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Sustained release of epidermal growth factor accelerates wound repair

(rat/granulation tissue/neovascularization/collagen)

ANNE BUCKLEY*, JEFFREY M. DAVIDSON*†‡, CRAIG D. KAMERATH†, TERRILL B. WOLT†, AND STEPHEN C. WOODWARD*†§

*Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT 84132; and †Research Service (151C), VA Medical Center, Salt Lake City, UT 84148

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ABSTRACT **Epidermal growth factor (EGF) is a potent mitogen *in vitro*, but its biological role is less clear. The vulnerary effects of EGF were evaluated in a model of wound repair, the polyvinyl alcohol sponge implanted subcutaneously in rats. EGF was purified to homogeneity by reverse-phase HPLC and quantified by receptor binding assay and amino acid analysis. Preliminary data showed moderate promotion of granulation tissue formation by daily injections of 10 μg of EGF. To test the hypothesis that long-term exposure to EGF is required for complete cellular response, the factor was incorporated into pellets releasing 10 or 20 μg of biologically active EGF per day, and the pellets were embedded within the sponges. Slow release of EGF caused a dramatic increase in the extent and organization of the granulation tissue at day 7, a doubling in the DNA content, and 33% increases in protein content and wet weight, as compared with placebo controls. Although collagen content was also increased by almost 50%, the relative rate of collagen synthesis remained the same, suggesting that the morphological and biochemical increase in collagen resulted from increased numbers of fibroblasts rather than a specific stimulation of collagen synthesis. These results indicate that the local sustained presence of EGF accelerates the process of wound repair, specifically neovascularization, organization by fibroblasts, and accumulation of collagen.**

Epidermal growth factor (EGF) stimulates a variety of biological phenomena, including proliferation of skin and corneal epithelia in organ culture, proliferation and differentiation of epidermis and corneal epithelial cells *in vivo* (1), neoangiogenesis in the rabbit cornea (2), and the synthesis of DNA, RNA, protein, and hyaluronic acid in various cell lines in culture (1). Many cell types, including dermal fibroblasts, possess EGF surface receptors and will proliferate in response to EGF in cell culture (3). By contrast to the many observations *in vitro*, the function of growth factors during wound repair is not clear. This is in part the result of the difficulty of delivering growth factors and quantifying the responses to them *in vivo*.

The process of healing surface wounds includes reepithelization, neovascularization, granulation tissue development, collagen elaboration, maturation and remodeling of the scar, and contraction (4). During the early phases of repair, the local accumulation of collagen strongly correlates with the accretion of tensile strength (5); hence, measuring the content and concentration of collagen at a repair site permits an estimate of the rate of healing.

The tendency of mice and other animals to lick open wounds, as well as the known presence of EGF in saliva, led Niall *et al.* to examine the effect of EGF on the contraction of open granulating wounds (6). Applying EGF to the wound accelerated the rate of contraction, and ablating salivary glands retarded the process. Further evidence that EGF may be a vulnerary agent (promoter of wound healing) was reported by Hiramatsu *et al.*, who found that EGF promoted an increase in the collagen content of cotton-pellet granulomas induced in rats (7). Whether or not EGF induces cells to synthesize more collagen has been in dispute. Huey *et al.* demonstrated that human gingival fibroblasts do not change their rate of collagen synthesis when exposed to EGF (8); however, an EGF-mediated decrease in collagen synthesis and collagen fiber formation was reported in osteoblastic-like cells (9). The suggestion that EGF might accelerate wound repair has received further recent support in the finding that EGF potentiated the activity of β-transforming growth factor by increasing protein, DNA, and collagen contents of wound chamber fluids in rats (10).

The present study was undertaken to determine how mouse EGF would affect the accumulation of fibroblasts, collagen, and newly forming capillaries at a site of wound repair in the rat. EGF was administered by a slow-release system. This system was devised because it has been suggested that the effect of growth factors can be modulated by controlling their availability to cell surface receptors (11) and because of our earlier observation that cartilage-derived growth factor (CDGF), an accelerator of wound repair, disappeared rapidly from sites of injection (12).

