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The effect of EGF application in gel form on histamine content of experimentally induced wound in mice

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Summary. The factors participating to the wound healing are complex and still obscure. Among these factors, epidermal growth factor (EGF) and histamine by increasing reepithelization and reparation tissue strength via enhancing collagen deposition to the wound site have a beneficial effect. This study was performed to investigate the effect of EGF dosage forms on the histamine content of the experimentally induced wound and some wound healing criters in the mice.

Histological investigation of reepithelization, wound tensile strength for healing and collagen maturation, and histamine levels were assessed in the present study. Thirty two mice were divided into control, and EGF treated groups. Controls included three subgroups; untreated (n = 5), 0.9% NaCl applied (n = 5), and gel applied (n = 5). Experimental groups were treated with two forms of EGF; EGF, solution form in 0.9% NaCl (n = 5) and the gel form in 0.2% w/w in carbopol 940 (n = 7). The discrepancy between these forms were evaluated. This evaluation was done by the application of two forms of EGF for 15 days on experimentally induced wound healing.

Gel form of EGF by sustained release from bioadhesive polymer is found to be more effective than the soluble form, on the healing of the wound, by acceleration of reepithelization and increment of wound tensile strength. The tensile strength of the wound indicates the rate of repair and collagen maturation. It has been observed that when physiological saline and carbopol 940 exposed to incision without EGF causes a significant increase in tissue histamine content.

According to the results of the present investigation; the histamine content is found to be decreased by EGF gel dosage form treatment, therefore preventing abnormal collagen formation has a beneficial effect on wound healing.

Keywords: EGF – Carbomer – Histamine – Wound healing

Introduction

One of the important factor in wound healing is epidermal growth factor (EGF). This factor has been shown to stimulate wound healing in skin (Pessa et al., 1987), cornea (Gönül et al., 2000) and gastric mucosa (Akbulut et al., 2002). It is well known that many types of cells, including dermal fibroblasts contain EGF receptors. Stimulation of these receptors by the growth factor respond by proliferation in cell culture (Ksander, 1989).

It has been shown that, one of the endogenous mediator for wound healing was histamine (Fitzpatrick and Fisher, 1982). Although, histamine, a chronic inflammation and fibrosis mediator, stimulates both angiogenesis and cell proliferation, which are vitally important processes in wound healing (Enerbsach and Norrby, 1989). However, the role of this amine in wound healing is controversial. Cohen et al. (1972) have indicated that oral administration of the antihistaminic agents to certain keloid patients seems to have a beneficial effect as an adjuvant therapy. Dabrowski and Maslinski (1981) and Schittek et al. (1984), have shown that the effect of histamine on wound healing is concentration-dependent. Histamine at high concentrations inhibits, while low concentrations of the amine stimulates the collagen synthesis.

Kikuchi et al. (1995), have studied the effect of growth factors on type I collagen metabolism in keloid and normal fibroblasts. According to the results of these investigators, keloid fibroblasts are more significantly affected by EGF and histamine when compared with normal fibroblasts. Recently it has been shown that the mast cell mediators inhibited 3H thymidine incorporation of keratinocytes in cell cultures as well as whole skin cultures in the absence of EGF/BPE (bovine pituitary extract) (Huttunen et al., 2001). This effect was not observed in the presence of EGF/BPE.

The aim of the present study was to investigate the possible interactions between EGF and histamine levels and secondly to clarify either the solution or the gel form

of EGF was effective in experimentally induced wound healing.

Material and methods

The experiments were performed on C57 BL-6J-Ola mice of either sex weighing 20–30 g. The animals were kept in normal laboratory conditions, housed individually in separate cages and fed with a standard diet and tap water *ad libitum*.

