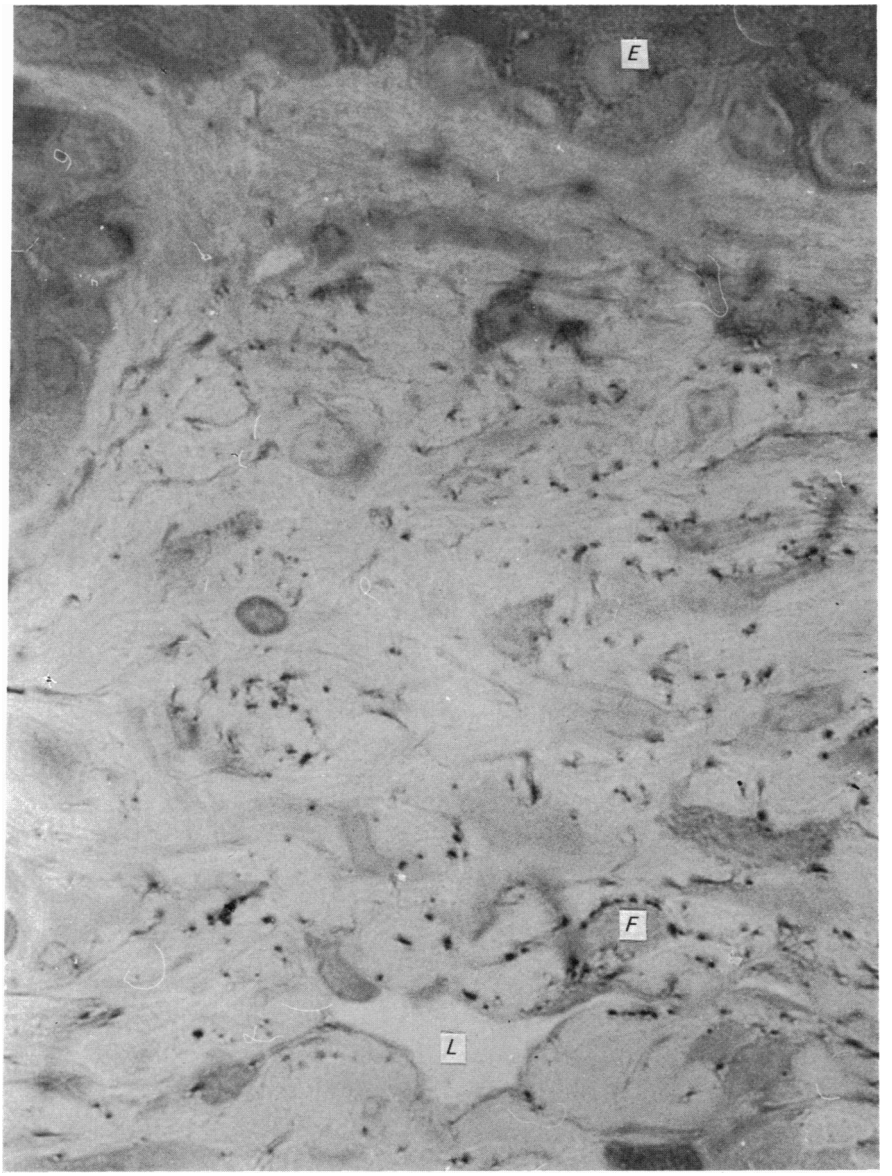

Fig. 3. Regenerating mouse dermis 30 days post-wounding stained with orcein. Elastic fibres surround a lymphatic vessel (*L*) and lie in a row partly outlining a fibroblast (*F*). However, they are absent just below the epidermis (*E*). × 1250.

i.e. immediately deep to the epithelium (Fig. 3). The elevated numbers of elastic fibres found at this time were much more pronounced in mice than in rats, but nonetheless, quite apparent in the rats. During this period, elastic fibres were commonly seen surrounding lymphatic vessels within the wound space (Fig. 3), although they were occasionally detectable there at least 10 days earlier.

