JOURNAL OF SURGICAL RESEARCH 59, 236–244 (1995)

Muy buen modelo

Dato ya conocido

Effect of Growth Factors on Cell Proliferation and Epithelialization in Human Skin

in vitro

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Submitted for publication May 27, 1994

The failure of chronic wounds to heal remains a major medical problem. Recent studies have suggested an important role for growth factors in promoting wound healing. We investigated the mitogenic effect of basic fibroblast growth factor (FGF), insulin-like growth factor-1 (IGF-1), and epidermal growth factor (EGF), comparing their effects with those of media alone (MEM) in a human skin explant model. A stable organ culture system for maintaining the histologic structure of human epidermis for 10 days *in vitro* was developed. DNA synthesis was measured on Days 1, 3, and 7 of organ culture using [^{3}H]thymidine ([^{3}H]thy) uptake and expressed as cpm/mg dry weight (mean ± SEM). FGF, IGF-1, and EGF were each capable of stimulating [^{3}H]-thy uptake on Day 1 of culture (2372 ± 335 FGF, 2226 ± 193 IGF-1, 4037 ± 679 EGF vs 1108 ± 70 MEM, $P < 0.05$). IGF-1 and EGF also stimulated [^{3}H]thy uptake on Days 3 and 7 of culture. The organ culture system was further employed to observe epidermal outgrowth. Longest keratinocyte outgrowth from the explant periphery (simulating epithelial regeneration from the wound edge) was observed on Day 7. EGF resulted in maximum stimulation of epithelial outgrowth (440 ± 80 μm), followed by FGF (330 ± 56 μm), IGF-1 (294 ± 48 μm), and MEM (189 ± 50 μm). We postulate, therefore, that FGF, IGF-1, and EGF are important mitogens for wound healing and that EGF in particular is capable of stimulating epithelialization. IGF-1 and EGF may play significant roles in both the early and late wound environments, while FGF may be most important during the early events of tissue repair.

INTRODUCTION

The healing of an adult skin wound is a complex affair of some considerable clinical interest. Endogenous mitogenic peptides including basic fibroblast growth factor (FGF), insulin-like growth factor-1 (IGF-1), and epidermal growth factor (EGF) are released at the wound site and presumed to be an essential part of the wound healing machinery [1–6]. Further, each of these growth factors has been shown to enhance healing when added exogenously to healing wounds [7–10].

The majority of investigations on cutaneous wound healing have concentrated on dermal repair and wound bed remodeling, with little emphasis on epidermal regeneration [11, 12]. Epidermal wound healing is a complex process involving both keratinocyte migration and proliferation from the wound edge under growth factor influence and direction [14]. We developed an *in vitro* model of wound healing, especially epidermal wound healing, to study the temporal relationship of FGF, IGF-1, and EGF on the rate of DNA synthesis in a human skin explant model. Organ culture *in vitro* is one of the most useful experimental models to study wound healing and tissue repair. It closely reflects the wound environment and provides a precisely controlled milieu to study the healing process. In addition, cell–cell interaction is maintained, allowing observation of the behavior of epidermal cells in three dimensions. This procedure has been tried in various ways [13–16]. Satisfactory results have not always been obtained because the epidermis of the explants has tended to become necrotic in various stages of explant culture. Here we report the use of a suitable skin organ culture system that is stable for 10 days and is appropriate to study the effects of various growth factors and drugs. This model allows the study of specific variables affecting the healing process in the absence of circulating factors or innervation.

Our aim was to assess and compare the effects of three growth factors, FGF, IGF-1, and EGF on wound healing phenomenon in our explant model. We utilized our model in two ways. First, we evaluated the effectiveness and time course of growth factor application on cell proliferation in the wound as determined by measurement of [^{3}H]thy uptake on Days 1, 3, and 7 of organ culture. Our second approach was to study epithelialization in response to growth factor treatment. The cut ends of the

Presented at the Annual Symposium of the Association of Veterans Administration Surgeons, Reno, Nevada, May 5–7, 1994.

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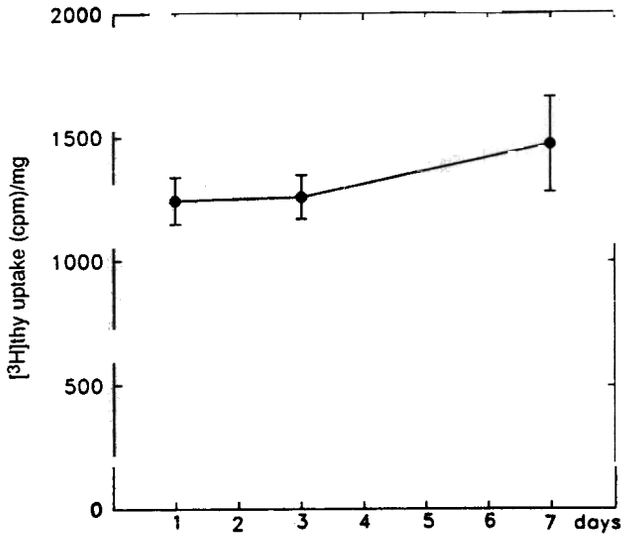


FIG. 1 Time course of [^{3}H]thy uptake by explants incubated in MEM.

explants act as wound edges, and keratinocyte outgrowth from the explant periphery (simulating epithelial regeneration from the wound edge) was used as a yardstick of the ability of the various growth factors to promote epithelialization.

