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Tissue expansion improvement: the role of epidermal growth factor

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In order to achieve the most satisfactory results in tissue expansion, a cream containing human recombinant epidermal growth factor (EGF) was applied daily to the expanding skin. The capacity of EGF to stimulate tissue repair has been evaluated on expanded skin flaps, comparing surgical procedures carried out with and without EGF. The present clinical, morphological, immunohistochemical and morphometric results demonstrate that EGF used together with the tissue expander may improve skin repair after mechanical stretching. In EGF-expanded skin flaps, clinical results show a reduction in teleangectasiae and histological evidence of a more regular texture in the dermal tissue, together with a significant thickening of the epidermal malpighian layer. Without EGF some signs of immaturity, such as abundance of fibronectin, persist for longer. EGF seems to be a helpful support to improve the tissue expansion process.

1. Introduction

The aim of using tissue expanders is to increase tissue availability. In fact, their use leads to a progressive increase in the cutaneous surface, enabling large integumentary skin losses to be re-epithelialized surgically using the adjacent cutis. The advantages of this process are basically twofold.

1. The possibility of using skin contiguous with the lesion to be repaired, thus having analogous colour, texture, thickness and elasticity.
2. The possibility of minimizing the scarring "reaction" at the level of the donor area, which gets covered by the excess of expanded skin [1].

The main factor to be considered in order to achieve these aims is the nature of the structural changes caused in the tissue by the mechanical stress. However, such physical stretching is not necessarily a negative factor, even though it is responsible for the alterations in the histo-architectural characteristics of the tissue or for the drop in blood perfusion levels [2, 3].

In fact, the elasticity of the skin and its capacity to expand are just two of the factors favouring the application of this surgical technique, since besides these there is an *ex-novo* skin synthesis [4–6].

Kinetic studies have demonstrated how tissue expansion is associated with a proliferation of epithelial cells [7–9] and an elevated incorporation of metabolic precursors of collagen fibrils [4, 5]. Therefore, it is currently thought that, in association with a passive skin-stretching phase and gain in the adjacent cutis resulting from the progressive mechanical forces applied, a reactive–neoformative process also takes place during expansion, leading to the organization of a definite, stable tissue gain [4, 5].

To encourage this reactive–synthetic phase of skin stretching, the current trend is therefore towards using appropriate biomolecules, applied during the expansion, to stimulate and accelerate the reconstitutive processes of the cutis, which may thus move to its new position and grow more easily.

β-Aminoproprionitrile, dimethylsulphoxide and fibrin glue have been used by other authors [10–12]. In one of our previous experimental studies we applied the expander together with a natural polysaccharide, chitosan, noted for its ability to favour orderly tissue reconstruction [13]. The present research was aimed at investigating the joint use of the expander and EGF, in the form of a cream to be applied externally each day during the expansion. In fact, increasing evidence seems to support the role of EGF in stimulating tissue repair processes with accelerated formation of new epidermis and dermis [14, 15].

In this paper we report the clinical assessment and the morphological, immunohistochemical and morphometric data for the expanded skin in the presence and absence of EGF cream, the application of which causes no trauma and is easy to repeat. Indeed, the possibility of repeated application would seem to be a determining factor in obtaining a biologically efficacious result [15].

2. Materials and methods

We studied 11 patients (age range 20–50 years) who

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received a skin expander, positioned in various parts of the body (thorax, forearm or leg) for about 2–3 months. Four of these patients also received one application per day of EGF (in the form of cream) during the expansion. Standard procedures [1] were used to perform the expansion.

After positioning the flap obtained by expansion, the patients were followed up clinically for a further 2–6 months. Before applying and after removing the expander, samples of cutis were taken in both control patients and those treated with EGF. Biosynthetic human EGF as 1 % cream (solution of 0.581 mg ml^{-1} purified peptide) was provided by the CIGB (Centro de Ingenieria Genetica y Biotecnologia, Havana, Cuba) [16].

The tissue fragments were subjected to immunohistochemical, ultrastructural and morphometric investigation.

2.1. Immunohistochemistry

All 11 cases were studied using antibodies to identify extracellular matrix proteins in frozen samples.

2.1.1. Extracellular matrix

Polyclonal antibodies to laminin (kindly provided by Dr Triche, Bethesda, Maryland, USA) and fibronectin (code 341643, Calbiochem-Behring) were applied to 4 μm serial, unfixed cryostat sections using an indirect immunofluorescence technique. Sections were air-dried for 2 h, incubated (for 30 min) with primary and secondary antibodies (anti-rabbit FITC, code F 205 DAKO and anti-mouse FITC, code 232 DAKO) and washed (for 15 min) in phosphate-buffered saline (PBS). Sections were mounted on PBS–glycerol and observed with a Leitz Orthoplan microscope.

2.2. Electron microscopy

2.2.1. Transmission electron microscopy (TEM)

Specimens were fixed in 2.5 % glutaraldehyde, in 0.1 % cacodylate buffer (pH 7.4), post-fixed in 1 % OsO_4 in cacodylate buffer, dehydrated in increasing ethanol concentrations, and embedded in Araldite. Semithin sections and ultrathin sections were cut using a Reichert Ultracut E microtome, and were stained with 2 % toluidine blue. Ultrathin sections were counterstained with uranyl acetate and lead citrate and observed with a Zeiss EM 109 electron microscope.

