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Effect of a Gelatin Hydrogel Incorporating Epiregulin on Human Keratinocyte Growth

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Abstract

Ionic hydrogels are biocompatible interesting candidates for tissue-engineering applications, such as the creation of artificial skin, as they can also be used, along with growth factors and cells grown *in vitro*, for developing bioengineered tissues to be implanted. Among the growth factors that can be used to induce keratinocytes growth *in vitro*, epiregulin, a broad-specificity epidermal growth factor (EGF) family member, has been shown to be more effective than EGF and transforming growth factor-alpha (TGF-α) in promoting re-epithelization *in vitro*. To produce a drug-delivery hydrogel for epiregulin, bovine gelatin was cross-linked with poly(glutamic acid) (PLG) in the presence of epiregulin (5–50 ng/ml). Spontaneously immortalized human keratinocytes (HaCaT) were seeded on unloaded and epiregulin-loaded hydrogels and cell adhesion was evaluated after 6 h. Moreover, cell proliferation and stratification, cytokeratins (K5, K10), differentiation markers (filaggrin and transglutaminase-1 (TG-1)) and matrix metalloproteinases (MMP-2, MMP-9 and MMP-28) expression were evaluated after 7 days. The presence of epiregulin induced an increase in cell proliferation, stratification and K5 expression along with MMP-9 and MMP-28 expression, while all differentiation markers expression (K10, filaggrin, TG-1) was decreased. These data indicated that a simple hydrogel loaded with epiregulin could be an effective tool for skin tissue engineering.

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Keywords

Skin, tissue engineering, ionic hydrogel, epiregulin, human keratinocyte

1. Introduction

Skin is the largest organ of the body in vertebrates and represents a physiological barrier against exogenous substances, pathogens and mechanical stress. Defects in this barrier integrity cause water and protein loss, and allow bacteria to invade the underlying tissue: in fact, after major trauma, such as burns, the absence of ker-

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atinocytes and the biological barrier they form causes different clinical problems such as infections and wound hypertrophy [1, 2].

Tissue engineering has provided many alternate therapies, including cultured allogeneic and autologous engineered skin, to conventional wound closure in patients suffering from major trauma where donor sites are limited and require multiple harvesting procedures [3].

Studies on the development of artificial epidermis started from the observation that keratinocytes can be grown in culture to produce thin epithelial sheet grafts using different experimental procedures [4]. A more useful technical approach to the development of artificial epidermis concerns the use of suitable biocompatible and biodegradable scaffolds, such as collagen, hyaluronic acid and biopolymers capable to favour keratinocytes' growth [5, 6].

Among biocompatible and biodegradable scaffolds, hydrogels (HGs) planning has lead to new results in skin tissue engineering because of their biocompatibility due to their high hydrophilicity along with their use as drug-delivery systems for growth factors [7–9].

We focused our research on one ionic hydrogel (HG) prepared by cross-linking gelatin, a natural biomaterial derived from collagen denaturation, with poly(L-glutamic acid) (PLG) [7], already successfully used for *in vivo* delivery of fibroblast growth factor 2 (FGF-2).

Cutaneous wound healing is a complex and dynamic process involving a number of precisely coordinated events including inflammation, cell migration, cell proliferation, angiogenesis, matrix deposition and remodelling [10, 11].

Cell proliferation and migration induced by the coordinate release of growth factors at the site of injury is a key point in re-epithelization of wounds.

Keratinocyte function is tightly regulated *via* intracellular signalling pathways mediated by the members of EGF family, that efficiently regulate keratinocytes' growth and differentiation *via* auto- and cross-induction pathways [12, 13].

In particular EGF and TGF-α have been shown to play important roles in promoting re-epithelialization of wounds [14–17]. Recently the newest member of the EGF growth factor family, epiregulin (Epi), has been shown to be both more potent and more effective than EGF or TGF-α in promoting *in vitro* wound closure by enhancing both migratory and proliferative cellular activities [18].

Epi, initially isolated from the conditioned medium of transformed fibroblasts [19], is a 46-amino-acid single chain polypeptide that exhibits 24–50% amino-acid sequence homology to other EGF receptor (EGFR) ligands [20].

In this paper the behaviour of immortalized human keratinocytes (HaCaT) seeded on gelatine-PLG hydrogels loaded with Epi has been investigated for 1 week.