MATERIALS AND METHODS

Chemicals

FGF, IGF-1, and EGF were obtained from Collaborative Biomedical Products (Bedford, MA). Iscove's modified Dulbecco's medium (MEM), fetal bovine serum (FBS), and antibiotic/antimycotic (penicillin, gentamycin, and nystatin) were obtained form GIBCO (Long Island, NY). NCS-II solubilizer for liquid scintillation counting, BCS-NA Biodegradable scintillation cocktail, and methyl [^{3}H]thy were obtained from Amersham (Arlington Hills, IL).

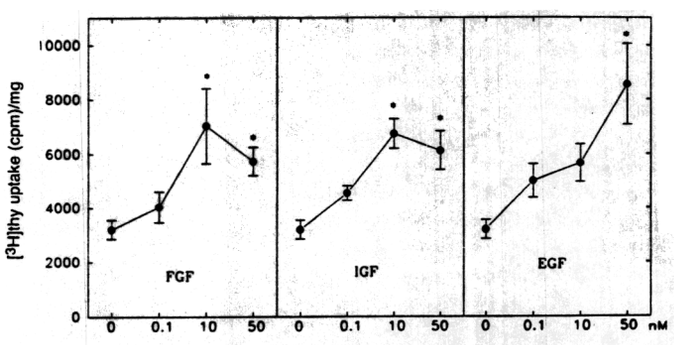


FIG. 2. Dose-response curves performed for FGF, IGF-1, and EGF (*$P < 0.05$ compared to MEM).

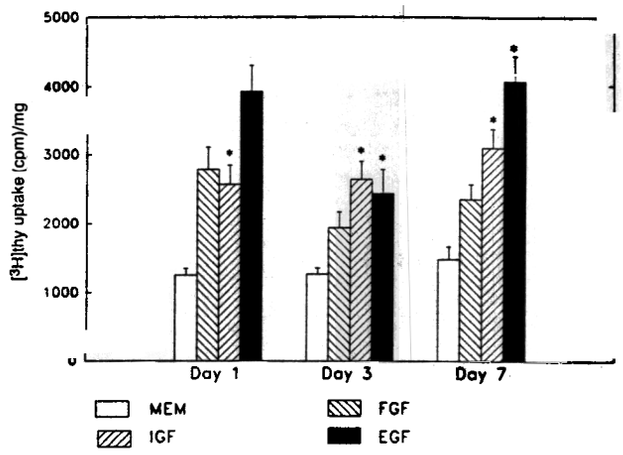


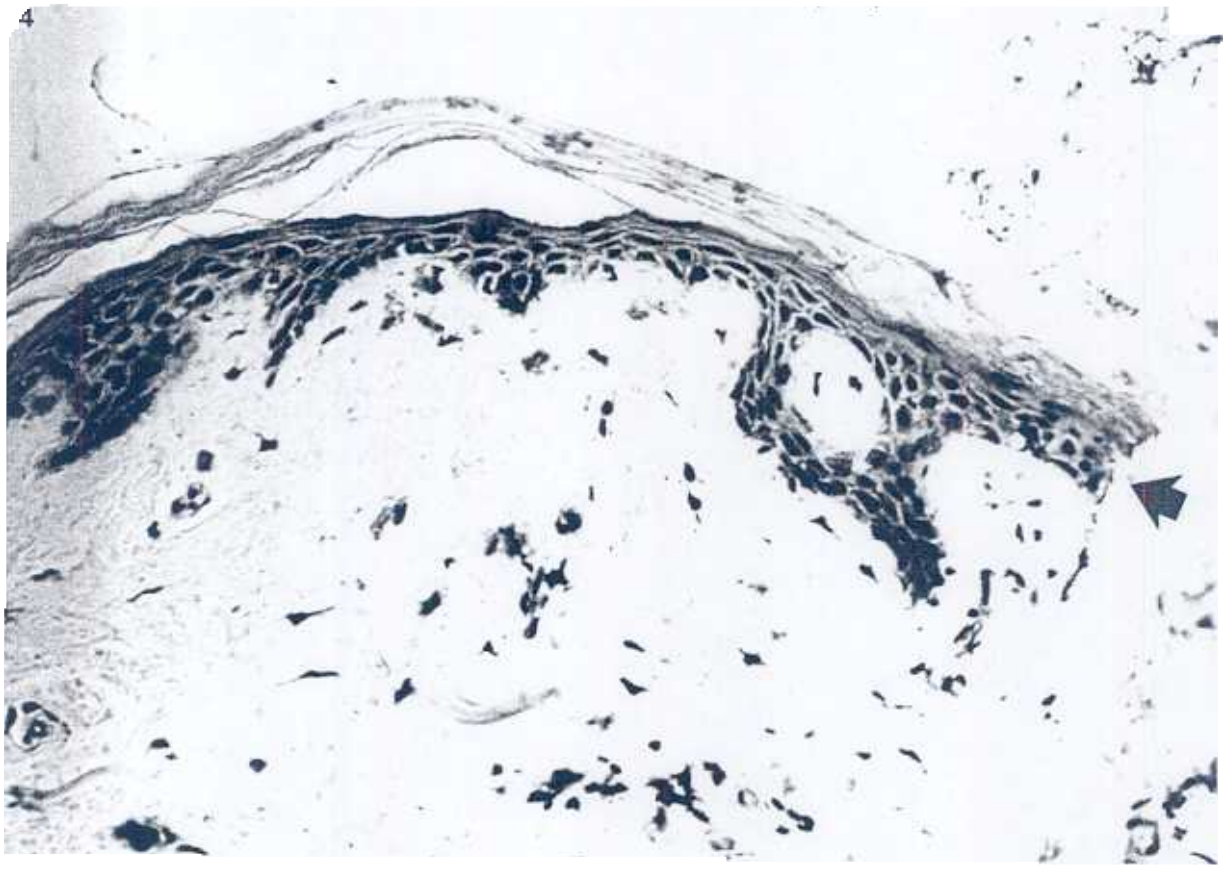
FIG. 3. Time course of [^{3}H]thy uptake by explants stimulated with FGF, IGF-1, and EGF compared to control (*$P < 0.05$ compared to MEM).