2.2.2. Scanning electron microscopy (SEM)

Specimens were fixed as described above, dehydrated in alcohols, critical-point dried in liquid CO_2, mounted on metal stubs with a conductive glue and coated with a thin layer of gold to improve conductivity. The samples were observed with a Philips 505 scanning electron microscope.

2.3. Morphometry

Semithin sections of epidermis and dermis were examined using a Zeiss light microscope to select 40 fields in each sample. The images recorded were fed into an IPS-Kontron image analyser to measure parameters such as the area of vessels and the number of connective tissue cells and dermis vessels. The images analyser was calibrated for the magnification specific to the working conditions, and the objects measured were considered in suitable chosen areas of references.

The thickness of the epithelial layers for each patient was also measured. Cells were analysed at $\times 100$ per unit area (u.a., equal to 2343.98 μm^2) and the vessels at $\times 40$ u.a.$^{-1}$ (u.a. equal to 14 353.95 μm^2). Epithelial thickness was measured at $\times 40$ magnification.

After testing the homogeneity of the data to ensure that they belonged to a single statistical population, Student's *t*-test was used to compare the data pool obtained from control and EGF-treated areas.

3. Results

3.1. Clinical assessment

No significant clinical differences appeared between the expanded flaps with and without EGF after positioning and at 2–6 months of follow-up. This is in agreement with previous observations that the expander flaps, once positioned, return to normal in 1–3 months.

However, this study revealed that teleangiectasiae of the repositioned flap occurred less frequently in the presence of EGF than in its absence.

3.2. Immunohistochemistry

3.2.1. Fibronectin

In the presence of EGF the widespread presence of fibronectin was observed as a regularly distributed reticular tissue network at the dermal level (Fig. 1a). In the absence of EGF, however, the distribution of this stromal glycoprotein in the expanded derma was less homogeneous, being more densely packed in some zones, whereas in others it was organized more loosely around rough trabeculae of collagen fibres (Fig. 1b).

3.2.2. Laminin

Following EGF treatment the immunohistochemical reaction to identify the glycoprotein laminin appeared localized at the dermo-epidermal junction and the walls of the dermal vessels, which in general displayed a quite regular outline (Fig. 2a). In expanded tissue without EGF treatment the pattern of the immunohistochemical staining was similar to that with EGF, even though the vascular profile was sometimes more irregular, and in some cases the microvessels had a dilated lumen (Fig. 2b).

3.3. Transmission electron microscopy

3.3.1. Expansion with EGF

The morphological analysis of the cutis expanded in the presence of EGF showed a correct structural

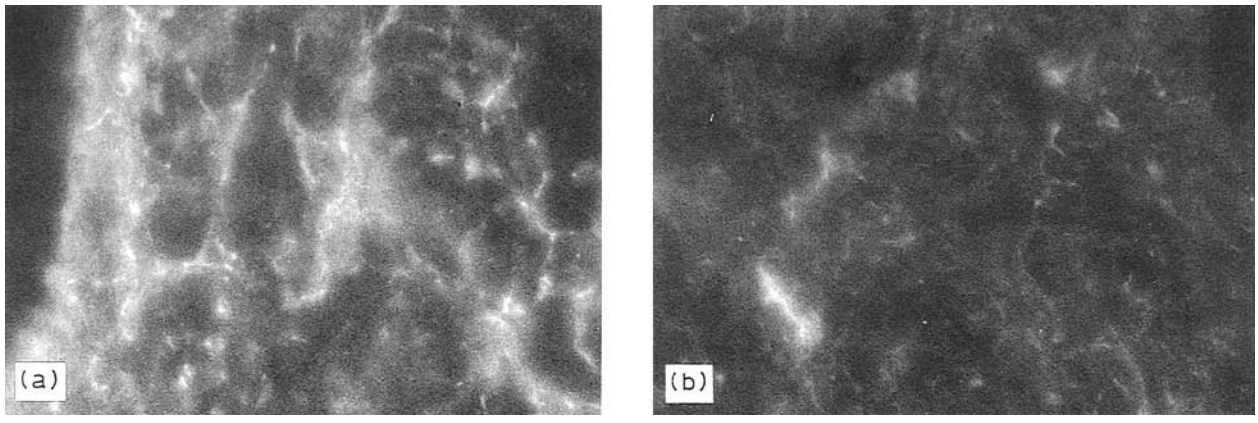


Figure 1 (a) Expansion with EGF; the fibronectin immunoreaction shows a reticular pattern (×250). (b) Expansion without EGF; the fibronectin immunoreaction; stromal area presenting a faint staining (×250).