Human keratinocytes adhesion, proliferation and stratification were monitored both by cell morphology and viability assays; the expression of cytokeratins (K5, K10), differentiation markers (filaggrin and transglutaminase-1 (TG-1)) and keratinocyte-specific matrix metalloproteinase-28 (MMP-28, epilysin) was evalu-

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ated by RT-PCR while the production of two matrix metalloproteinases involved in migration of keratinocytes (MMP-2, MMP-9) was assayed by gelatin zymography.

2. Materials and Methods

2.1. Synthesis of Gelatin Hydrogels (HG)

A 10% (w/v) gelatin solution was prepared by adding 100 mg gelatin type B (from bovine skin, Sigma (approx. 300 bloom; p*I* 9.0)) to 1 ml phosphate-buffered saline solution (PBS, pH 7.4). The mixture was heated to 40°C for 10 min to allow complete gelatin dissolution. Following gelatin dissolution 10 mg PLG (Sigma, MW 50 000–100 000) was added. EDC (1-[3-dimethylaminopropyl]-3-ethylcarbodiimide, Sigma-Aldrich)/NHS (N-hydroxysuccinimide, Sigma-Aldrich) solutions (4:1 molar ratio in PBS) were added. After the cross-linking reaction, hydrogels were washed in PBS to remove the unreacted by-products and the urea derivative of EDC [7]. In order to obtain HG enriched with Epi, various amounts of human recombinant (*Escherichia coli* derived) growth factor (R&D Systems, 5–50 ng/ml in PBS) were added during cross-linking process. Hydrogels were produced in sheets (surface 10 cm^2, width approx. 1 mm) and then cut in 1-cm^2 square samples.

2.2. *Epiregulin* In Vitro *Release Assay*

Epi release from hydrogels has been evaluated by enzyme-linked immunosorbent assay (Uscn Life Sciences) on both Epi-free (control) and 50 ng/ml enriched samples. Briefly, Epi-free and Epi-loaded hydrogels (HG surface 0.8 cm^2, volume 64 μl, 3.2 ng Epi) have been incubated in PBS at 37°C and at fixed time points (1, 2, 3, 7 days) aliquots of PBS (100 μl) have been recovered and stored at −20°C until analysis. PBS aliquots of 100 μl for each sample have been assayed following manufacturer's instructions and the optical density (OD) was read at 450 nm on a microplate reader. Results were expressed as mean values ± SD.

2.3. Human Keratinocyte Adhesion and Proliferation

Spontaneously immortalized keratinocytes (HaCaT), isolated from human adult skin [21], were a kind gift of Dr. M. De Andrea from the Microbiology Laboratory, University of Eastern Piedmont 'A. Avogadro'. Cells were grown in culture flask (75 cm^2) in DMEM medium (Euroclone) supplemented with 10% heat inactivated foetal bovine serum (FBS) (Euroclone), penicillin (100 U/ml), streptomycin (100 mg/ml) and L-glutamine (2 mM) (Euroclone) in a humidified atmosphere containing 5% CO_2 at 37°C. A volume of 200 μl of HaCaT cells suspension (5×10^5 cells/ml) in serum-free DMEM was added to HG samples (sample surface area 1 cm^2).

After incubation, unattached cells were removed by washing with PBS and 1 ml fresh medium (DMEM with 10% FBS) was added. Cell adhesion was assessed by counting cells in 10 fields (field area 3 mm^2) and expressed as adherent cells/cm^2.

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After 3 and 7 days cell proliferation was quantified both by morphological observation taking 10 random microscope images across the sample surface using a Leica DM2500 microscope and using a fluorimetric method based on the quantification of resazurin reduction by viable cells (CellTiter-Blue, Promega). Briefly, 25 μl dye was dissolved in 0.5 ml complete cell growth medium and after 4 h the OD was measured at 620 nm.

Cell proliferation was expressed as OD variation ± SD. At day 7 hydrogel samples were fixed for 15 min at room temperature using a solution of formaldehyde (3.7%) and sucrose (3%) in PBS (pH 7.4). After fixation samples were embedded in cryoblock medium and 25-μm sections were cut using a Leica CM 1850 Cryostat. Sections were stained with 2 μg/ml Hoechst 33342 solution in order to stain cell nuclei and then observed under UV light using a fluorescence microscope (Leica DM2500).