Wound Healing Model

DNA synthesis. Full thickness skin was obtained from the amputated lower extremities of human subjects after voluntary consent and in accordance with institutional guidelines. Weight and size standardized pledgets of skin (6 × 8 mm) were trimmed of excess adipose tissue and incubated dermal side down in MEM ± growth factor with 0.25% bovine albumin, 1% antibiotic/antimycotic, and 1% FBS at 37°C in 5% CO_2. Medium containing the appropriate growth factors was changed every 48 hr. DNA synthesis was measured on Days 1, 3, and 7 of culture by pulsing the explants with [^{3}H]thy (3.6 μCi/ml) for 18 hr before harvest. At the end of the incubation periods, the explants were washed 3× with cold phosphate buffered saline (PBS), desiccated for 48 hr, weighed to obtain dry weight, partially rehydrated, and solubilized by incubating with 300 μl of tissue solubilizing reagent for 48 hr at 45°C. The solubilized homogenates were cooled, neutralized with 10 μl of glacial acetic acid, and counted in 2 ml of scintillation liquid. Results are expressed as [^{3}H]thy uptake (cpm)/mg dry weight (mean ± SEM).

Epidermal outgrowth. Epithelial outgrowths were observed from the cut ends of the explants incubated in MEM ± growth factor in 5% FBS, with maximum growth seen on Day 7. Explants incubated longer than 7 days did not result in increased epidermal outgrowth. Hematoxylin and eosin stained sections were analyzed and epithelial outgrowths measured in micrometers using ocular microscopy. Measurements were made from the cut ends of the explants and confirmed by an independent pathologist unaware of the treatment groups. We conducted four separate experiments using MEM, FGF, IGF-1, and EGF. The results of three experiments with the longest outgrowths on Day 7 were analyzed.

Immunohistochemistry. The skin explants were fixed in 10% buffered formalin, dehydrated in serial al-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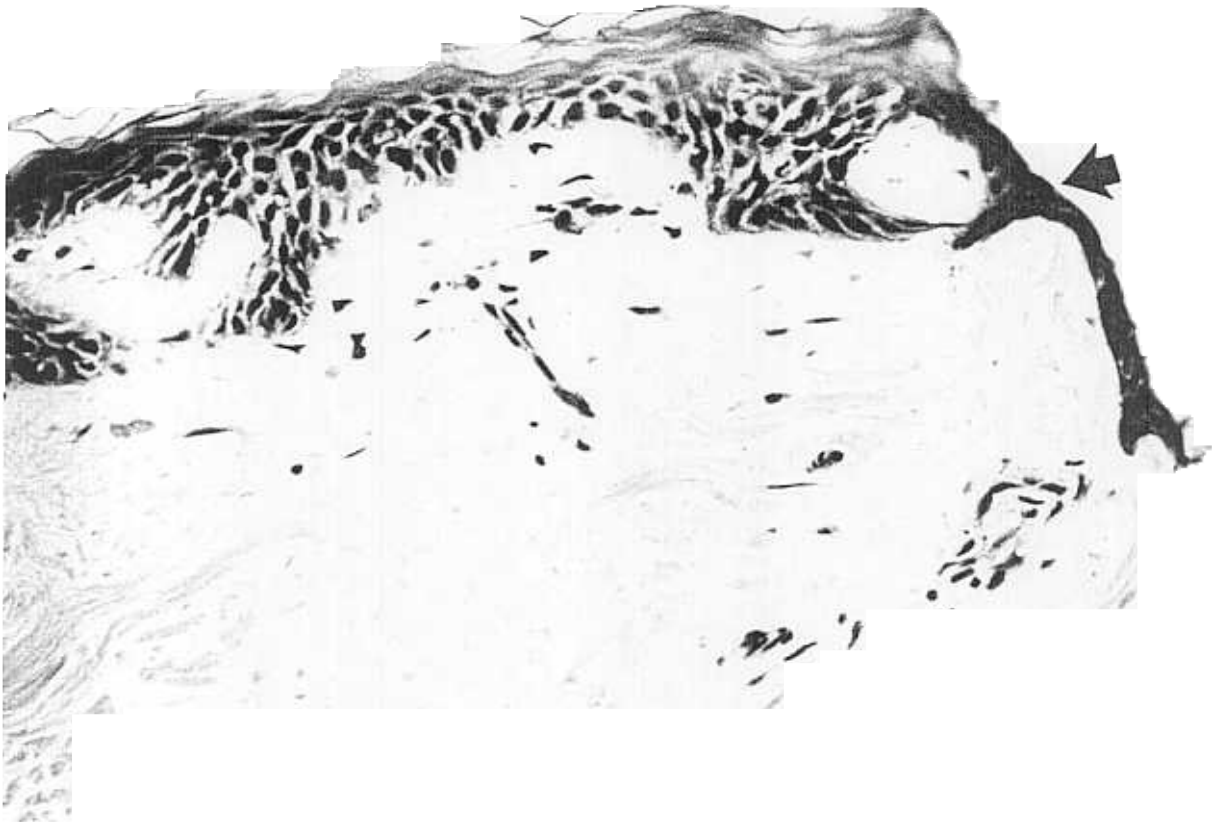