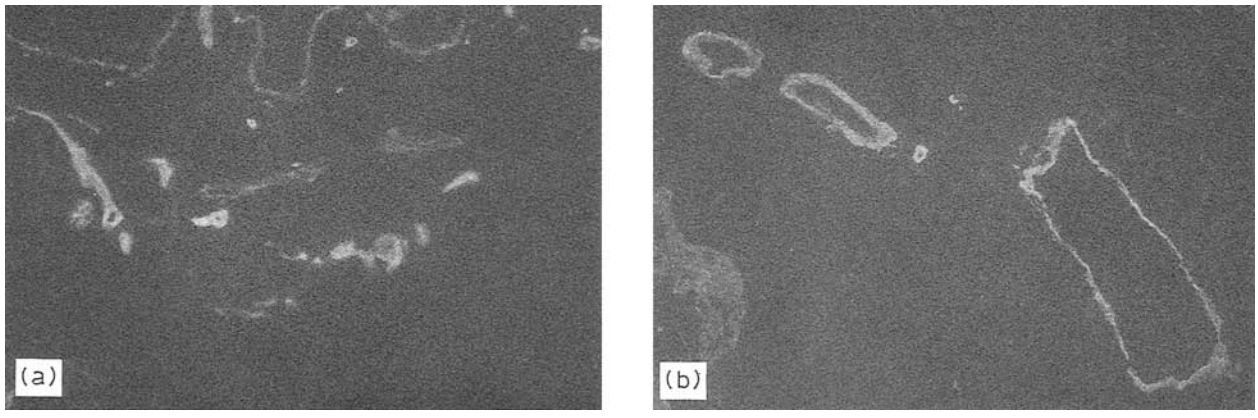


Figure 2 (a) Expansion with EGF. The laminin immunoreaction clearly highlights the dermal–epidermal junction and the wall of the microvessels (×100). (b) Expansion without EGF. The immunohistochemical reaction for laminin outlines microvessels with dilated lumina (×100).

organization of the epidermis with progressive maturation of the keratinocytes towards the more superficial layers. These cells were linked by numerous desmosomes in which tonofilaments come together (Fig. 3a and b).

At the dermal level, bundles of collagen fibres forming an ordered, regular histo-architectural tissue network could be identified (Fig. 4a). The microvessels had regular features, with endothelial cells overlying a basal lamina and a few pericytes (Fig. 4b). The interstitial mesenchymal cells looked typically fibroblastic, with abundant dilated rough endoplasmic reticulum (Fig. 4c).

3.3.2. Expansion without EGF

In the absence of EGF the submicroscopic features of the epidermis were similar to those of expanded cutis with EGF although, at times, circumscribed zones with irregular, haphazard keratinocyte interconnections were observable (Fig. 5a and b).

In general the basal lamina was clearly identifiable as separating the basal cells from the underlying derma. Conversely, at the dermal level a poor structural organization could be observed which distinguished this dermis from that expanded in the presence of EGF. The stromal tissue presented some areas having oedematous features with a scanty number of collagen fibres, whereas in other zones an evident accumulation of collagen bundles was noted (Fig. 6a and b).

In general, a regular microvascular bed was present. The stromal cells had a fibroblastic–fibrocytic appearance.

3.4. Scanning electron microscopy

3.4.1. Expansion with EGF

This investigative technique revealed the presence of collagen fibres with a well-ordered assembly of fibrils arranged in bundles having a precise direction and in an orderly fashion (Fig. 7a).

3.4.2. Expansion without EGF

The collagen bundles seemed to be composed of loosely and irregularly arranged fibrils (Fig. 7b).

3.5. Morphometric analysis

3.5.1. Expansion in the presence of EGF (Table I)

After EGF application the expanded cutis displayed significant structural differences from the unexpanded