2.4. Reverse Transcriptase Polymerase Chain Reaction

Total cellular RNA was extracted using Qiagen RNeasy MicroKit following the manufacturer's protocol. Complementary DNA (cDNA) was prepared from total RNA using 2.5 μM random primers (Sigma-Aldrich) in a final volume of 20 μl. The reaction mixture was heated at 70°C for 5 min and then 50 mM $MgCl_2$, 5× reaction buffer (Promega), 10 mM dNTPs mix (Sigma-Aldrich), 0.20 U ribonuclease inhibitor (Qiagen) and RT enzyme (Promega) were added. The reaction was performed at 42°C for 1 h. The resulting cDNA was used for the polymerase chain reaction (PCR). Synthetic oligonucleotide primers specific for human epilysin (MMP-28), filaggrin (Fil), transglutaminase-1 (TG-1), cytokeratin 5 (K5), cytokeratin 10 (K10) and β-actin were designed using the primer-blast tool software from NCBI. the forward and reverse primer sequences and the expected size of PCR products are shown in Table 1. Reaction products were separated on an 1.8% TBE-agarose gel and stained with ethidium bromide. DNA molecular weight marker (Sigma-Aldrich) was used as a size marker.

2.5. Gelatin Zymography

To detect MMP-2 and MMP-9 activity, conditioned media from HaCaT cells grown on control and Epi-enriched HG were separated by electrophoresis on SDS-polyacrylamide gels containing 0.2% gelatin. Samples were loaded on zymograms without denaturation. After running, gels were washed at room temperature for 2 h in 2.5% Triton X-100 solution and incubated overnight at 37°C in 0.5 M Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$ buffer. Gels were then fixed in MeOH/acetic acid (50:10) solution and stained in 0.5% Coomassie blue in MeOH/acetic acid (40:10) solution. Images of stained gels were acquired after appropriate destaining. Gelatinolytic activity was detected as white bands on a dark blue background and quantified by densitometric analysis using ImageJ software.

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Table 1.
Primers designed for human cytokeratine 5 (K5), citokeratine 10 (K10), epilysin (MMP-28), filaggrin, transglutaminase-1 (TG-1) and β-actin

Gene	Primers	Product size (bp)
K5	Forward: 5′-CAC CAA GAC TGT GAG GCA GA-3′ Reverse: 5′-CAT CCA TCA GTG CAT CAA CC-3′	274
K10	Forward: 5′-GAA CCA CGA GGA GGA AAT GA-3′ Reverse: 5′-AAC TGT TCT TCC AGA GCG GA-3′	398
MMP-28	Forward: 5′-AGA GCG TTT CAG TGG GTG TC-3′ Reverse: 5′-AAA GCG TTT CTT ACG CCT CA-3′	180
Filaggrin	Forward: 5′-TGA TGC AGT CTC CCT CTG TG-3′ Reverse: 5′-TGT TTC TCT TGG GCT CTT GG-3′	338
TG-1	Forward: 5′-ACG TTA CTG GGA GCA GCA GT-3′ Reverse: 5′-TGC CAT AGG GAT GGT CTC TC-3′	351
β-actin	Forward: 5′-ACA CTG TGC CCA TCT ACG AGG GG-3′ Reverse: 5′-ATG ATG GAG TTG AAG GTA GTT TCG TGG AT-3′	360

2.6. Statistical Analysis

The statistical analysis of data was performed using the Graph Pad Prism 2.01 software for Windows using the ANOVA test followed by Dunnett's post-hoc test. Values of $P < 0.05$ were considered statistically significant.

Experiments were done in triplicate and data were expressed as mean values ± SD.

3. Results

3.1. Epiregulin In Vitro *Release*

In vitro Epi release from 50 ng/ml enriched and epiregulin-free hydrogels has been evaluated by ELISA. As shown in Fig. 1, an initial burst in Epi release could be observed after 1 day, resulting in 170.3 ± 39.54 pg/ml Epi released, a very small quantity compared to the Epi loaded (3200 pg/sample). After this initial burst Epi release drops down on days 2 and 3 (79.8 ± 19.81 pg/ml and 60.8 ± 24.67 pg/ml, respectively), and after 7 days Epi concentration released in the medium by the hydrogel still drecreased, reaching 28 ± 12.72 pg/ml ($P < 0.001$ compared to day 1, $P < 0.05$ compared to day 2).

3.2. Cell Adhesion and Proliferation

As shown in Fig. 2, Epi incorporation into HG did not significantly modify HaCaT adhesion after 6 h. In fact, the average number of cells adhered on HGs was 56 700 ± 5800 cells/cm^2 in control samples, while it was 46 027 ± 6200, 50 348 ± 5900 and 45 111 ± 7100 cells/cm^2 for 5, 10, 50 ng/ml Epi-enriched HG, respectively.