Experimentally induced wound was done by the incisions made with 1 cm width through the skin after scrubbing with iodine swab sticks, and reached the subcutaneous muscle layer as described previously (Çelebi et al., 1993; Dinçer et al., 1996), on each side of lumber spine of the mouse, under diethylether anesthesia. The wounds were then closed immediately end to end with 5/0 atraumatic silk sutures. After this procedure each animal received single dose of 400 IU penicilline procaine intraperitoneally. The sutures were removed on the day of 7.

The animals were treated by two forms of EGF for 15 days beginning on the first day of the incision was done in separate groups. The right side of the wound of each animal was used for the application of EGF and the left side was used as control by application of vehicles 5 μl twice a day.

Two forms of EGF were prepared in different carrier systems and these forms were applied locally to the lesions. The aqueous form of EGF (100 μg/ml) was prepared in physiological saline (EGF + PS), the gel form was prepared as 100 μg/ml in 0.2% w/w carbopol 940 (EGF + Gel) and two forms were used 5 μl twice a day to each animal. As a vehicle in one group, physiological saline in the second group, carbopol 940 was used.

After 15 days treatment the animals were sacrified by using overdose anesthetic agent. Part of the wound tissue specimens were dissected for histologic examination and transferred to a beaker containing 2.5% buffered glutaraldehyde for fixation.

After fixation for 2 hours the tissues, used for histological examination, were postfixed in 1% osmium tetroxide, dehydrated in serial alcohols and embedded in araldite. The thick sections of 0.5 mm were then stained with

Table 1. Effects of EGF dosage forms and vehicles on histamine contents of full thickness skin wounds

Groups	n	Histamine (μg/g tissue)
1. Skin control	5	27.78 ± 4.08
2. Wounded skin		
Untreated	5	$47.46 \pm 5.99^{*}$
PS treatment	5	$133.01 \pm 24.36^{**}$
PS + EGF treatment	5	$35.07 \pm 3.74^{*}$
Gel treatment	5	$46.62 \pm 4.91^{*}$
Gel + EGF treatment	7	$0.20 \pm 0.10^{**}$

PS, 09% NaCl solution 5 μl twice a day × 15 days
PS + EGF, 100 μg EGF/ml PS 5 μl twice a day × 15 days
Gel, Carbopol 940 (0.2% w/w) 5 μl twice a day × 15 days
Gel + EGF, 100 μg EGF/ml gel 5 μl twice a day × 15 days
* $p < 0.05$; ** $p < 0.001$

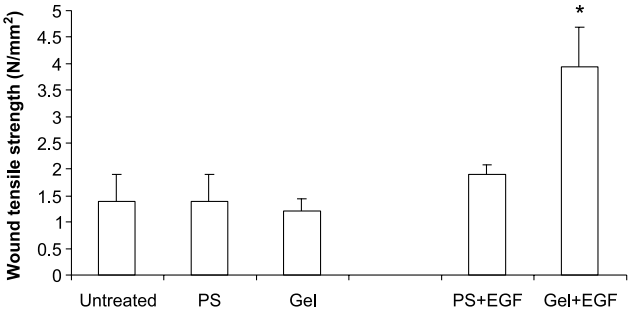


Fig. 1. Effect of EGF dosage forms on the wound tensile strength. *PS*, 09% NaCl solution 5 μl twice a day × 15 days; *PS + EGF*, 100 μg EGF/ml PS 5 μl twice a day × 15 days; *Gel*, Carbopol 940 (0.2% w/w) 5 μl twice a day × 15 days; *Gel + EGF*, 100 μg EGF/ml gel 5 μl twice a day × 15 days; * $p < 0.05$

Fig. 2. **a** Untreated control; The epidermis has began developing in the lesion area, in some areas is missing (arrows). Note thick collagenous fibers (*CF*) in the dermis. Toludine blue. ×400. **b** Electron microscopic view of the untreated control group; epithelium has not developed already (arrow). Note thick crust layer (*CL*). Lead citrate. ×9000