MATERIALS AND METHODS

Materials. HPLC chemicals were from the following sources: trifluoroacetic acid (CF_3COOH) and acetonitrile (HPLC grade), Burdick and Jackson (Muskegon, MI); phenylisothiocyanate, Beckman; triethylamine [$(C_2H_5)_3N$] ("Sequanal" grade), glacial acetic acid and sodium acetate (both Phix grade), and Iodo-Gen (1,3,4,6-tetrachloro-3,6-diphenylglycouril), Pierce. The amino acid standard kit and hydroxypyroline and hydroxylysine were from Calbiochem, and purified collagenase was from Advance Biofactures (Lynbrook, NY). All tissue culture reagents were purchased from Flow Laboratories or GIBCO. Polyvinyl alcohol sponges ("Ivalon") from which the sponge discs were fabricated were supplied by Unipoint (High Point, NC). Slow-release pellets were manufactured by Innovative Research of America (Rockville, MD).

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Abbreviations: EGF, epidermal growth factor; CDGF, cartilage-derived growth factor; CF_3COOH, trifluoroacetic acid; $(C_2H_5)_3N$, triethylamine; H/E, hematoxylin/eosin.

‡To whom reprint requests should be addressed at: Research Service (151C), VA Medical Center, Salt Lake City, UT 84148.
§Present address: Department of Pathology, Vanderbilt University, Nashville, TN 37232.

Purification of EGF. EGF was extracted from the submaxillary glands of adult male Swiss Webster mice that had been primed with 10 μg of testosterone 2 weeks prior to sacrifice in order to increase the glandular EGF concentration. Glands (30 g per batch) were extracted according to Savage and Cohen (13) and this crude extract was purified by HPLC procedures (14). The purity of EGF was determined by $NaDodSO_4$/PAGE, amino acid analysis, and sequence analysis, and its concentration was measured by amino acid analysis. $NaDodSO_4$/PAGE resulted in a single stained band with a molecular weight of ≈6000; furthermore, NH_2-terminal sequence analysis (data not shown) showed agreement of the first 50 amino acids with the published sequence of EGF-I (14, 15). Yields averaged 5 mg per batch. The biological activity was assessed by (*i*) a receptor-binding assay using A431 cell membranes (16) and (*ii*) *in vitro* mitogenesis (increased DNA content) of human foreskin fibroblasts and granulation tissue fibroblasts (17).

Wound Model. Four polyvinyl alcohol sponge discs (9.5 mg, 2 mm in thickness, 10 mm in diameter) were implanted subcutaneously beneath the ventral panniculus carnosus in male Sprague–Dawley rats (18). EGF was administered by slow release (10 or 20 μg/day) from cholesterol/methylcellulose/lactose pellets (0.5 × 3 mm discs) that were embedded on edge in the center of the sponge disc prior to implantation. The rate of release and the activity of EGF were confirmed by incubating a pellet in medium and estimating (by membrane assay) the amount of EGF secreted daily. Growth factor was released at a rate of 10 or 20 μg/day and EGF's ability to bind to its receptor was unimpaired. Each of the animals in the slow-release study received two sponges implanted with EGF pellets and two sponges implanted with placebo pellets. Animals were sacrificed by CO_2 asphyxiation at days 3, 7, and 14 after implantation, and the sponges were removed for histologic and biochemical evaluation.

Histology. Immediately after removal, sponges were fixed in buffered formalin. Sections were prepared and stained either with hematoxylin/eosin (H/E), periodic acid–Schiff, or Masson's trichrome to reveal various cellular and matrix elements.

Biochemical Procedures. After sacrifice, implanted sponges were cleanly dissected from the loosely adherent fat and muscle and weighed (wet weight). Sectors were cut from each sponge disc for DNA, protein, and collagen determinations.

Collagen Biosynthesis. The remainder of each sponge was minced finely in medium (Dulbecco's modified Eagle's medium) containing antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml). Minces were incubated as described (12). More than 95% of the newly synthesized collagen was recovered by combining three vigorous washes of the minces in phosphate-buffered saline together with incubation medium. Relative collagen synthesis was quantified from the amount of radioactivity released from a CCl_3COOH precipitate of medium with saline extracts into CCl_3COOH-soluble material after limit-digestion with highly purified bacterial collagenase (16). Total incorporation was determined as the sum of acid-insoluble radioactivities in the remaining tissue, the medium, and the saline extracts after precipitation with CCl_3COOH, as described (12).