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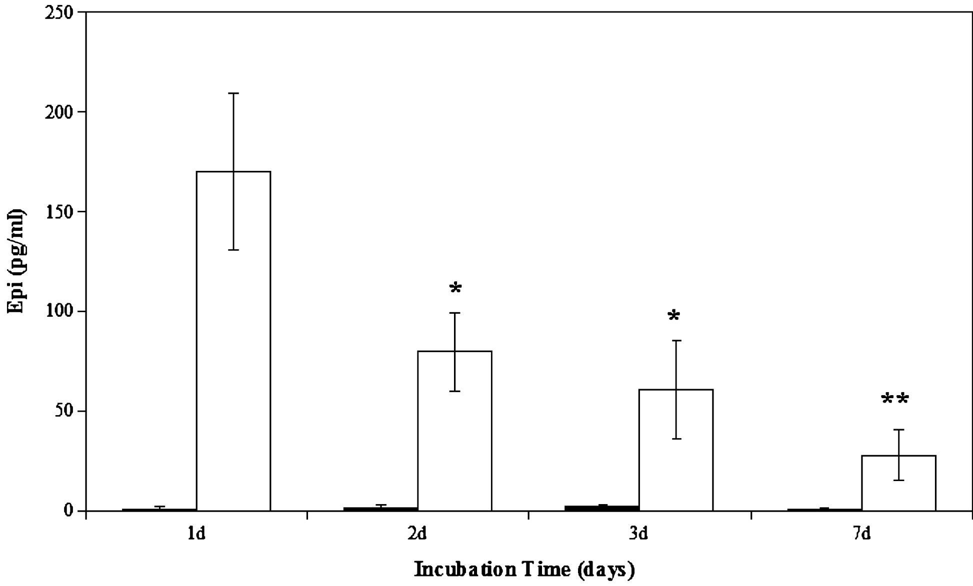

Figure 1. Quantification of *in vitro* Epi release by ELISA assay at 1, 2, 3, 7 days. Black bars, Epi-free HG; white bars, Epi-loaded HG. $^{**}P < 0.001$; $^{*}P < 0.05$, compared to day 1.

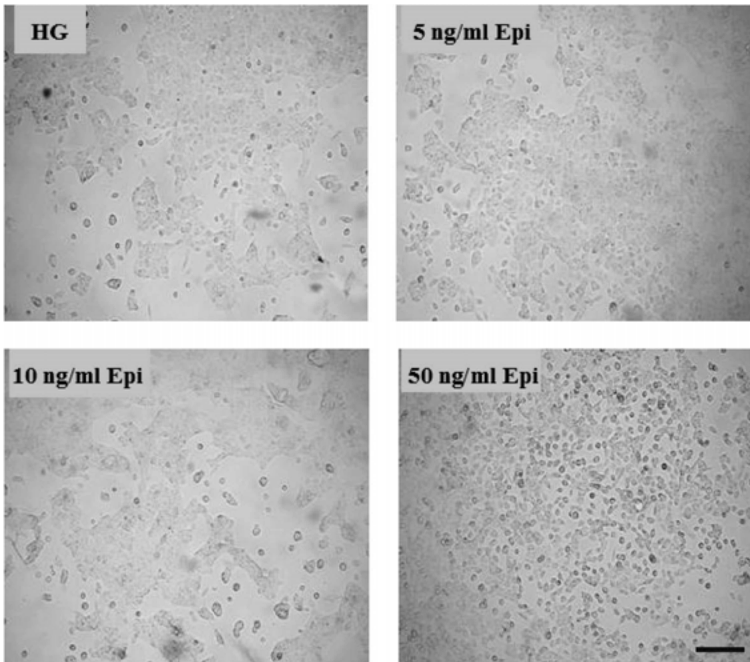

Figure 2. HaCaT adhesion on control and Epi-enriched hydrogel at 6 h post-seeding. Scale bar = 60 μm.