Fig. 3. The control group. Mast cells (arrows) ×1000

Fig. 4. PS; Developing epidermis (*E*), Crust Layer (*CL*) on the upper surface, Collagenous Fibers (*CF*). Toluidine blue. ×1000

Fig. 5. The PS group. Mast cells (arrows) ×1000

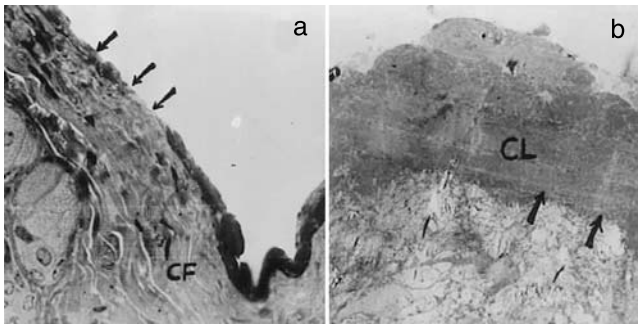
Fig. 6. **a** EGF + PS; almost totally mature epidermis with irregular cell shape and layers that has not already reached the normal state (*E*), extremely active fibroblasts (*F*) and thick collagenous fibers (*CF*). Mast cells (arrows). Toluidine blue. ×400. **b** An electronmicroscopic picture from PS + EGF group. Developing epidermis (*E*). Tonofilaments in the basal cell cytoplasm (arrows) and keratohyalin granules in the surface flat cells (*KG*) can be observed. Keratinization (K) is complete on the upper surface, Collagenous fibers (*CF*) are remarkable in the dermis. Lead citrate. ×9000. **c** A light microscopic pictures from EGF + Gel group. Epidermis (*E*); the keratinized layer (*KL*) on its surface and the flat cell layer of upper part (arrows). Toluidine blue. ×1000. **d** In the electron microscopic view of EGF + Gel group; developing epithelium (*E*); basal cells (*BC*), intermediate cells (*IC*), flat cells (*FC*) can be observed. Arrows show the keratohyalin granules in the flat cell layers. Lead citrate. ×9000

Fig. 7. The EGF + PS group. Mast cells (arrows) ×1000

Fig. 8. The EGF + Gel group. Mast cells (arrows) ×1000

Fig. 9. **a** Gel; lesioned area (arrow). Very thin epidermis (*E*) consist of a few layers of cell. Cuboid cells in the basal layer (*BL*), thick collagenous fibers (*CF*) in dermis and a few mast cells (MC) can be seen. Toluidine blue. ×1000. **b** An electron microscopic picture from the Gel group. Epidermis (*E*) consist of a few cell layers. Vacuoles in the cytoplasm of basal cells are either big (arrow) or small (double arrows). Keratohyalin granules (*KG*) in the flat cells of the upper layer can be seen together with the crust layer (*CL*) on the epidermis. Remarkable collagenous fibers (*CF*) are present in dermis. Lead citrate. ×9000

Fig. 10. The Gel group. Mast cells (arrow) ×1000



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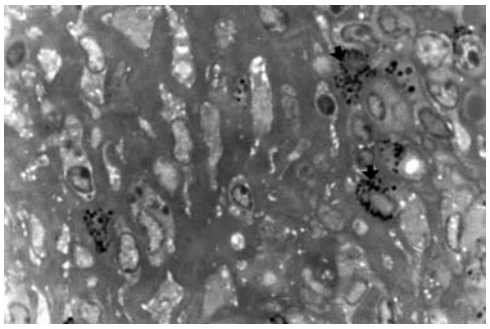

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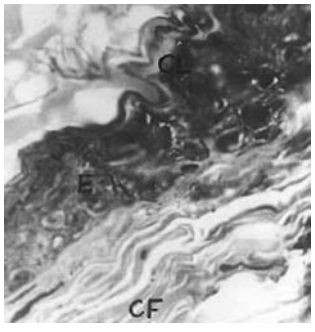