Collagen Content. Collagen (hydroxyproline) content of the sponge was determined after hydrolysis *in vacuo* in constant-boiling HCl (110°C, 16 hr). Hydroxyproline was detected as its phenylisothiocyanate derivative by reverse-phase HPLC.

For phenylisothiocyanate derivatization, a portion of sponge granulation tissue (≈30 mg) was hydrolyzed as described, dried, taken up in 1 ml of "redrying solution" [ethanol/water/$(C_2H_5)_3N$, 2:2:1], and 100 μl was redried under N_2. Twenty microliters of derivatization reagent [ethanol/water/$(C_2H_5)_3N$/phenylisothiocyanate, 7:1:1:1] was added, and the reaction was allowed to proceed at room temperature for 20 min. The sample was then lyophilized during centrifugation (Savant Speed-Vac) to remove the reagents. Samples were either frozen at this stage or diluted to 1 ml with buffer (5% acetonitrile in 5 mM disodium phosphate, pH 7.4). A solution containing amino acid standards was dried and coupled in the same manner. The coupling efficiency was >99% under these conditions.

Other Assays. DNA and protein contents of the sponges were determined on NH_4OH extracts (0.1 M, overnight at 4°C) of minced sponge by employing a fluorimetric DNA assay (19) and the Bio-Rad protein assay, respectively.

RESULTS

Preliminary data suggested that sequential daily injections of 10 μg of EGF resulted in a greater organization of the sponges by fibroblasts than in controls. Collagen content at day 20 was elevated in EGF test sponges to 8.7 ± 1.4 mg (mean ± SD) compared with 6.9 ± 1.3 mg per sponge in saline controls. In addition, EGF increased angiogenesis and caused vascular dilation (18, 20).

The EGF stimulus was transient. ^{125}I-labeled EGF rapidly disappeared from its injection site, the interior of the sponge. By 4 hr after injection, only 10% of the initial radioactivity could be recovered from the interior of the sponge (data not shown). To overcome this transient exposure, slow-release pellets were used.

Slow Release of EGF. Sponges containing slow-release pellets with EGF or placebo pellets were implanted as described. Three days after implantation of sponges containing EGF or placebo pellets, little histologic organization was found. The sponges contained predominantly fibrin and inflammatory cells and no difference due to EGF release was observed at 3 days. By contrast, a striking difference was observed at day 7. EGF-treated sponges were almost completely organized by granulation tissue (Fig. 1*A*), whereas placebo controls were only about 50% organized by granulation tissue (Fig. 1*B*). In addition to mediating a pronounced increase in cellularity and increased visible collagen, slow-release EGF stimulated the presence of many more capillaries, often widely dilated (Fig. 1 *C–E*). Fourteen days after implantation all of the sponges were fully organized; thus, no morphologic difference could be demonstrated between EGF test sponges and placebos. Only a minimal inflammatory response, concluded by day 7, occurred adjacent to EGF-containing or placebo pellets (Fig. 1*F*).

The dramatic changes induced by slow-release EGF at day 7 were confirmed quantitatively. Slow release of EGF (10 μg/day and 20 μg/day) mediated a marked increase in DNA content (Fig. 2*A*), protein content (Fig. 2*B*), and wet weight (Fig. 2*C*) at this time period. The lower dose of growth factor (10 μg/day) increased DNA content, protein content, and wet weight over controls by 100%, 33%, and 28%, respectively. These effects were not enhanced by a higher dose (20 μg/day). No biochemical differences between control and experimental sponges were found at day 3 or day 14, but the histologically observed increase in collagen due to slow release of EGF was also confirmed biochemically (Fig. 3*A*). At day 7, sponges receiving 10 μg of slow-release EGF per day contained 1.14 ± 0.11 mg of collagen, whereas placebo controls contained only 0.81 ± 0.08 mg ($P < 0.002$). These findings are in agreement with the histologically observed degree of organization. The higher dose of growth factor produced no further increase in collagen content. The increase in collagen content (41%) was not reflected in an increase in relative collagen synthesis (Fig. 3*B*). At all time periods, EGF-treated sponges in short-term explant culture showed relative rates of collagen synthesis similar to controls—namely, 4–6% of total protein synthesis.