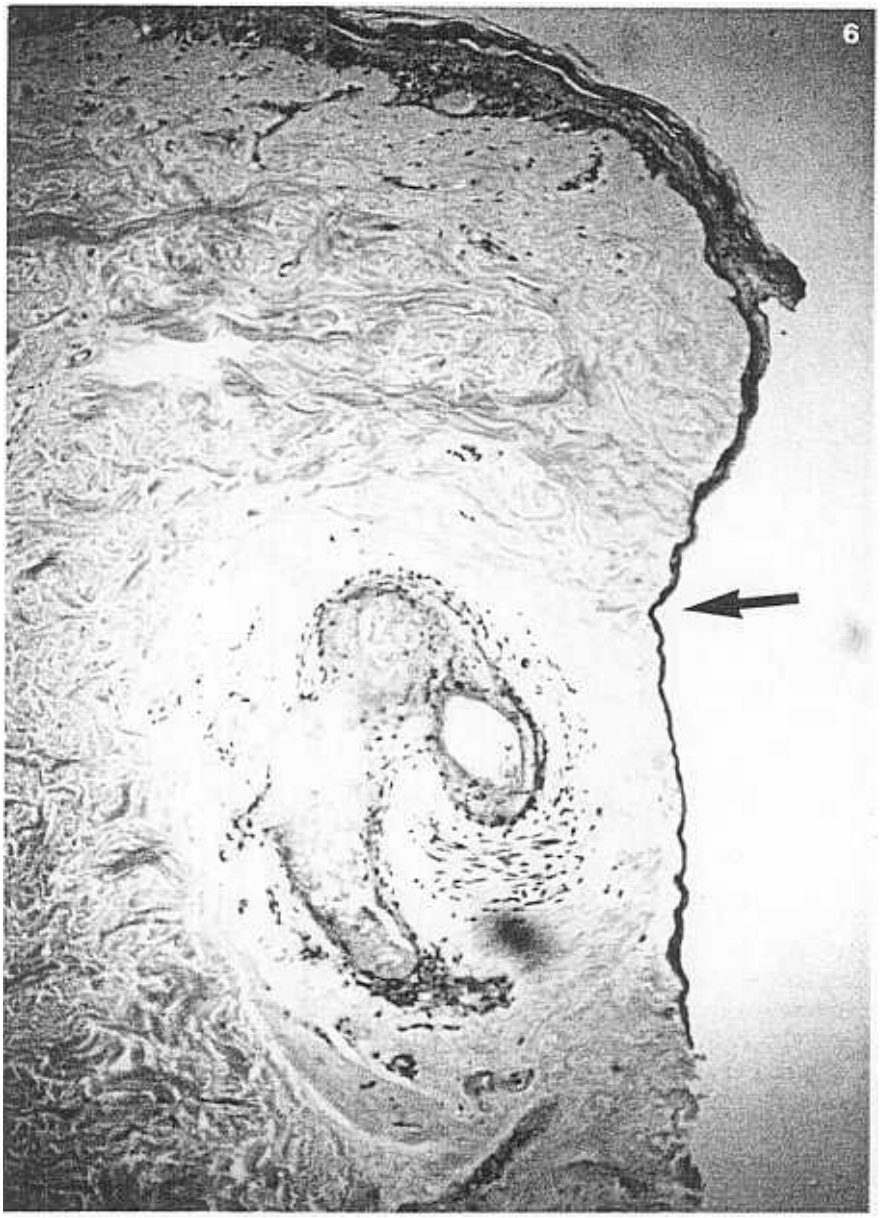

FIG. 6. Histologic appearance of an explant on Day 7 of culture. The outgrowth from the basal cells of the epidermis now extends 502 μm down the adjacent cut end (arrow), epithelializing about 80% of the dermis. Both the outgrowth and the rest of the epidermis show normal histology (MEM, ×180).

cohol baths, and embedded in paraffin. Five-micrometer sections were cut and mounted on polylysine-pretreated slides. Staining was performed for α-cytokeratin using mouse monoclonal anti-α-cytokeratin (Sigma Immunochemicals Pan Cytokeratin Kit). The slides were deparaffinized in xylene, rehydrated in descending serial alcohol baths, and exposed to blocking serum to inhibit nonspecific binding of the primary antibody. The slides were then incubated with the α-cytokeratin antibody at 37°C for 60 min. Normal mouse serum was used as a negative control. The slides were washed and a biotinylated secondary antibody (goat anti-mouse immunogloblin in

FIG. 4. Histologic appearance of an explant at the beginning of incubation. The cut end of the explant is seen on the right (arrow). No outgrowth is seen at this time. The rest of the epidermis is normal in appearance (×365).

FIG. 5. Histologic appearance of an explant on Day 3 of culture. The basal cells of the epidermis show hyperplasia and an epidermal outgrowth (arrow) measuring 82 μm extends from the basal cells along the adjacent cut dermis (MEM, ×365).

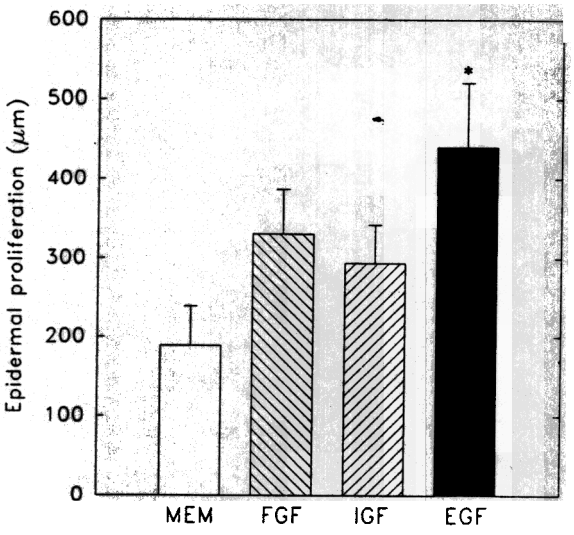

FIG. 7. Epidermal outgrowth in response to growth factor treatment measured on Day 7 (*$P < 0.05$ compared to MEM).

buffered saline) was applied and the slides were incubated at 37°C for 30 min. Following washing, ExtraAvidin-conjugated peroxidase was applied for 30 min. A third washing was followed by application of a substrate reagent (acetate buffer, AEC Chromogen, and 3% hydrogen peroxide) for about 10 min while chromogen development was monitored. The slides were then counterstained with Mayer's hematoxylin.