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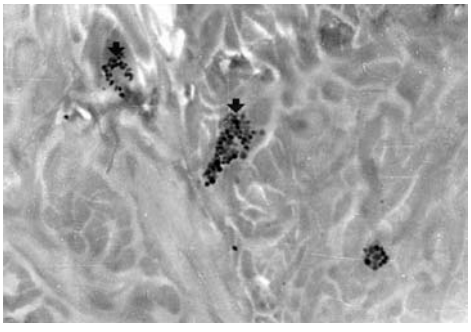

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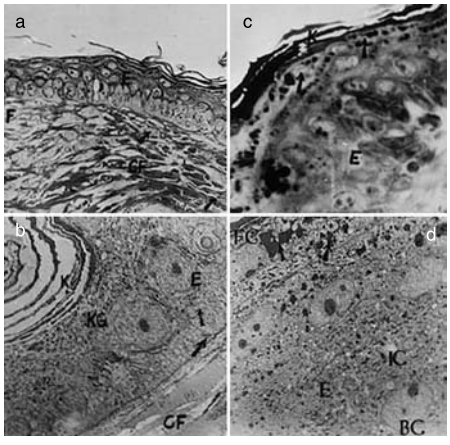

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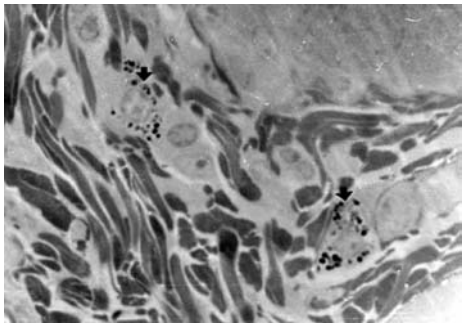

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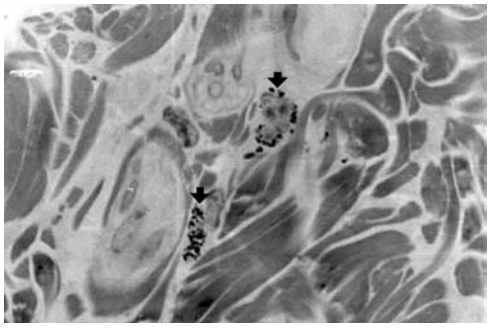

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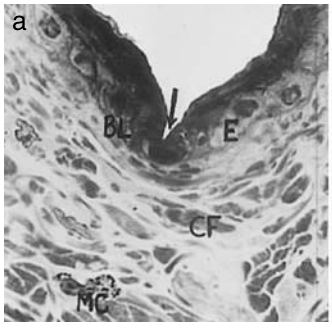

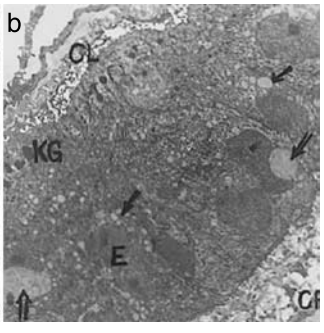

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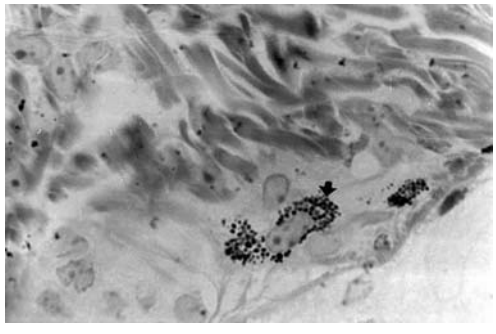

Fig. 10.

toluidine blue and examined under Olympus® BH2 light microscope. The thin sections of 200–300 Å were examined under Carl-Zeiss® EM9S2 electron microscope.