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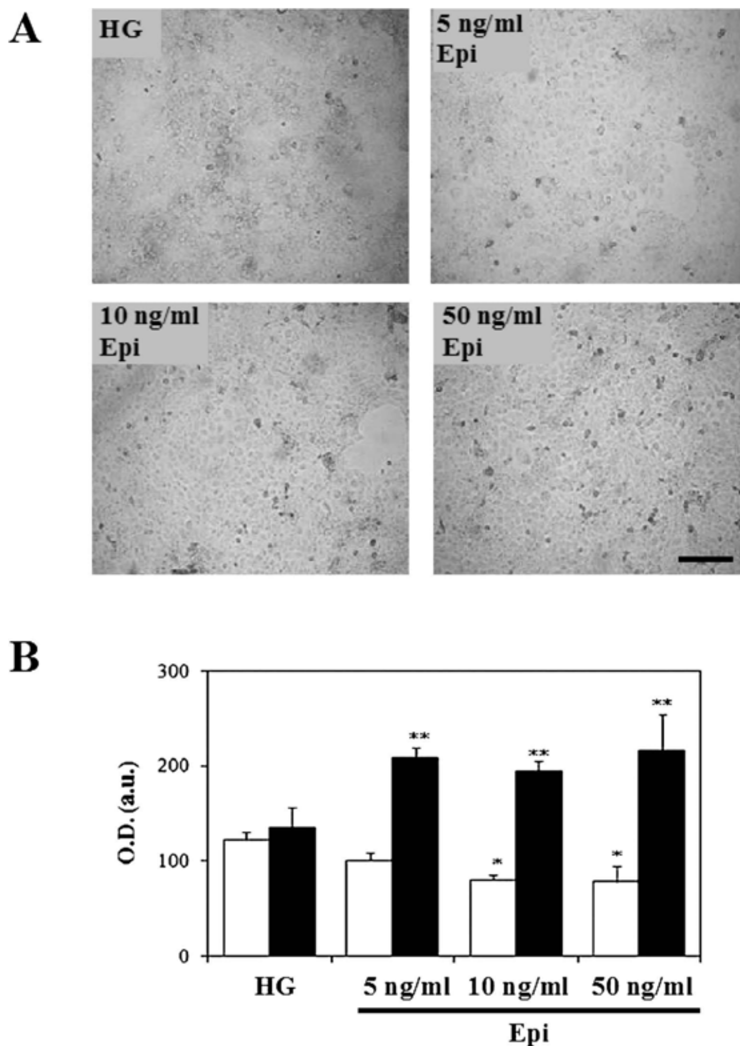

Figure 3. (A) HaCaT morphology on HG and Epi-enriched HG at 7 days post-adhesion. Scale bar = 60 μm. (B) Quantification of HaCaT proliferation at 3 (white bars) and 7 (black bars) days post-adhesion by colorimetric assay (CellTiter-Blue). ** $P < 0.001$; * $P < 0.05$, compared to control samples.

At 3 days post-adhesion, a slight decrease in HaCaT proliferation occurred compared to cells seeded on hydrogels containing the highest Epi concentrations ($P < 0.05$ compared to control samples), but this effect was completely reverted at 7 days post-adhesion. In fact, as shown both in Fig. 3A and B, proliferation of HaCaT cells seeded on Epi-enriched HG was higher than that observed for HaCaT cells seeded on control HG ($P < 0.001$). It is noteworthy that the maximum proliferative effect was already reached at the lowest Epi concentration.

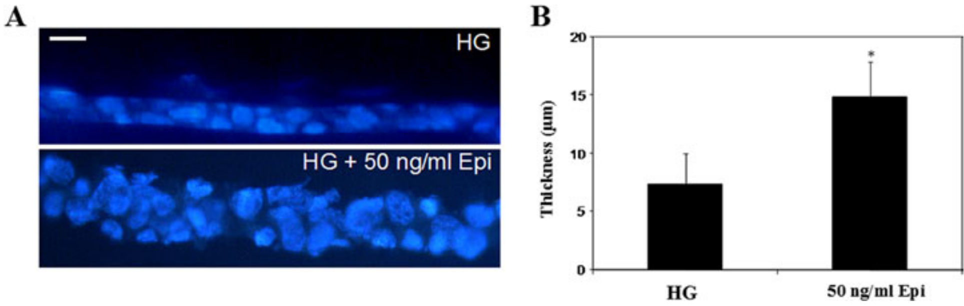

Figure 4. (A) Representative fluorescence image of a transversal section (25 µm) of HaCaT epithelium at 7 days post-adhesion on control and 50 ng/ml Epi-enriched hydrogel, respectively. Scale bar = 12 µm. (B) Quantification of the epithelial thickness. $^{*}P < 0.05$. This figure is published in colour in the online edition of this journal, which can be accessed *via* http://www.brill.nl/jbs

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At 7 days post-adhesion HaCaT cells grown both on control and Epi-enriched HGs formed a continuous epithelium. In order to evaluate its structure samples were fixed, included in cryoblock medium and 25-µm sections were cut using a cryostat. Slides were then stained with Hoechst 33342 solution in order to stain keratinocytes nuclei as shown in Fig. 4A.