Electron microscopy. Transmission and scanning electron microscopy were performed on freshly obtained skin specimens and skin-incubated for 7 days. Briefly, the skin specimens were washed with PBS ×3 and fixed with phosphate-buffered 2% paraformaldehyde–1% glutaraldehyde for 15 min. Fresh fixative was added for another 60 min at room temperature. After fixation, the specimens were washed ×3 with buffered sucrose (0.1 *M* phosphate buffer, 5% sucrose solution, pH 7.4) for 5 min and postfixed at 4°C for an additional 60 min in phosphate-buffered 2% osmium tetraoxide. Dehydration was carried out using a graded series of ethanols. Specimens for scanning electron microscopy were critically dried using liquid CO_2, coated with gold/palladium using a sputter coater, and examined and photographed with a Model DSM 960 Zeiss scanning electron microscope. Specimens for transmission electron microscopy were embedded in Durcurpan ABCD (Sigma) and cured according to the manufacturer's specifications. Semithick sections were then stained with 0.5% Toluidine blue (GIBCO). Finally, ultra-thin longitudinal and cross sections were mounted on 300 mesh copper grids (6 grids/area). The specimens were enhanced with uranyl acetate and lead citrate and viewed under the transmission electron microscope (Zeiss EM 10 CA) at 80 kV. Photographs were taken with electron microscopy film (Kodak, Estar, Thick base, 4489, Rochester, NY).

Statistical Analysis

One-way analysis of variance was used to compare different groups. All results were obtained from at least three experiments, with each treatment group having 5–6 explants. $P < 0.05$ was accepted as being significant.

RESULTS

[^3H]Thy Uptake and DNA Synthesis

[^{3}H]Thy uptake and DNA synthesis were measured on Days 1, 3, and 7 of explant culture in media without added growth factors. The explants showed constant rates of DNA synthesis at all three time points investigated, demonstrating good explant viability and model stability (Fig. 1).

Dose–response curves were performed on Day 1 for each of the growth factors using concentrations of 0.1, 10, and 50 n*M*. FGF and IGF-1 treatment resulted in maximal stimulation of DNA synthesis at a concentration of 10 n*M*, while EGF stimulated maximally at 50 n*M* (Fig. 2). These concentrations were used for subsequent experiments.

DNA synthesis was then measured on Days 1, 3, and 7 of explant culture with growth factor treatment and compared with explants incubated in media without growth factors. FGF, IGF-1, and EGF each resulted in stimulation of DNA synthesis on Day 1 of culture compared to that in media alone (FGF, 2372 ± 335; IGF-1, 2226 ± 193; EGF, 4037 ± 679 vs MEM, 1108 ± 70, $P <$ 0.05). IGF-1 and EGF in addition significantly stimulated DNA synthesis on Days 3 and 7 of culture (IGF-1, 2363 ± 169 Day 3, 2705 ± 531 Day 7; EGF, 1874 ± 323 Day 3, 3725 ± 660 Day 7 vs MEM, 1434 ± 151 Day 3, 1394 ± 280 Day 7, $P < 0.05$). Although FGF tended to increase DNA synthesis on both Days 3 and 7 when compared to that of baseline, the results were not significant. (Fig. 3).

Epidermal Outgrowth

Epidermal outgrowth was observed from the cut ends of the explants, extending out from the basal epidermal cells and epithelializing the adjacent exposed dermis. These outgrowths increased in length over time with maximum growth seen on Day 7, when about 80% of the exposed cut dermis was found to be epithelialized (Figs. 4, 5, and 6). EGF treatment resulted in maximum stimulation of epithelial outgrowth (440 ± 80 μm vs 89 ± 50 μm MEM, $P < 0.05$), followed by FGF (330 ± 56 μm) and IGF-1 (294 ± 48 μm) (Fig. 7). The epithelial nature of the outgrowths was demonstrated by staining with anti-α-cytokeratin antibodies. Transmission and scanning electron microscopy performed on Day 7 specimens showed that the outgrowths were composed of multiple layers of keratinocytes arising from the basal layer of the epidermis (Figs. 8, 9, and 10). Emulsion autoradiography