The full thickness skin flaps 25 mm^2 in dimensions were dissected for mechanical testing. The wound was located in the middle of the flap. The skin was secured with the clamps exactly 1.5 mm away from and parallel to the wound, giving total distance of 3 mm between the clamps and wound centre. The tensile strength was determined via force displacement transducer (FT.03) on a Grass Polygraph Model 7.

The other part of the tissue was used for measuring histamine level. Histamine content of the tissues, sampled including 2–3 mm of the edges of the wound, were examined spectrofluorometrically by a fluorescent OPT (O-phtaldialdehyde) reaction as described previously (Shore et al., 1959).

Statistics

Statistical analysis were done by using Student's t-test. All values were expressed as mean ± SEM. $p < 0.05$ was considered as significant.

Results

Histamine levels in normal skin tissue was found to be $27.78 \pm 4.08\ \mu g/g$. The incision wounds causes a significant increase in tissue histamine levels and found to be $47.46 \pm 5.99\ \mu g/g$ ($p < 0.05$). Interestingly it has been observed that physiological saline treatment, as a vehicle, caused a further increase in tissue histamine levels and found to be $133.01 \pm 24.36\ \mu g/g$. However, carbopol 940 pretreatment did not change the histamine content of the tissue exposed to injury.

EGF + PS treatment for 15 days causes a significant decrease in tissue histamine levels ($35.07 \pm 3.74\ \mu g/g$) when compared with only physiological saline treated group. EGF + carbopol 940 treatment causes a significant decrease in tissue histamine levels when compared with carbopol 940 alone treated, wound developed untreated and control groups. These results are summarized in Table 1.

The effect of two forms of EGF on wound tensile strength was examined. The tensile strength taken as a parameter of collagen synthesis did not change by both vehicle treatment when compared with untreated group. However, EGF + carbopol 940 treatment causes a significant increase in tensile strength ($p < 0.001$) when compared with EGF + PS treated group. The results are shown in Fig. 1.

According to microscopic examination, epithelization has nearly begun around and inside of the lesioned area in the untreated wound-developed skin tissue. With higher magnification it has been observed that epithelization has begun on the wound margins but the total formation of flat laminated epithelium has not totally formed in the same specimen (Fig. 2a). Electron microscopic examination of the wound indicates that epithelization has not already begun but a thick crust was placed over the dermis (Fig. 2b). Less intensive granules were observed in the cytoplasm of the mast cells (Fig. 3).

In PS treated group, it was observed that regeneration of the epithelium has begun from basal layer with an irregular shape and the abundance of collagenous fibres was striking in the dermis and the neo-dermis was also covered with a thick crust (Fig. 4). Mast cells with the intensive granules filling the cytoplasm were observed in the same group (Fig. 5).

In the group treated with EGF + PS, it has been shown that, epidermis was almost totally mature; formed of 3–5 cell layers. On the flat cells of laminated epithelium surface; keratohyalin granules were also striking. Dermis seemed to be normal together with extremely active fibroblast and thick collagen fibres (Fig. 6a). In electronmicroscopic examination it was observed that, epithelium layers were less, tonofilaments were striking in the cytoplasm of the basal cells and keratohyalin granules were seen in the apical layer cells together with total keratinization (Fig. 6b). Degranulated mast cells with less amount were shown in Fig. 7.

In the group treated with EGF + carbopol 940 for 15 days after development of wound, epithelization of wound was observed to be regularly developed and upper layer cells had keratohyalin granule formation and keratinization microscopically (Fig. 6c) in the wounded skin. On the electron microscopic images, epithelization was found to be completed; cells were multilayered as in normal skin and fully mature. Basal cells were short and prismatic while middle layer cells were polygonal. Keratohyalin granules in the upper cells and keratinization could also be discriminated. Dermis was impressive with abundant connective tissue (Fig. 6d). Mast cells with less granules were observed in this group (Fig. 8).