During the final 12 weeks of observation (30th to the 150th day post-wounding), the number of elastic fibres appeared to decrease slightly in all parts of the wound space, but still remained high when compared to unwounded regions. In contrast,

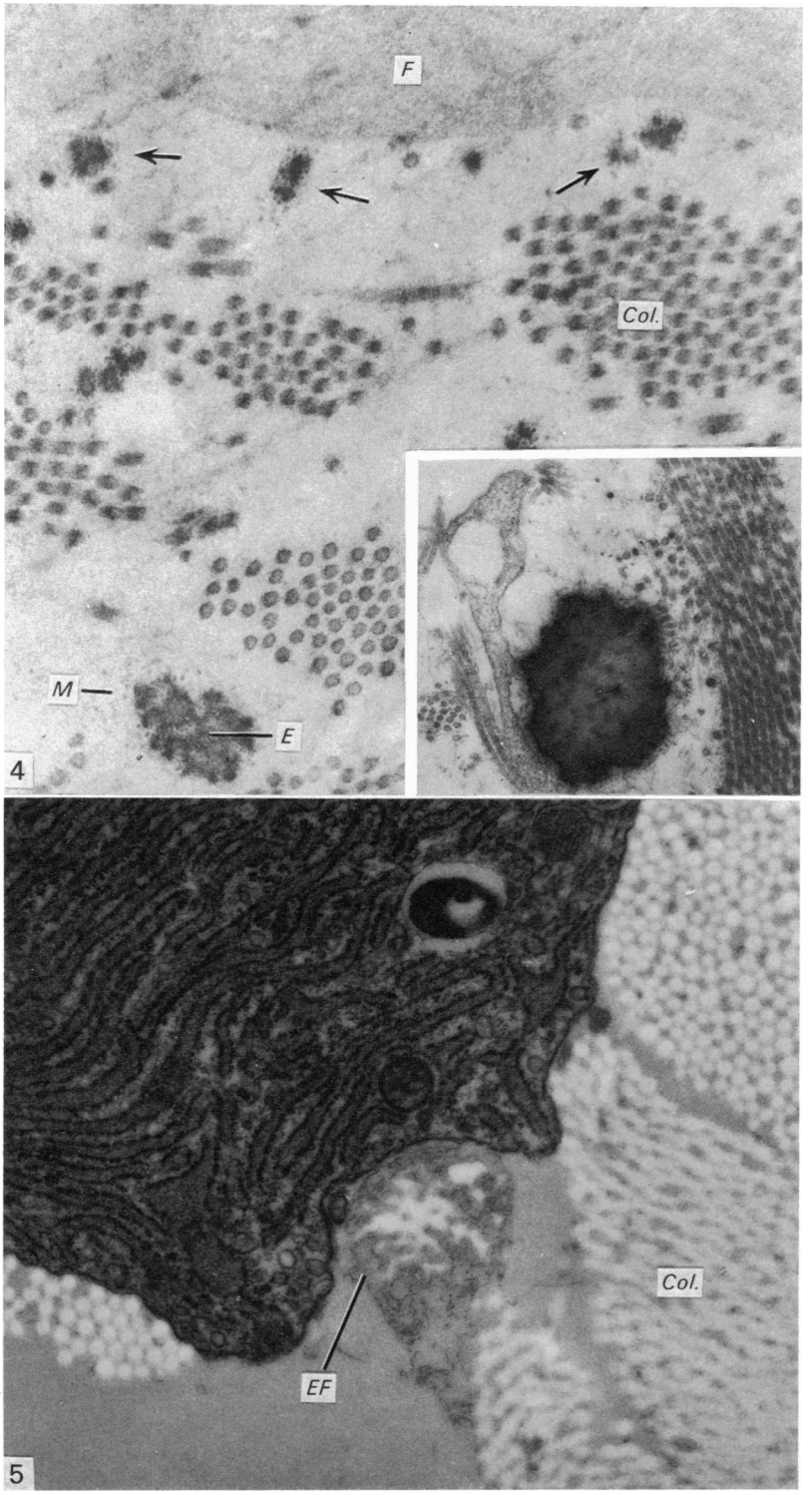

Fig. 4. New elastic fibres stained with alcoholic phosphotungstic acid. Both the microfibrils (*M*) and central component (*E*) appear darkly staining, as does the collagen (*Col*). Just adjacent to a faintly staining fibroblast (*F*) are a number of smaller elastic fibres (arrows). ×22000. The insert is an elastic fibre from a non-wounded animal processed in the same manner. ×14000.

Fig. 5. A new elastic fibre (*EF*) stained with lead citrate and uranyl acetate. With these ions the central component is not stained as are often the collagen fibres (*Col*). ×22000.