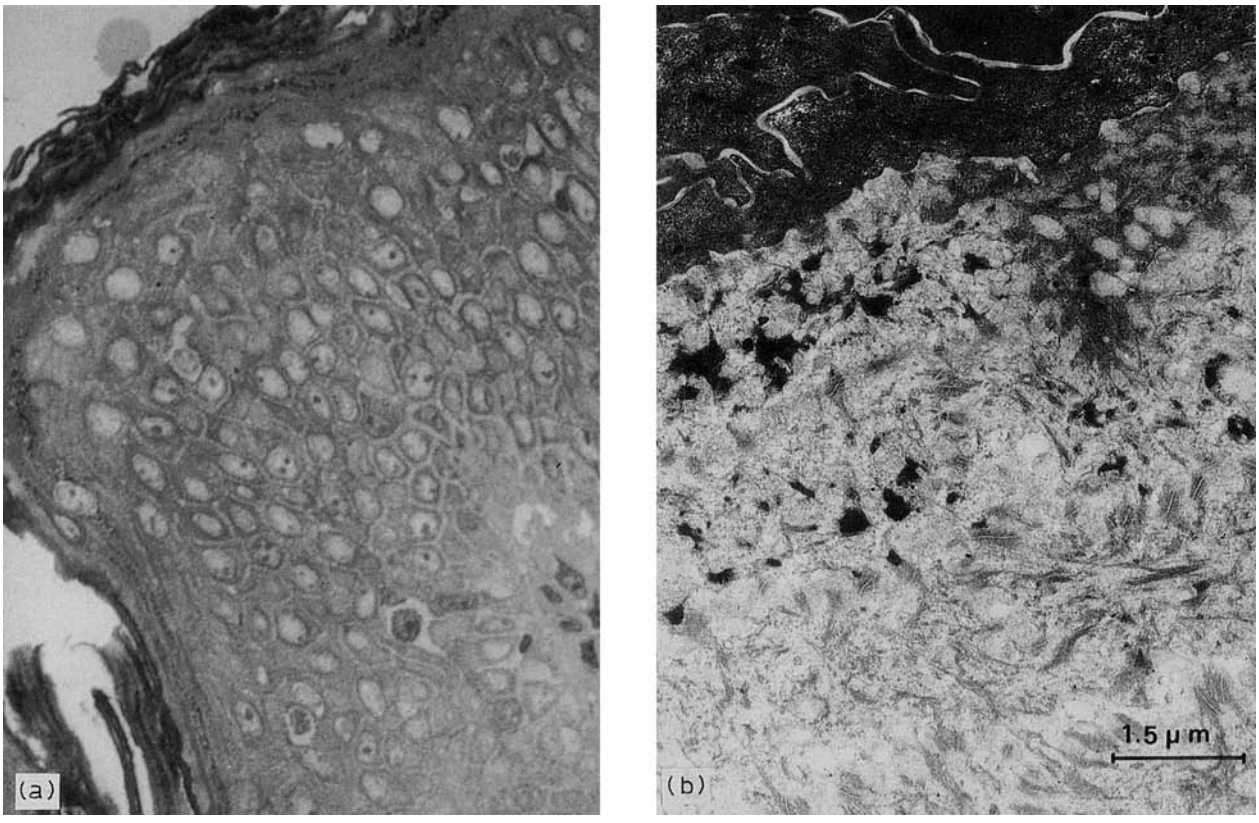


Figure 3 (a) Expansion with EGF. In the light microscope the epidermis appears to consist of cells showing a correct maturation towards the more superficial layers (×250). (b) Expansion with EGF. The ultrastructural examination also confirms the correct maturation of the keratinocytes towards the more superficial layers.

cutis, but only at the dermal level, where the number of microvessels appeared higher than in the unexpanded cutis (2.13 and 1.30 u.a.$^{-1}$, respectively; $P < 0.001$), whereas the area occupied by them was smaller than in the controls (481.97 and 663.16 u.a.$^{-1}$, respectively; $P < 0.05$).

3.5.2. Expansion in the absence of EGF (Table II)

The morphometric analysis of the cutis, both before and after expansion without EGF, showed a significantly reduced thickness in the more superficial (granulous and horny) layers of the epidermis in the expanded cutis compared with the unexpanded case (30.84 and 39.13 u.a.$^{-1}$, respectively; $P < 0.01$). No significant differences between the two conditions were observed in the malpighian layer. In the expanded dermis there was a significant increase in the number of vascular structures (2.50 and 1.63 u.a.$^{-1}$, respectively; $P < 0.05$) and the area of these vessels also appeared greater than that observed in cutis without expansion (843.71 and 409.55 u.a.$^{-1}$, respectively; $P < 0.001$). However, the number of cells present in the dermis of the expanded zone seemed to be similar to that observed in the cutaneous areas not subjected to expansion.

3.5.3. Expansion in the presence and absence of EGF (Table III)

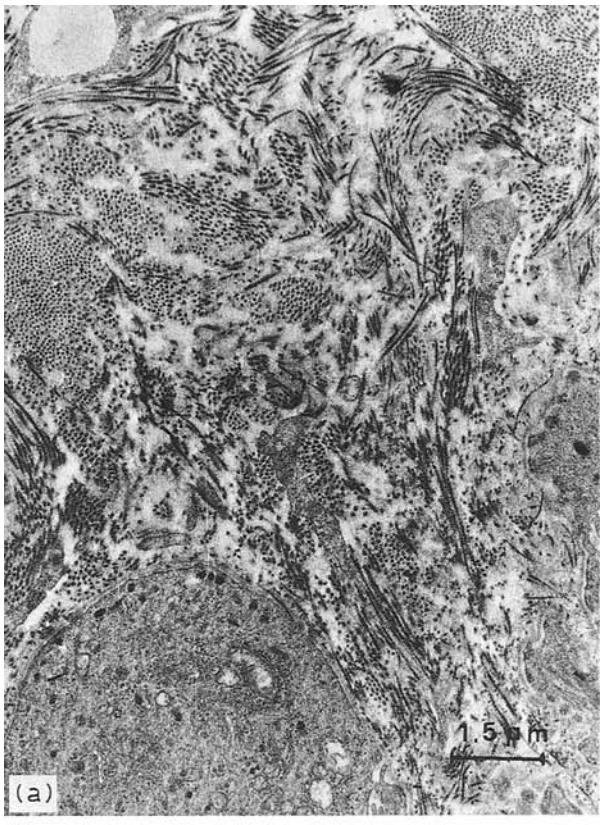
Comparing these two expansion conditions, the thickness of the malpighian layer was seen to increase significantly after EGF application, compared with cutis expanded without EGF (48.81 and 41.06 u.a.$^{-1}$, respectively; $P < 0.05$). At the dermal level, in the absence of EGF the vascular structures occupied an area significantly greater than in the presence of EGF, perhaps also due to the vascular lumen, sometimes quite dilated (481.97 and 843.71 u.a.$^{-1}$, respectively; $P < 0.05$) (see above).