Epithelium grown on all the Epi-enriched HGs was thicker than that grown on control HG. In fact, morphometrical analysis performed on 50 ng/ml Epi-enriched HG sections (Fig. 4B) highlighted that epithelium grown on this HG was 14.9 ± 2.9 µm thick, while the one grown on control HG was only 7.3 ± 2.8 µm thick ($P < 0.05$). Similar results were obtained for both 5 and 10 ng/ml Epi-enriched HGs (data not shown).

3.3. Proliferation and Differentiation Markers Expression in Cells Grown on Epi-Enriched HGs

Total RNA was extracted from keratinocytes grown for 7 days on control and Epi-enriched (5, 10, 50 ng/ml) HGs. RNA was reverse-transcribed and amplifyed by PCR to assess cytokeratin 5 (K5), cytokeratin 10 (K10), filaggrin, transglutaminase-1 (TG-1) and epilysin (MMP-28) expression. β-actin was used as housekeeping gene.

As shown in Fig. 5A and B, Epi addition to HG greatly modified cytokeratin expression in HaCaT cells. In particular K5 expression, which is usually correlated with proliferating keratinocytes, was doubled at 7 days by 50 ng/ml Epi as observed by densitometric analysis, while the differentiation marker K10 expression was halved.

According to this anti-differentiation effect, also the expression of other two differentiation markers (filaggrin and TG-1) appeared reduced in a dose dependent manner (Fig. 6A and B), while the expression of epilysin (MMP-28), a matrix metalloproteinase expressed by basal and suprabasal keratinocytes [22], was slightly increased in HaCaT cells grown on Epi-enriched HGs.

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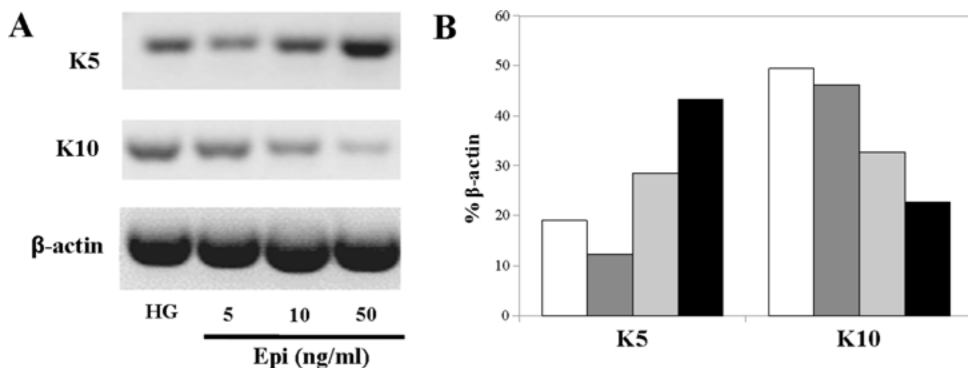

Figure 5. (A) Representative TBE-agarose gel for RT-PCR amplified K5, K10 and β-actin transcripts in HaCaT cells 7 days post-adhesion on control and Epi-enriched hydrogel. (B) Densitometric quantification of K5 and K10 expression. β-actin was used as housekeeping gene for data normalization. Cells were grown on hydrogels enriched with different concentrations of Epi: no Epi (control, white bars), 5 ng/ml (grey bars), 10 ng/ml (light grey bars) and 50 ng/ml (black bars).