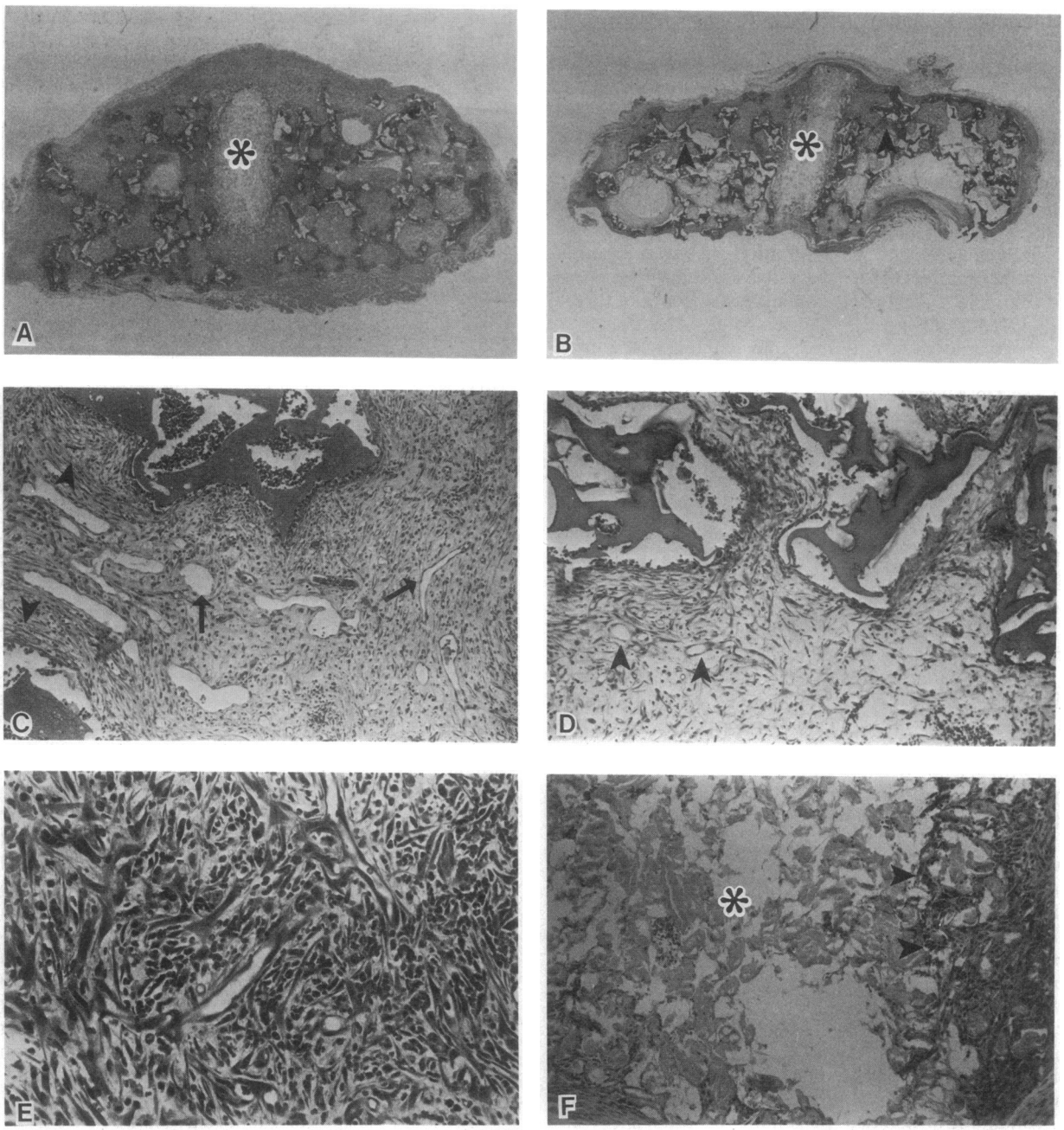

FIG. 1. Morphological features of sponges after sustained release of EGF. (*A*) EGF pellet sponge (10 μg/day) at 7 days. Pellet (*) is in the center of the tissue. The sponge (staining darkly) is almost completely organized by vascular granulation tissue (H/E, ×35.) (*B*) Placebo pellet sponge at 7 days. Pellet (*) is indicated. Granulation tissue has penetrated only to the extent marked by pointers. (H/E, ×35.) (*C*) EGF pellet sponge (10 μg/day) at 7 days, 200 μm from surface. Note dilated capillaries (arrows), dense cellularity, visible collagen fibers (arrowheads), and complete organization. (H/E, ×100.) (*D*) Placebo pellet sponge at 7 days, 200 μm from surface. Compare with *C* to observe fewer capillaries (arrowheads), less dense cellularity, and less visible collagen than in *C*. The placebo pellet evoked a minimal chronic inflammation response on fragmentation. (H/E, ×100.) (*E*) Center of high-dose (20 μg/day) EGF pellet sponge at 7 days. Note dense cellularity, proliferating capillaries, and organizing fibroblasts. (H/E, ×200.) (*F*) EGF-containing pellet (*) at 7 days. A foreign-body giant cell response (arrowheads) surrounds fragments of the pellet. The same reaction was observed in controls. No other inflammatory components are present. (H/E, ×80.)

DISCUSSION

The biological function of EGF is uncertain, and we explored the hypothesis put forward by Carpenter (21) that this mitogen may promote wound repair. Purified, injected EGF qualitatively accelerated wound repair in a carefully studied model of this process, the sponge granuloma (18). The cellular interactions of EGF suggested that the