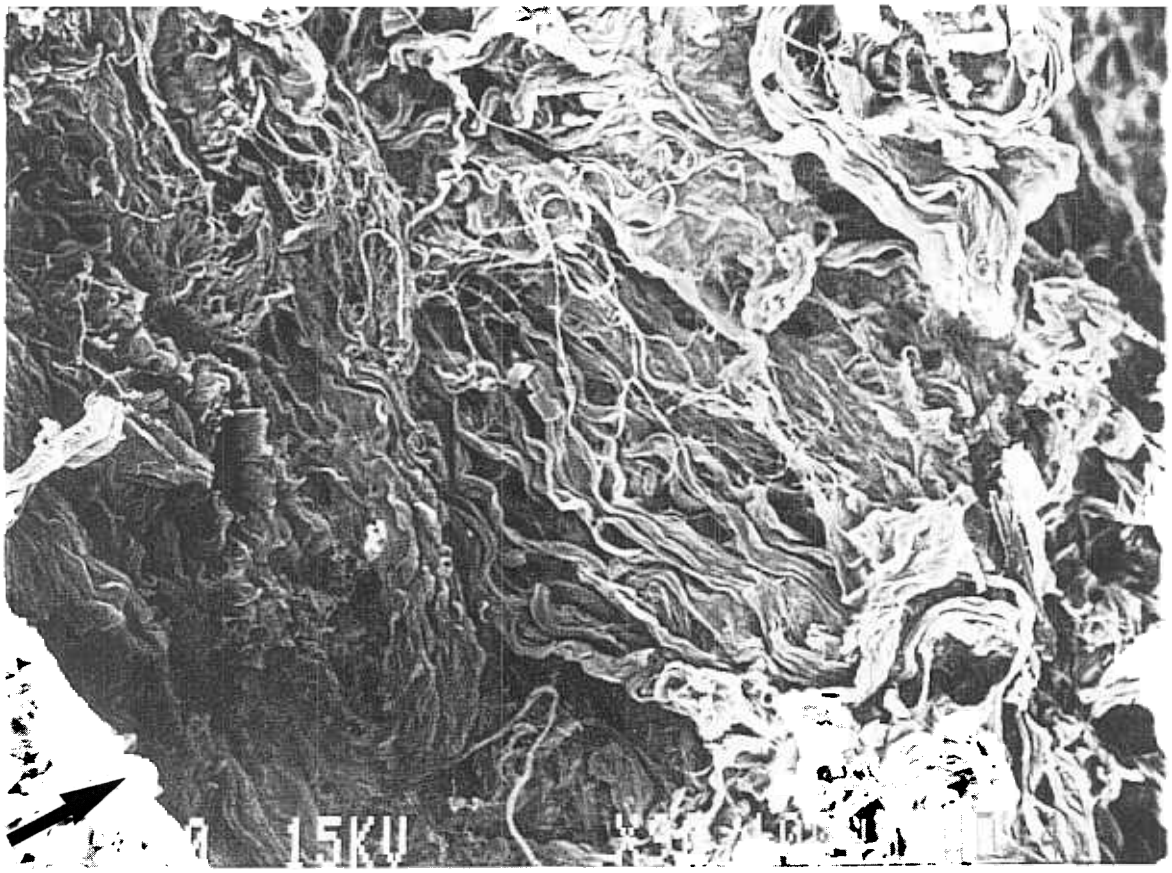

FIG. 8. Scanning electron micrograph of an explant at the beginning of incubation. The keratinized cut end of the explant is seen on the left (arrow), with exposed collagen strands of the dermis seen beyond it. No epidermal outgrowth is seen at this time (×1000).

of [^{3}H]thymidine incorporation showed that the vast majority of the thymidine uptake was in the germinal layer of the epidermis (Fig. 11).

DISCUSSION

Growth factors are believed to play an important role in cellular proliferation and wound healing and are capable of stimulating mitosis of quiescent cells in a nutritionally complete medium lacking serum [2]. We used a human skin explant model to explore two features of growth factor effect: cellular proliferation as measured by [^{3}H]thy uptake and epithelial outgrowth measured morphometrically.

Our results show that proliferation of cells in human skin explants occurs at a constant rate over 7 days and is increased by growth factor treatment. Light and electron microscopy confirm good preservation of tissue and epidermal viability, demonstrating model stability. Regarding proliferation, we hypothesized that different growth factors would differ in their effectiveness in stimulating cellular proliferation and in the time course of their effect. Although significant [^{3}H]thy incorporation in human skin organ cultures incubated with EGF for 48 hr has been reported [19], we extended these studies by comparing the effect of EGF with other growth factors and at different time periods. Hence we examined the temporal effect of FGF, IGF-1, and EGF on the rate of DNA synthesis on Days 1, 3, and 7 in human skin in an attempt to identify particular periods during wound healing when an individual factor would be most effective. Our results show that FGF, IGF-1, and EGF each induced a "mitotic phase" as early as 24 hr after organ culture. This mitotic phase persisted on Days 3 and 7 in explants treated with IGF-1 and EGF and was diminished somewhat in FGF-treated cultures in that it did not reach statistical significance in our experiments. FGF may thus be exerting its maximum effect in the early stages of wound healing, while IGF-1 and EGF appear to increase mitogenesis for at least 7 days in culture, establishing their potential importance in both early and late wound environments.

Most growth factors stimulate mitosis of more than one cell type involved in wound healing. However, each factor exhibits some degree of selectivity. EGF is an effective mitogen for epidermal cells. FGF is an effective mitogen for fibroblasts, keratinocytes, and endothelial cells, and IGF-1 primarily stimulates fibroblasts and