Treatment with carbopol 940, as a vehicle, causes a partial renewed epithelium from basal layers. Epidermis was formed of 1 or 2 layers, the basal cells had an appearance of cuboid shape. The collagen fibres of the dermis were found to be remarkably thick by striking of the mast cells in the connective tissue (Fig. 9a). Electronmicroscopic views of the same group it has been observed that many small or large vacuoles were present in the cytoplasm of the epithelial cells. Keratohyalin granules were seen in the newly formed cells of the uppermost layer (Fig. 9b). Mast cells with intensive granules were observed (Fig. 10).

Discussion

EGF, is an important factor on wound healing by its regulatory effect (Mian et al., 1992). Topical application of this factor has been shown to accelerate the regeneration of epidermis *in vivo* (Schultz et al., 1987). According to the findings of Brown et al. (1986), and Laato et al. (1986), daily application of EGF solution locally in experimentally-induced wounds, causes an enhancement of epithelization with the accumulation of granulation tissue, collagen and glycosaminoglycans. There were many studies on the regulatory role of EGF on collagenesis and wound healing (Pessa et al., 1987; Gönül et al., 2000). But the role of this factor in the formation of abnormal healing is still obscure. Recently Kikuchi et al. (1995) have shown that EGF and histamine together cause an enhancement in the growth of keloid and normal scar fibroblasts in culture.

There is an abundant observation on the participation of mast cells in fibroblast proliferation (Trabucchi et al., 1988; Lipshits and Zviahintseva, 1997; Artuc et al., 1999). Activation of these cells cause an increase in the release of histamine and by this mediator induce a growth-promoting potential (Jordana et al., 1988). There are some evidence indicating that histamine is increased, in the reparatively growing tissues, in skin wounds as a result of increased histidine decarboxylase activity (Kahlson et al., 1960). It is well known that this amine has an important role in chronic inflammation, scatrics and fibrosis. Antihistaminic agents were shown to have a beneficial effect on abnormal collagen production which indicates that histamine production or release from the mast cells may have a role in the abnormal collagen formation (Topol et al., 1981; Bairy et al., 1990). In addition it has been shown that histamine caused an inhibition in 3H-tymidine incorporation of keratinocytes and whole cultures in a dose dependent manner, when in absence of EGF (Huttunen et al., 2001).

According to the results of the present study, EGF has a beneficial effect on wound healing by accelerating epithelization, increasing tensile strength and decreasing histamine content. Increase in tensile strength is more pronounced when EGF was prepared in carbopol 940 which is a gel form. Gel preparations have a high viscosity than saline. Therefore topically applied gel form may increase the bioavailability of EGF in the wounded area by increasing the contact time of the factor. That might be the reason why EGF in gel form is more effective in wound healing than the aqueous saline form (Ludwing et al., 1990). These results are in accordance with the previous findings on alkali burned corneal wound healing in mice (Gönül et al., 1995).

In addition, EGF + gel treated group granules of the mast cells were found to be decreased and wound tissue histamine levels were also found to be significantly reduced. This finding may be related to the reducing effect of EGF on histamine content of the mast cells and tissue levels. The effect of EGF might be due to the inhibition of the granulation of histamine in mast cells or to the increase of the metabolism of the amine either in mast cells or in tissues. This observation also supports the findings of Kuna et al. (1993) who reported that EGF has a negative effect on the release of histamine from basophils. Our results are in accordance with the findings of Huttunen et al. (2001) who observed the inhibitory effect of histamine on 3H-tymidine incorporation of keratinocytes and skin culture was reversed by EGF. Therefore EGF by decreasing histamine levels and mast cell contents may prevent the epithelial outgrowth and abnormal collagen formation due to histamine.

In conclusion, it can be assumed that local application of the EGF in gel form, by sustained release, enhancing epithelization and tensile strength, decreasing histamine content of the tissue therefore preventing abnormal collagen formation has a beneficial effect on wound healing.

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