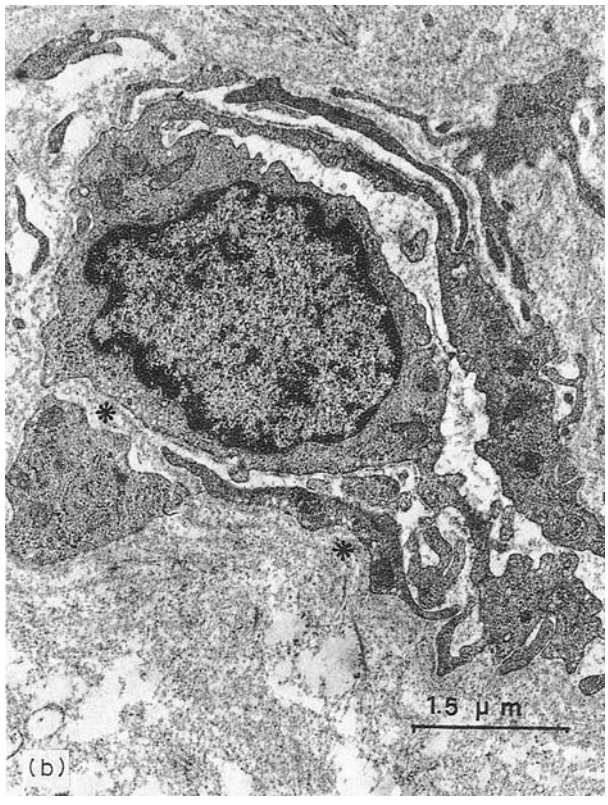
4. Discussion

Attempts to improve the quality of expanded skin have been made in various directions. The type of expansion and method used are two of the factors most often considered [2, 3, 17], although it must not be forgotten that the response to the process of cutaneous stretching may be more or less efficient according to the specific site of the cutis involved [2].

Currently, however, the aim of obtaining expanded cutis of elevated biostructural quality might be better achieved by using biomolecules that favour the reparative and/or synthetic processes of the dermal–epidermal tissues, thus helping to maintain good cutaneous trophism also during the expansion process.

Therefore, EGF seems to provide valid support to tissue expansion seen during both the "passive" phase linked to mechanical traction and the "active" one of *ex novo* tissue synthesis [15]. This result may be due to the capability of EGF to induce cellular proliferation (epithelial and fibroblastic), angiogenesis and rapid tissue organization. These biological characteristics of





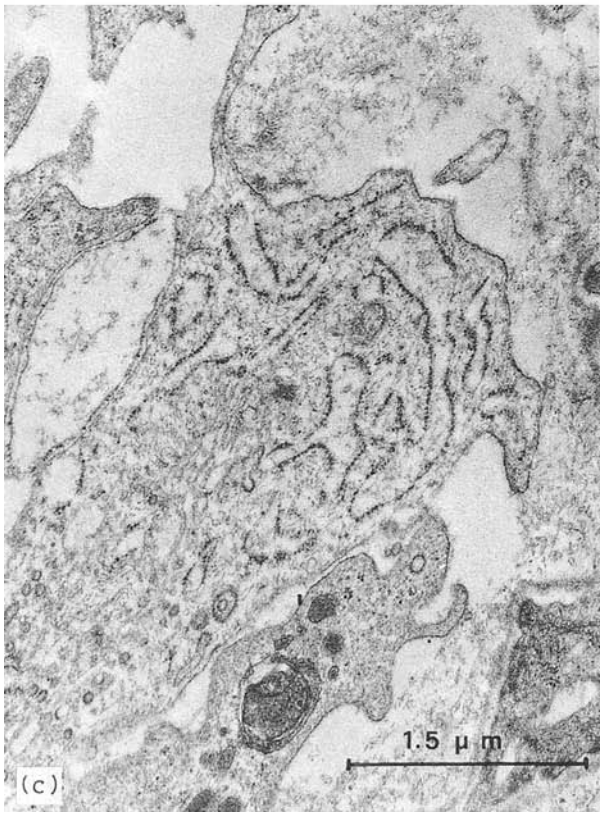


Figure 4 Expansion with EGF. (a) In the dermis the bundles of collagen fibres form an ordered histo-architectural tissue network. (b) Typical microvessel with a wall consisting of some differentiated endothelial cells overlying a basal membrane (*) and a few pericytes. (c) Ultrastructure of a mesenchymal–fibroblastic cell, showing abundant rough endoplasmic reticulum with dilated cisternae.