wound-healing properties of EGF might be enhanced by constant exposure of the tissue to the growth factor, in this case, from a slow-release pellet embedded in the sponge. The pellets released 400–800 ng of EGF per hour, as compared with endogenous plasma levels of 1.4 ng/ml (22) and (mouse) skin content of 60 ng/g of wet weight (23). Indeed, this method of delivery of growth factor resulted in a much more dramatic stimulation of wound repair than did sequential daily injection. This stimulation was demonstrated by a more rapid influx of granulation tissue and neovascularization. Control and experimental granulation tissue had reached the same level of complex organization 2 weeks after implantation.

Activity of EGF. EGF appeared to promote the rate of wound repair in the sponge granuloma model. The effect of daily injections was discernable but not dramatic. Topical application of EGF has also been reported to enhance the

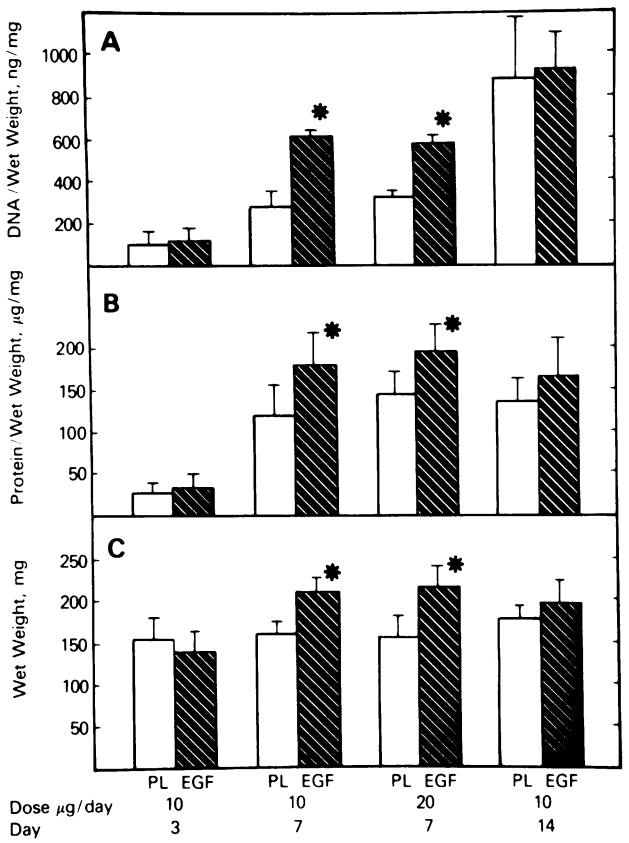

FIG. 2. Effects of slow-release EGF on granulation tissue growth. Pellets formulated to release either 10 or 20 μg of purified EGF per day for at least 15 days were embedded in the center of the sponge immediately prior to implantation. Animals were sacrificed on days 3, 7, and 14 and the wet weights of the sponges were noted (*C*). Analyses of DNA (μg, *A*) and protein (μg, *B*) content (expressed per mg of wet weight) were made. EGF results are shown as hatched bars and are compared with placebo-treated (PL) pellets (open bars). Standard deviations of the mean of 10 sponges are shown and statistically significant increases ($P < 0.02$; one-tailed, paired *t* test) are shown by an asterisk.