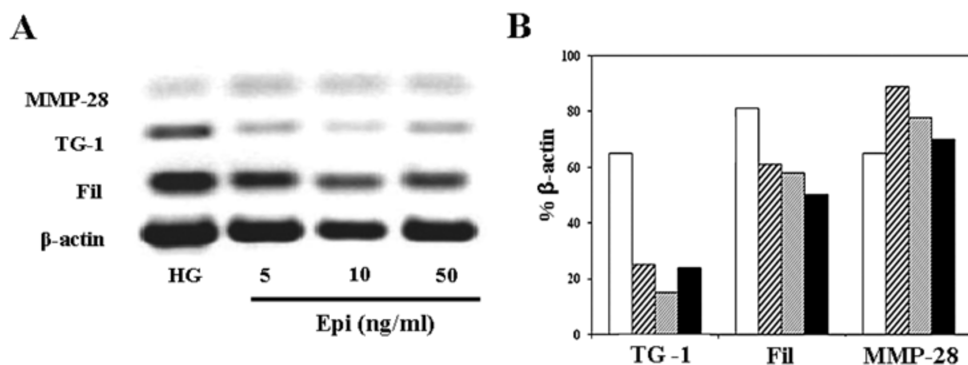

Figure 6. (A) Representative TBE-agarose gel for RT-PCR amplified MMP-28, TG-1, Filaggrin and β-actin transcripts in HaCaT cells at 7 days post-adhesion on control and Epi-enriched hydrogel. (B) Densitometric quantification of MMP-28, TG-1 and filaggrin expression. β-actin was used as housekeeping gene for data normalization. Cells were grown on hydrogels enriched with different concentrations of Epi: no Epi (control, white bars), 5 ng/ml (striped bars), 10 ng/ml (grey bars) and 50 ng/ml (black bars).

3.4. MMP-2 and MMP-9 Activity

The presence and activity of MMP-2 and MMP-9, two Zn^{2+}-dependent preoteases known to be involved in cell migration [23], was assessed by means of gelatine zymography in conditioned cell-culture medium from cells grown both on control and Epi-enriched HGs for 7 days, as shown in Fig. 7A.

HaCaT cells grown on Epi-enriched HG produced a significantly higher amount of MMP-9 starting from 10 ng/ml Epi concentration, while MMP-2 expression was reduced in a similar manner. In particular, densitometric analysis indicated that, in cells grown on 50 ng/ml Epi-enriched HG, both an increase of more than 350% of

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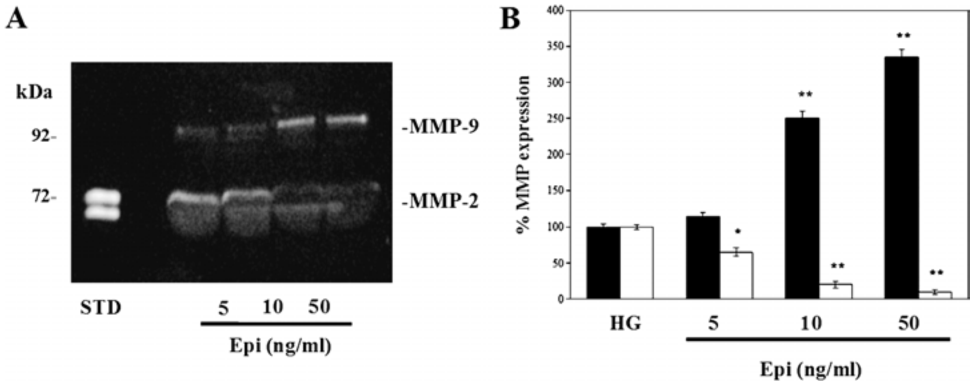

Figure 7. (A) Gelatin zymography highlighting MMP-2 and MMP-9 activity in conditioned cell-culture medium from HaCaT grown on control or Epi-enriched hydrogels for 7 days. (B) Densitometric quantification of MMP-2 and MMP-9 activity at 7 days post-adhesion. White bars represent MMP-2, black bars represent MMP-9 activity, respectively. $^{*}P < 0.05$; $^{**}P < 0.001$.

MMP-9 and a 80% decrease in MMP-2 production occurred compared to control sample (Fig. 7B, $P < 0.001$). Those data indicated that the presence of Epi in HG fostered a proliferative behaviour in HaCaT cells.

4. Discussion

Tissue engineering provides solutions for tissue loss, or restoration of tissue or organ function with constructs that contain specific populations of living cells [24].

Since tissue loss at the skin level is a common occurrence due to a plethora of events such as lacerations, cutaneous disease, neoplasia, infection, burns and other trauma, adequate cutaneous constructs that could act as effective skin replacements, capable of mimicking native skin, are highly desirable [25].

Many natural biopolymers, such as glycosaminoglycans, collagen, fibronectin and hyaluronan, are currently used as potential scaffolds for keratinocyte adhesion and proliferation, but the lack of blood vessels, their reduced mechanical capabilities and the absence of differentiated structures, such as sebaceous glands, melanocytes and Langerhans cells, impair their use as natural skin substitutes.