EGF, documented both *in vivo* and *in vitro* [15, 18–20], have already been exploited experimentally and on patients using murine and recombinant EGF to enhance wound healing [14, 15].

In agreement with the above, the histo-architectural features that we evaluated with submicroscopic and immunohistochemical investigations and quantified with morphometric studies, highlight how EGF improves the structural organization processes of the tissue during tissue expansion. In fact, this biomolecule stimulates the formation of a well-ordered stromal tissue in the expanded cutis flap, with a well-developed microcirculation and vascular structures made up of differentiated endothelial cells lying on a clearly identifiable basal lamina and surrounding lumens generally of limited size. These last features, almost pointing to the existence of a good vascular tone, appear less frequently in expanded cutis without EGF treatment, where the vessels may be seen to have a more twisted, dilated lumen.

The regular layout of the fibronectin network in the presence of EGF correlates well with our morphological ultrastructural observations that under the same conditions (plus EGF) show a regular histo-architectural organization of the stromal tissue. In fact, it is recognized that fibronectin tends to set up an "intelligent" template on which the collagen network of the dermis is arranged [21, 22].

In the presence of EGF the epithelium shows a significantly thicker malpighian layer, whereas superficial strata display no significant differences with and without EGF. These observations are in keeping with the *in vivo* and *in vitro* results of other authors, who have shown how human keratinocytes present EGF receptors which vary in number according to the

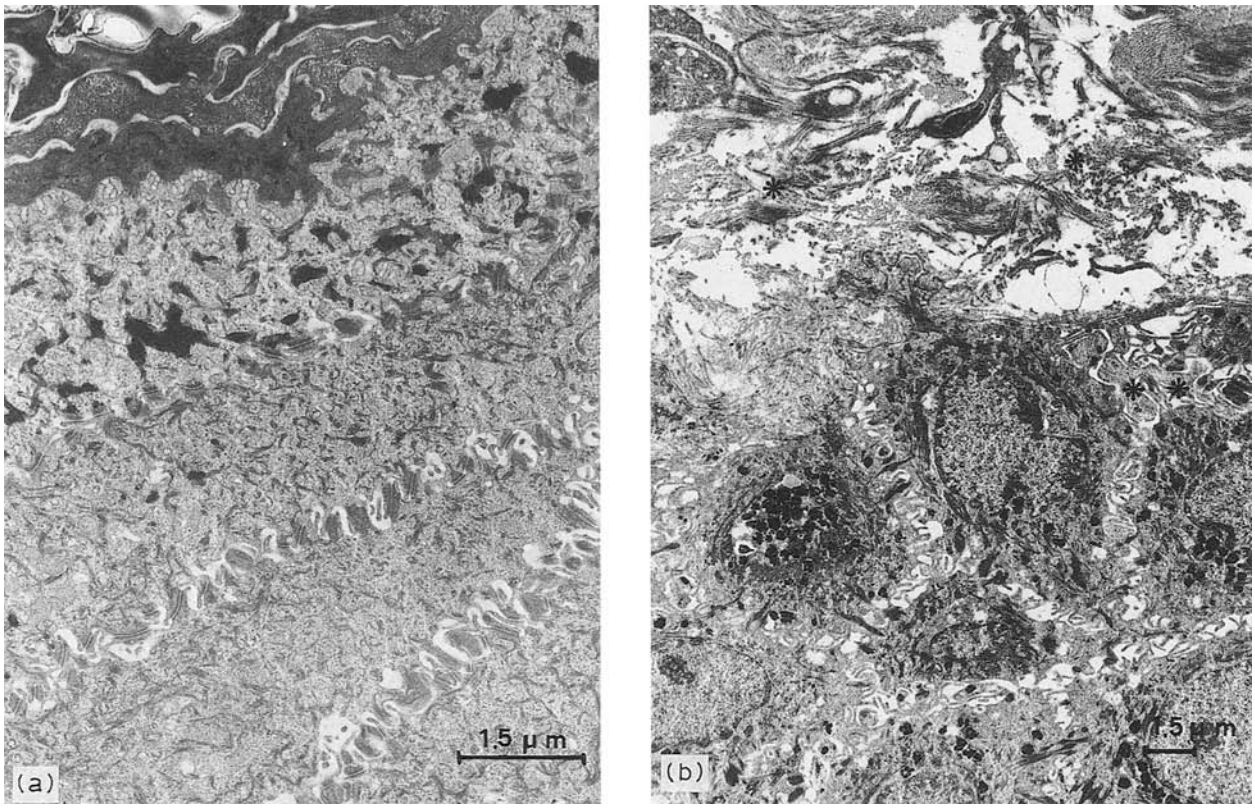


Figure 5 Expansion without EGF. (a) The epidermis shows correct keratinization. (b) Focal aspects of irregular interconnections between keratinocytes (**) Collagen fibres are haphazardly distributed in the dermis (*).