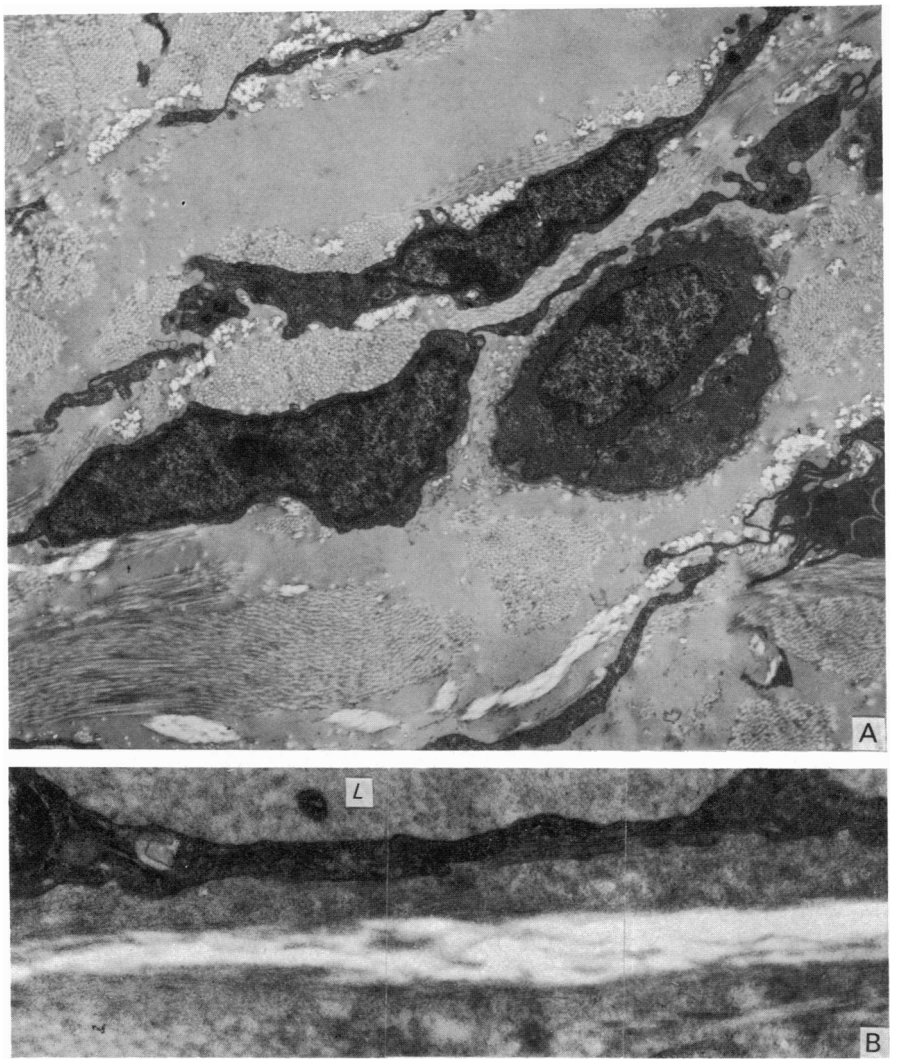

Fig. 6. (A) Thirty days post-wounding with mouse dermis. Uranyl acetate and lead citrate, × 8000. (B) An elastic fibre near a lymphatic vessel (*L*) from a non-wounded animal processed exactly as (A) for comparison, × 22000.

the diameter of these elastic fibres appeared to increase during the later phases of regeneration.

Replacement of elastic fibres: electron microscopy

Because of the extensive proliferation of elastic fibres in the regenerating dermis after 20 days post-wounding, this region was studied most extensively with the electron microscope. The ultrastructure of these new elastic fibres was the same as that of the elastic fibres of unwounded dermis (Fig. 4). Both were composed of electron-dense microfibrils surrounding and partially embedded within a variously stained central component (compare Figs. 4 and 5). However, the earliest elastic fibres of the wound space were usually smaller and contained relatively more of the microfibrillar com-