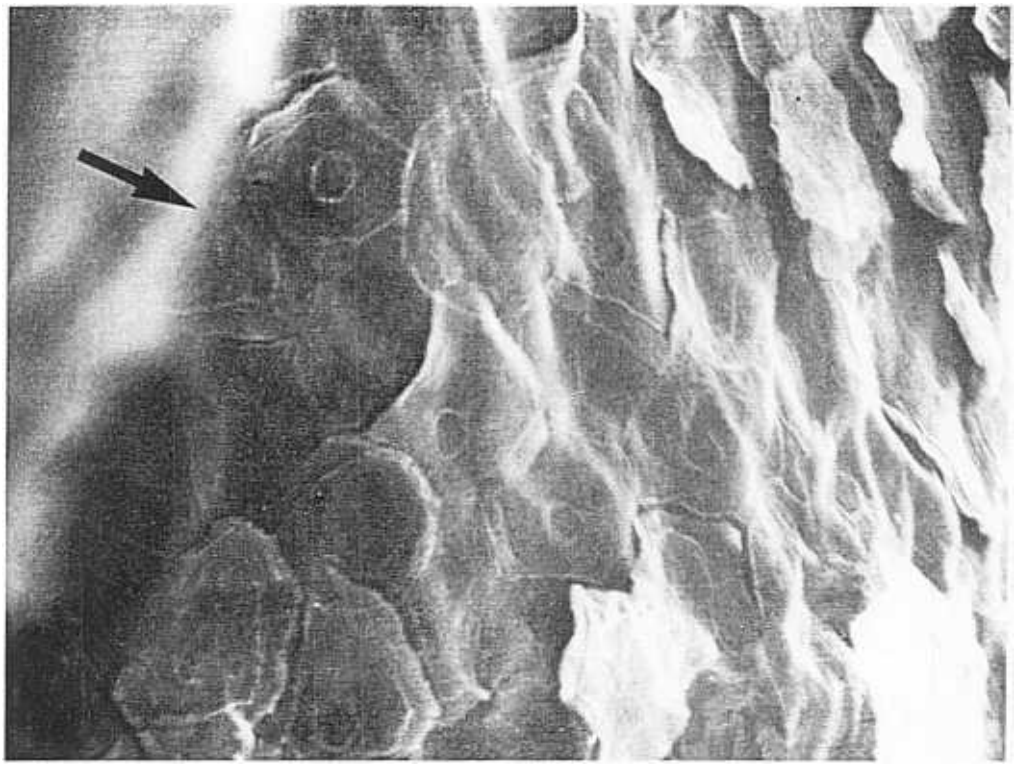

FIG. 9. Scanning electron micrograph of an epidermal outgrowth on Day 7 of culture. The keratinized cut end of the explant is seen (arrow), with epithelial cells extending beyond it. The epithelial cells have normal morphology with well-defined cell borders (×550).

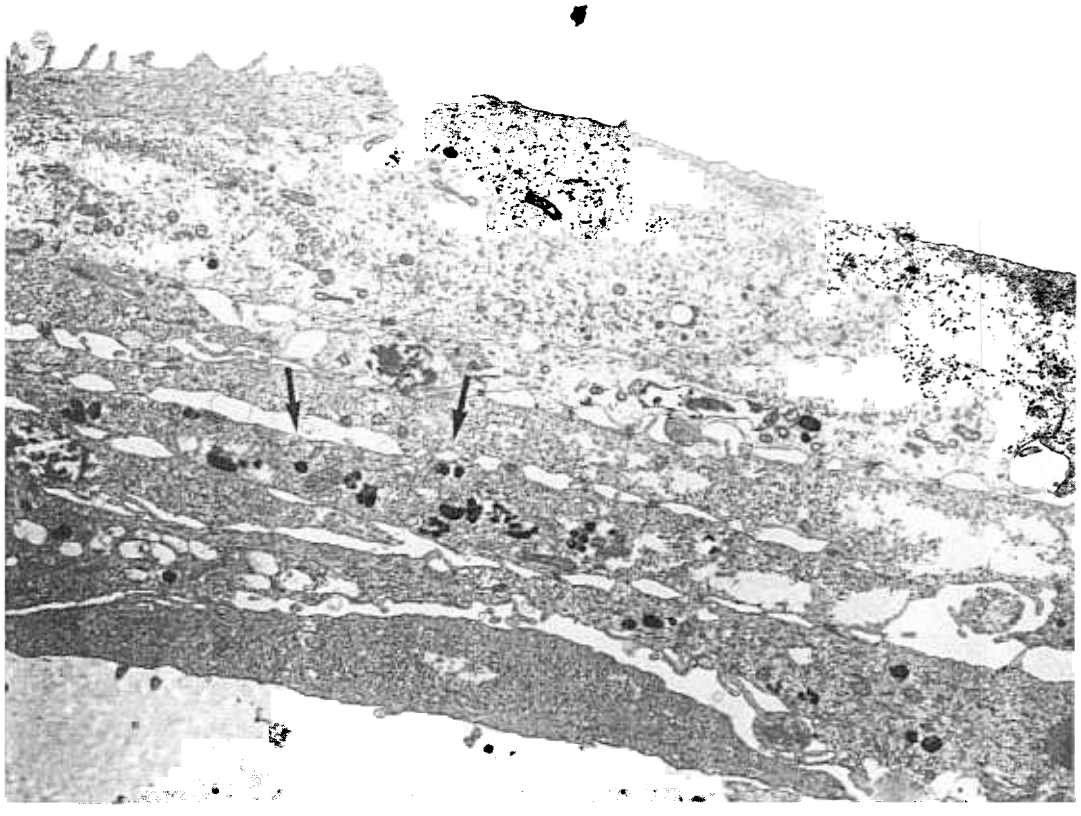

FIG. 10. Transmission electron micrograph of an epithelial outgrowth on Day 7. The outgrowth is seen to be comprised of multiple layers of keratinocytes arising from the basal layer of the epidermis. Characteristic keratin granulations (arrows) are seen (uranyl acetate and lead citrate, ×4120).