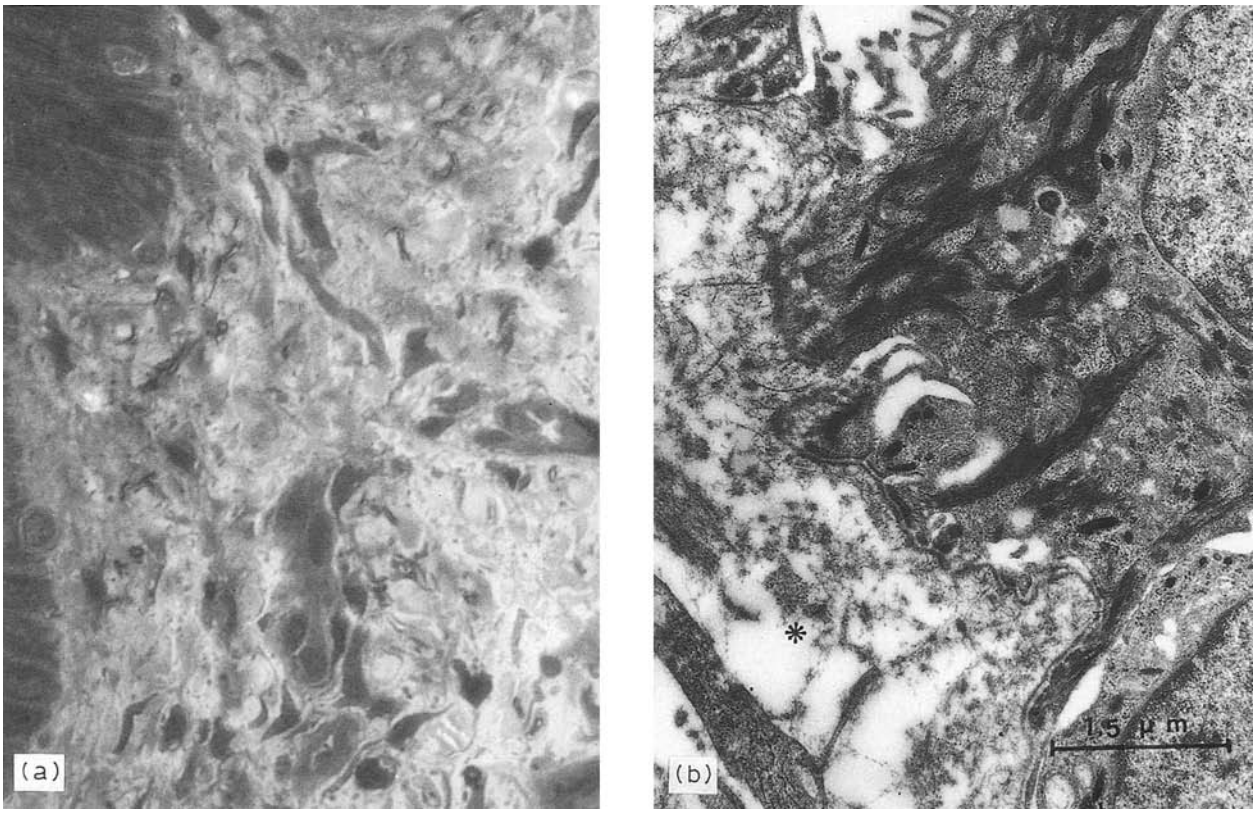


Figure 6 Expansion without EGF. (a) In the light microscope the dermis presents a non-homogeneous appearance. (b) Ultrastructural analysis demonstrates areas of oedema (× 630) at the more superficial levels of the dermis.

degree of cellular differentiation: they are more numerous at the malpighian layer (the zone to which cellular proliferation is normally confined) and then their numbers fall progressively [15, 25].

The fibroblasts also possess the EGF receptor and respond to exposure to this growth factor by increasing their mitotic rate [10]. It is therefore understandable how EGF stimulates the various cells to carry out