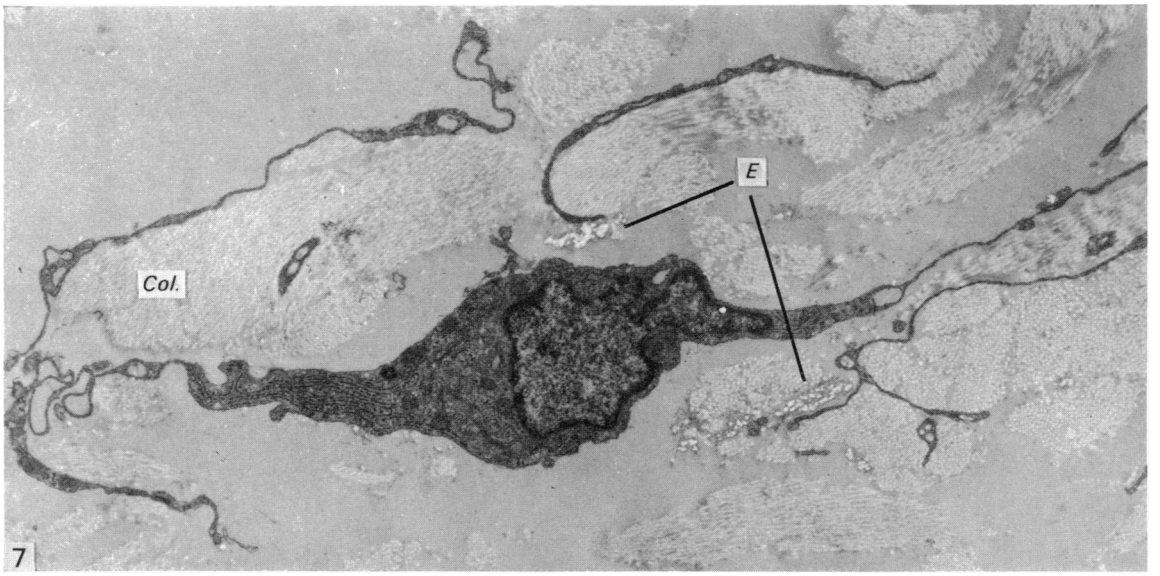

Fig. 7. In the deepest regions of the wound space fibroblastic attenuations are the most elaborate. Elastic fibres (*E*); collagen (*Col*). ×8000.

ponent than elastic fibres of non-wounded areas (Figs. 4 and 5). The wound space elastic fibres were often found immediately adjacent to the cell membranes of fibroblasts. In the light microscope this relationship was also apparent (Fig. 3). Frequently within a cluster of new elastic fibres a size gradation could be found.

Both components of the elastic fibre were not always seen. Occasionally, what appeared to be only the microfibrillar element was observed. This finding was not restricted to biopsies taken at the earliest stages of elastic fibre regeneration, for such bundles could be found at least 2 months post-wounding.

At about 1 month post-wounding the number of elastic fibres appeared to be at its peak (Fig. 6A). The fibres at this stage were generally larger than those found 10 days earlier, owing to an increase in the central component relative to the microfibrillar, and they more closely resembled elastic fibres in non-wounded animals (Fig. 6B). Elastic fibres were often found associated with long attenuations of fibroblasts which could be so slender as to have room for only a few ribosomes, or one cistern of endoplasmic reticulum, although the elastic fibre itself might be large and well developed (Fig. 6A).

During the first 2 months of wound healing the ultrastructure of the fibroblasts was characteristic of cells actively engaged in protein synthesis. They were rich in rough endoplasmic reticulum, polyribosome clusters and commonly showed a well developed Golgi apparatus.

As already mentioned these cells commonly possesssed long, attenuated processes (Figs. 6A and 7). An examination of the axial ratios of these structures indicates that, in three dimensions, some must be spindle or tube shaped and others in the form of flattened sheets. Often they were found to be elaborately convoluted, especially in the deepest regions of the wound space where the connective tissue is loosest (Fig. 7).

By the third month post-wounding all these characteristics were much less pronounced and the fibroblasts assumed a more quiescent appearance. At this stage of

the healing process elastic fibres were usually situated between bundles of collagen as in unwounded skin, rather than in contact with fibroblasts. At 4 months post-wounding (the oldest scar studied) the appearances were substantially the same as in the previous biopsy findings.

DISCUSSION

Three general observations can be summarized with regard to elastic fibre replacement: (1) there is an ordered reappearance of elastic fibres which begins in the deepest regions of the wound space as early as 8 days after a full-thickness incision; (2) there follows a proliferation of new elastic fibres far above normal numbers, reaching a peak within the dermis at about 1 month post-wounding; (3) these elevated numbers of elastic fibres are retained in older scars for at least 4 months.