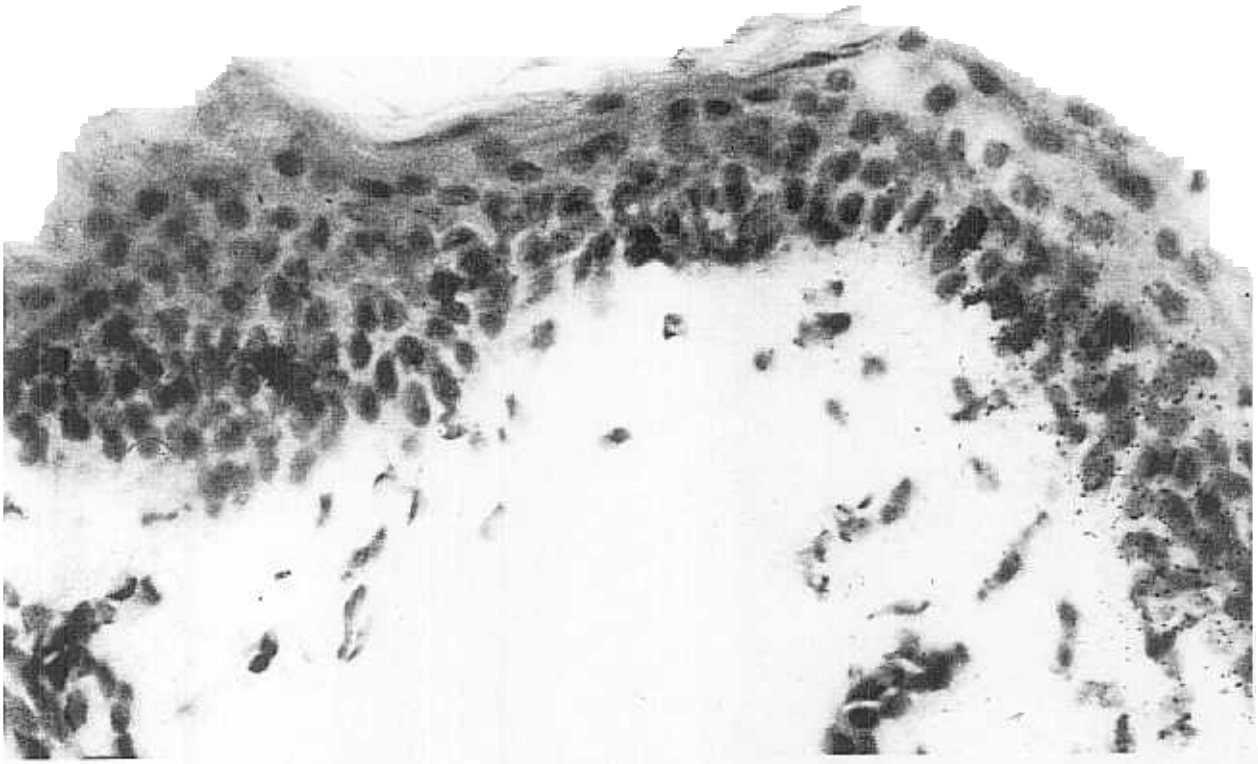

FIG. 11. Emulsion autoradiogram of [^{3}H]thymidine incorporation showing many proliferating cells in a Day 7 culture specimen of human skin. Most of the uptake is in epithelial cells in the germinal layer.

smooth muscle cells [4]. Although the proliferative effect seen in our explants may be due to the summation of DNA synthesis in all of these cell types, previous investigators have reported that for EGF-treated organ explants at least, no stimulation of [^{3}H]thy incorporation occurs in dermal cells [19]. However, further studies are needed to characterize which cell types respond to which growth factors in our model.

To address our second hypothesis, we studied epithelial outgrowth from the cut ends of the skin explants. Epidermal wound healing is an intricate process involving both migration and proliferation of keratinocytes [14]. We found that keratinocytes did grow from the cut ends of the explants in our model, resulting in epithelialization of the adjacent cut dermis. These outgrowths increased in size over 7 days with no further increase in length observed from 7 to 10 days. Of all the factors tested, the individual factor that produced maximum epidermal outgrowth was EGF. This is not surprising, since EGF has greatest affinity for epithelial cells. This EGF effect is consistent with previously published data [17, 21]. Treatment of explants with FGF and IGF-1 tended to increase epidermal outgrowth when compared to that in baseline, but was less effective than EGF. Labeling of the epidermis with α-anti-cytokeratin antibodies and electron microscopy of the specimens confirmed that the outgrowths were indeed epithelial in nature.

A human organ culture model has several important advantages over monolayer culture. No one cell culture or animal model fully authenticates the processes occurring in human tissue repair. Only humans can be used to reflect accurately the events of human wound healing. An *in vitro* model of wound healing provides a system in which primary epithelial cells in various stages of differentiation can be maintained and studied. In addition, the normal relationship between the epidermis and the dermis, as well as basement membrane–epidermal interactions, remain undisrupted [14]. An *in vitro* explant of skin also eliminates confounding systemic circulatory factors and permits elucidation of the effects of local factors on wound healing [15]. We have used 1% FBS in MEM as control, a concentration of serum sufficient to support growth, yet low enough to observe the effect of exogenously added growth factors. Controls for each experiment were obtained from the same individual, minimizing result variability.

It is increasingly apparent that growth factors such as FGF, IGF-1, and EGF play important roles during both

normal and impaired tissue healing. Although usually released endogenously at the site of injury, it may be advantageous to augment growth factor levels to achieve accelerated normal healing or to correct impaired healing. Intermittent topical application of growth factors has not been as successful as initially hoped [22]. These situations may necessitate higher and more prolonged exposure of cells to growth factors [23, 24]. Hansbrough's use of growth factors bound to collagen is one such strategy for prolonging and enhancing their action [25, 26]. Our *in vitro* model of human skin is especially suited to the evaluation of novel strategies of growth factor delivery as well as the potential of combining multiple growth factors with different mechanisms of action to potentiate their benefit.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the help of Lloyd D. McKie, M.D., FRCS, for help with statistical analysis and Nirmal Saini, M.D., for histologic and morphometric examination of specimens. Edward Flores and Billy Andrews deserve credit for excellent technical assistance. Fares Hakim, M.D., and Robert S. Redman, D.D.S., Ph.D. (Chief, VA Oral Pathology Laboratory), performed the emulsion autoradiography.

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