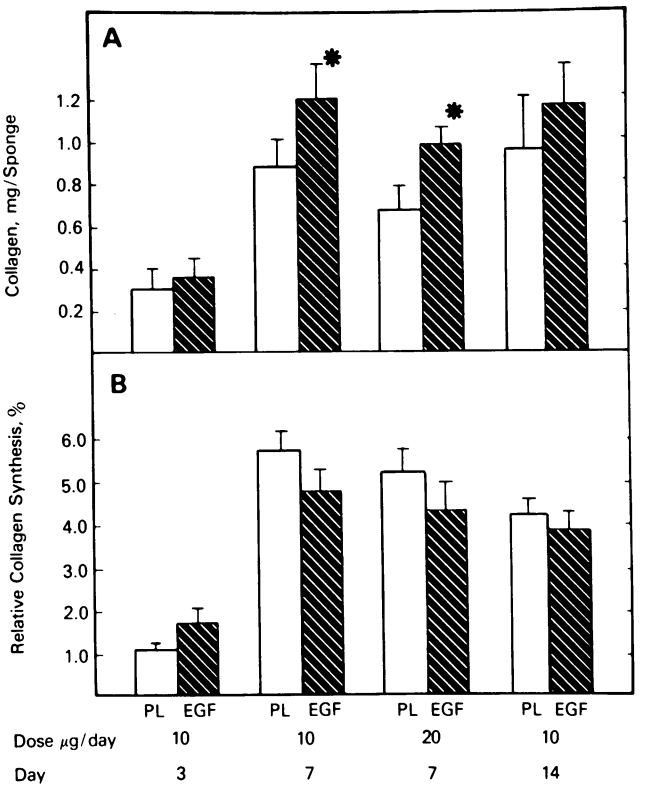

FIG. 3. Effects of slow release of EGF on collagen accumulation of granulation tissue. EGF (10 or 20 μg/day) was released from pellets embedded in sponge granulomas. Collagen content (*A*) was estimated at days 3, 7, and 14 by HPLC of hydrolyzed tissue after phenylisothiocyanate derivatization. Reverse-phase HPLC separation of phenylisothiocyanate amino acids was performed on an Altex Ultrasphere ODS 5-μm column (4.6 × 15 cm). Automatic injection was accomplished by a Waters Wisp 718 auto-injector and two Waters M-6000 pumps were controlled by a Waters 680 gradient controller. A Waters 440 absorbance detector set at 254 nm was connected via a Nelson Analytical 760 series interface to a Hewlett–Packard 9816 computer programed with the Nelson 3600 series chromatography software. Ten microliters of each dissolved, derivatized amino acid mixture was applied to the column and eluted in a gradient from 100% buffer A [0.05% (vol/vol) $(C_2H_5)_3N$ in 0.14 M sodium acetate, pH 6.4] to 46% buffer B (60% acetonitrile in 0.5% CF_3COOH) over 10 min at 1 ml/min using a convex curve (curve 5). An additional step of 100% buffer B was used to wash the column prior to reequilibration. Hydroxyproline was well-separated from all other phenylisothiocyanate-derivatized amino acids and was quantified by comparison of peak areas with that of phenylisothiocyanate-derivatized hydroxyproline from the standard amino acid mixture. Collagen content of each sample was estimated on the assumption that the average rat skin collagen α-chain contained 10% hydroxyproline residues. Values are expressed as mg of collagen per sponge. Collagen synthesis (*B*) was estimated as percent of total protein synthesis in granulation tissue. EGF results are shown as hatched bars and are compared with placebo-treated (PL) sponges (open bars). Standard deviations of the mean of 10 sponges are shown and statistically significant increases ($P < 0.02$; one-tailed, paired *t* tests) are shown by asterisks.

closure of surface wounds in mice (6). Hiramatsu *et al.* (7), using cotton pellets as a model for wound repair, also demonstrated that EGF could mediate an increase in wet weight and collagen content.