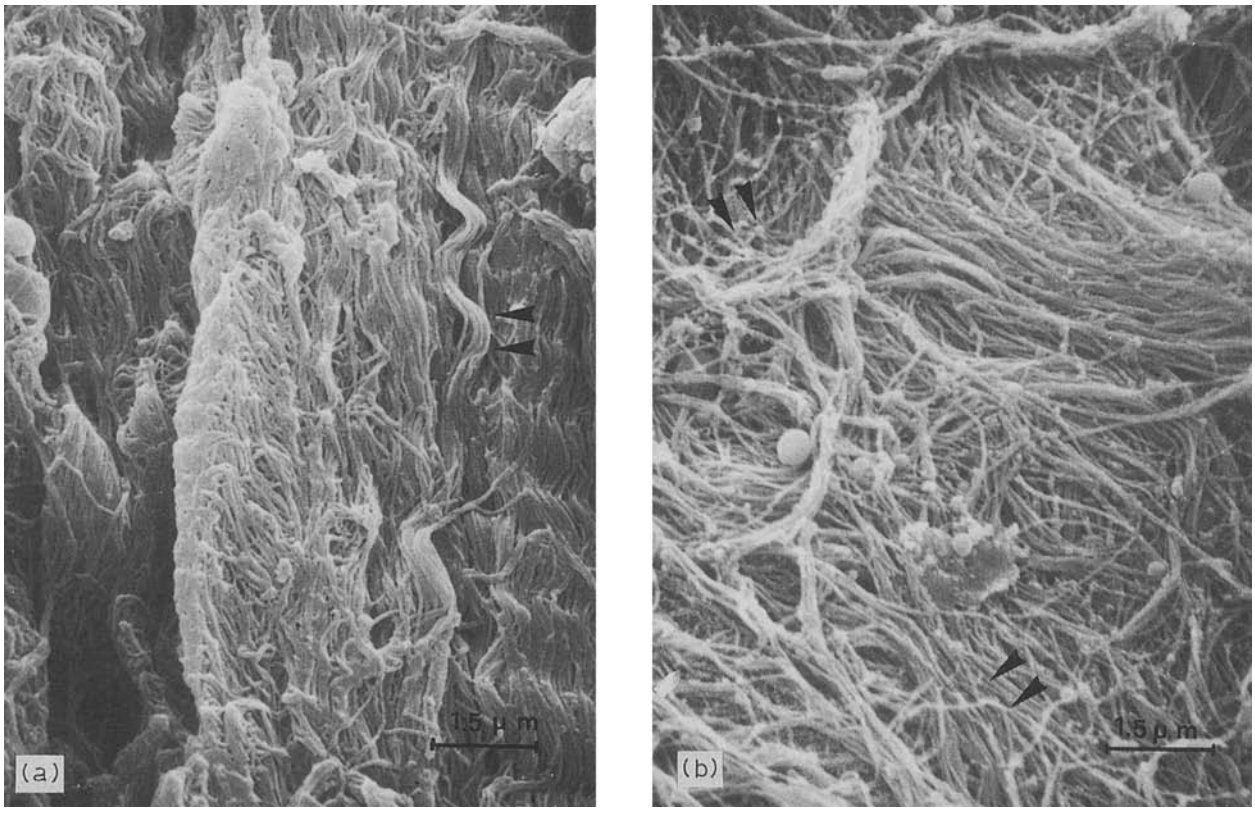


Figure 7 (a) Expansion with EGF. Scanning electron microscope showing bundles of collagen with fibres regularly assembled (▲▲). (b) Expansion without EGF. Collagen fibrils are arranged irregularly and interlace haphazardly (▲▲).

TABLE I

	Expansion with EGF	Control cutis	Significance[a]
Thickness of granulous and horny layer	29.61 ± 5.32	29.85 ± 5.58	NS
Thickness of malpighian layer	48.81 ± 17.75	45.54 ± 15.04	NS
Number of vessels[b]	2.13 ± 0.94	1.30 ± 0.92	< 0.001
Vessel area	481.97 ± 310.73	663.16 ± 371.12	< 0.05
Number of dermal cells[b]	3.58 ± 2.78	4.39 ± 2.87	NS

[a] Student's *t*-test between EGF-expanded areas and controls (NS, not significant).
[b] Referred to unit area (see text).

TABLE II

	Expansion without EGF	Control cutis	Significance[a]
Thickness of granulous and horny layer	30.84 ± 6.82	39.13 ± 13.20	< 0.01
Thickness of malpighian layer	41.06 ± 9.75	41.94 ± 15.52	NS
Number of vessels[b]	2.50 ± 1.20	1.63 ± 1.50	< 0.05
Vessel area	843.71 ± 718.00	409.55 ± 354.31	< 0.001
Number of dermal cells[b]	3.33 ± 2.25	3.72 ± 2.25	NS

[a] Student's *t*-test between expanded areas without EGF and controls (NS, not significant).
[b] Referred to unit area (see text).

TABLE III

	Expansion with EGF	Expansion without EGF	Significance[a]
Thickness of granulous and horny layer	29.61 ± 5.32	30.84 ± 6.82	NS
Thickness of malpighian layer	48.81 ± 17.75	41.06 ± 9.75	< 0.05
Number of vessels[b]	2.13 ± 0.94	2.50 ± 1.20	NS
Vessel area	481.97 ± 310.73	843.71 ± 718.00	< 0.05
Number of dermal cells[b]	3.58 ± 2.78	3.33 ± 2.25	NS

[a] Student's *t*-test between areas EGF-expanded with and without EGF (NS, not significant).
[b] Referred to unit area (see text).

the functions that each given cell type is programmed to execute. The action of EGF appears to be of paramount importance during embryonic development, and therefore it is not surprising to find it present in the amniotic liquid [15, 24].

Furthermore, EGF is seen to have great importance in maintaining the normal physiological function of various organs and tissues, besides restoring such normal function when structural damage has been caused for whatever reason. This could explain the presence of EGF as a soluble factor in plasma, urine, saliva, tears and sweat [15, 24].

In conclusion, the present clinical and morphological data on tissue expansion illustrate the capacity of EGF to favour skin morphogenetic processes as physiologically as possible. Therefore, this study must be considered as an attempt to move closer to the important goal of using biomaterials [11, 12] to produce an expansion that is guided correctly, causes very few traumas for the expanded flaps and has short clinical recovery times.

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