These results represent significant departures from the previous literature. Elastic fibres have never before been reported to appear so early and in the deepest regions of a healing skin wound. All of the previous investigations, except those of Feher *et al.* (1971), were confined to the regenerating *dermis* and did not include observations on the deepest regions of the wound space, although it had been shown by Hadfield (1963) and corroborated by Ordman & Gilman (1966) and others that, after full-thickness incisions, the earliest signs of tissue repair are found in the *subdermal* regions

The present study also indicates that those elastic fibres which populate the scar do not do so in a random fasion but show a definite pattern of appearance. New elastic fibres are seen in the deep and peripheral parts of the incision (anterior, posterior, and lateral) before they are found in the more central and superficial parts. Ordman and Gilman (1966), also studying healing skin incisions, showed a very similar pattern of *collagen* fibre replacement. These parallel findings indicate that fibroblast activity is not uniformly distributed throughout the wound space. A systematic study of fibroblast morphology at various locations and post-wounding times would, perhaps, detect correlative ultrastructural variations.

The significance of the attenuated processes of the fibroblasts is unknown and should be investigated. Possibly they are portions of migrating cells, having some role in wound remodelling. Williams (1970), in his study of burn wounds, observed similar structures, but did not speculate about their function.

Numerous investigators have studied the normal development of elastic fibres at the ultrastructural level, e.g. in ligmentum nuchae (Usuku, 1958; Greenlee, Ross & Hartman, 1967), tendon (Ferenbach, Sandberg & Cleary, 1966; Greenlee *et al.* 1966), lung (Rhodin & Dalhamn, 1955; Albert, 1972; Brissie, Spicer & Thompson, 1975) and aorta (Haust, 1967; Karrer, 1960). The electron microscopic observations in this paper are in general agreement with most of these descriptions. The only differences to be noted concern the order of appearance of the two elastic fibre components. Under the conditions of this experiment it is impossible to state, unequivocably, that the microfibrillar element was the first to be synthesized. Observations of isolated bundles of these microfibrils could have been the result of a tangential section through a mature fibre, or they might have been unrelated microfibrils of the extracellular space, similar in general appearance to those of the elastic fibre, but not in reality the microfibrils peculiar to the elastic fibre. Although the literature suggests that the microfibrils are the first to be synthesized, only Ross (1971) has shown it conclusively for smooth muscle cells in culture.

The most surprising and significant observation which was made concerns the

extent of elastic fibre proliferation and persistence in scars. The number of these fibres in regenerating dermis may be as much as 30 times that in adjacent unwounded tissue. In contrast, it has been shown that the collagen content of regenerating dermis does not significantly increase above that in unwounded regions (Madden & Peacock, 1971). This strongly suggests an important, previously unsuspected, role for elastic fibres in the repair process.

It has been shown that the physical properties of elastic fibres enable them to absorb and dissipate energy (Wood, 1954; Daly, 1969; Hoffman *et al.* 1974) in low stress regions. During the first few months of wound repair the rates of collagen resorption and deposition are elevated (Madden & Peacock, 1968, 1971), and the wound is more susceptible to breaking than is unwounded skin. For instance, at 1 month post-wounding, the tensile strength of the scar is, on the average, only between 20 and 40% of unwounded skin (Howes, Sooy & Harvey, 1929; Howes, Harvey & Hewitt, 1939; Dunphy & Jackson, 1962; Levenson *et al.* 1965; Forrester *et al.* 1969; Van Winkle, 1969). It may be that the large number of new wound space elastic fibres enables the scar to dissipate stresses to which it is normally subjected, as well as to contribute more directly to the tensile strength of the wound. Certainly, studies of scar tissue elasticity (now in progress) should answer many obvious questions. Whatever may be its functional significance elastic fibre regeneration in healing skin wounds, and tissue repair generally can no longer be ignored.

SUMMARY

The replacement of elastic fibres in healing skin wounds in mice and rats was studied with light and electron microscopy. New elastic fibres were seen 1 week post-wounding in the deepest regions of the scar. By 3 weeks post-wounding they could be found in the regenerating dermis. There followed a proliferation of these fibres to numbers far greater than those in unwounded skin. These elevated numbers persisted for the duration of the experiment (4 months). The common notion that elastic fibre formation is not a component of the repair of skin wounds is clearly erroneous.

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