Other cellular growth factors have wound-healing properties. β-Transforming growth factor, in concert with EGF, accelerated the accumulation of protein, collagen, and DNA in a wound chamber model (10), and the primary effect was presumably increased EGF receptor number (24). In addition, we have recently demonstrated the capacity of CDGF to accelerate wound repair (12).

Collagen Accumulation. There is a dispute in the literature as to whether EGF is capable of stimulating collagen synthesis. Collagen prolyl hydroxylase activity was stimulated by EGF in the cotton-pellet granuloma system of Hiramatsu *et al.* (7); however, the significance of this is uncertain since collagen synthesis was not directly determined in that study, and this enzyme activity is usually not rate-limiting for collagen synthesis (25). These authors also reported an EGF-mediated increase in this enzyme in cloned osteoblastic cells, yet a decrease in collagen synthesis was noted (26). In addition, EGF reduced collagen fiber formation and stimulated the production of type III collagen by osteoblastic cells (9).

Our results confirmed previous studies involving human gingival fibroblasts (8). As reported here, 7 days after implantation of sponges containing slow-release EGF, there was a significant increase in collagen content of sponges. The presence of more collagen, however, did not appear to be a result of specific stimulation of collagen synthesis, since relative synthetic rates were not influenced by EGF. Rather, collagen accumulation appeared to be the result of an equivalent rate of synthesis by many more cells or an increase in total protein production, which requires correction for precursor pool size. The formal possibility that absolute rates of production of collagen were altered remains to be tested.

Role of Continuous Exposure. Why was constant exposure to EGF more efficient in promoting the rate of wound repair

as compared with repeated injections? The interaction between EGF and cells that culminates in a mitogenic response is initiated by binding of EGF to cells at its high-affinity cell surface receptor (27, 28), yet initiation of DNA synthesis requires at least an 8- to 12-hr exposure to EGF. Throughout this period the number of EGF surface receptors is reduced by down-regulation (3), and, *in vitro*, the concentration of free EGF in the medium is simultaneously lowered as a result of cellular uptake and degradation. Previous studies showed that the total number of EGF receptors at the cell surface declined after the addition of EGF until a low, constant amount of receptor occupancy was achieved after about 6 hr (3, 29). It was concluded that this number of persistently occupied receptors determines the degree of mitogenic stimulation (30).

Despite high concentrations, injected ^{125}I-labeled EGF was rapidly cleared from the granulation tissue, possibly due in part to increased receptor-mediated endocytosis. Thus, when EGF was administered by multiple injections, the amount of EGF available to cells during the interval when receptor occupancy could have induced cell division was probably minimal. The more marked response seen with the slow-release system was likely due to the low, steady exposure to the growth factor. These results are consistent with the steady-state model of Knauer *et al.* (30), which proposed that the major regulatory step in EGF-stimulated growth (*in vivo*) may be at the level of EGF receptor occupancy and that the degree of occupancy is proportional to the continuous availability of the hormone. It is possible that the function of the so-called "EGF carrier protein" (31, 32) is to regulate the availability of the growth factor to the receptor, as in the case of the insulin-like growth factor (11).

EGF may simply exert its wound-healing properties via a mitogenic effect on granulation tissue, or it may involve recruitment or interactions among other factors and multiple cell types. Despite the fact that EGF has been shown to be mitogenic for many cell types *in vitro*, we have no direct evidence that the increase in cellularity that we observed *in vivo* was purely due to mitogenesis. Other growth factors are known to be potent chemoattractants: platelet-derived growth factor (33, 34) and CDGF (12). It is possible that EGF mediated its effect via a mixture of both mitogenesis and recruitment, although EGF itself is not a chemoattractant for fibroblasts (33) or inflammatory cells (34).

Additional complexities of EGF action *in vivo* include decreased receptor–ligand affinity induced by platelet-derived growth factor (35), increased affinity from retinoids (36, 37), or increased receptor number from β-transforming growth factor (22). With the development of the slow-release delivery system it should be possible to determine the mechanism by which EGF promotes the rate of wound healing *in vivo*.

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