Recently, among the ‘natural scaffold’ for tissue-engineering applications, ionic hydrogels have been extensively studied. Ionic hydrogels are polymeric materials with a porous tridimensional structure, made by cross-linking different natural and synthetic compounds, obtaining a highly hydrated structure highly biocompatible and biodegradable. Moreover, these scaffolds display a good swelling so they are interesting candidates for many biological applications [26].

In this study a gelatin-based hydrogel (HG) has been used. This HG has already been proven to be biocompatible [27] and to act as a good drug-delivery system for fibroblast growth factor 2 (FGF-2) [7]. Therefore, it could be used for cell-based drug delivery [28] in the case of burns or chronic wounds, so it has been loaded

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with a specific epithelial growth factor to enhance and/or to speed the formation of a functional epithelium.

The aim of this paper was to observe if this anionic hydrogel formed by cross-linking gelatin with PLG could be a real good substrate for human keratinocyte growth. Moreover we would like to test if HG loading with epiregulin (Epi), a member of the EGF family, produced by keratinocytes, could improve cell proliferation and/or differentiation.

Our data indicate that the anionic hydrogel, used as scaffold for keratinocyte growth, released a small amount of Epi in 7 days, thus acting as drug-delivery device and allowing HaCaT cells adhesion and growth. Moreover, even if the quantity of Epi released is low (in the order of pg/ml), after 1 week it is effective in stimulating keratinocytes' growth, maybe because of a releasing occurring very close to the cells.

Epidermal regeneration is a complex process in which residual epithelial cells proliferate and migrate in an integrated fashion to regenerate intact epidermis.

It has been reported that Epi stimulates human keratinocyte proliferation under both subconfluent and confluent culture conditions in the absence of exogenous EGF family growth factors [12].

Keratinocytes seeded on cell-culture-grade plastic dishes and control hydrogel reach confluence and stop proliferating. In our experimental model keratinocytes seeded on an Epi-enriched hydrogel start a 'vertical' growth, creating a stratified epithelium. The overall effect of Epi, present in the hydrogel and released during 7 days, was a block of the contact-mediated cell-growth inhibition, resulting in an increased cell proliferation, also in a vertical direction.

The increase in cell proliferation observed on Epi-loaded HG was confirmed by the increased expression of K5, a cytokeratine expressed by basal proliferating keratinocytes [29] and by the simultaneous decrease in K10 expression, a marker of keratinocyte differentiation [29].

Furthermore cells grown on Epi-loaded HG showed a lowering in the expression of filaggrin and TG-1.

Filaggrin is an abundant and important protein expressed in differentiating keratinocytes that functions to aggregate keratin interfilaments into aligned macrofibrils in the stratum corneum of the epidermis [29, 30], while TG-1 is a key enzyme mainly expressed in skin granular layer and it catalyzes the cross-linking of several proteins for the formation of the cellular envelope [31]. Its activity is fundamental for skin epithelium stability and function [32]. Therefore, Epi seems to lead keratinocytes toward a proliferative and less differentiated phenotype.

Another important aspect in wound healing is the ability of keratinocytes to migrate to reconstruct the epithelium continuity. Epi has been proven to be more potent than EGF in accelerating wound closure, but it also promoted greater thickening of the epidermis. This effect resulted in a more robust epithelial cover that could likely withstand increased stress and trauma and it relies primarily on the process of epithelialization for closure.

Key enzymes involved in wound healing are matrix metalloproteinases (MMPs), in particular gelatinase A (MMP-2) and gelatinase B (MMP-9), while another MMP, epilysin (MMP-28), was also involved in wound healing, even if its role is still unclear [22]. During wound healing a number of migratory and remodelling events that require the action of MMPs and their natural inhibitors, the tissue inhibitors of metalloproteinases, occur. Mounting evidence suggests that EGF family members play a role in regulating MMP expression in the wound milieu, in fact, treatment with Epi or EGF accelerates the repair process resulting in more mature wounds that require less ongoing proteolytic activity than their same day vehicle treated counterparts [20].

So far, no data are available from the literature on MMP-28 induction mediated by EGF or Epi.

MMP-28, also known as epilysin, is a proteolytic enzyme belonging to the MMP family, selectively produced by keratinocytes [22], the expression of which is upregulated during wound healing [33] and hypertrophic scaring [34].

Epilysin expression is induced during wound healing, in migrating keratinocytes and in basal stationary keratinocytes far behind the wound edge, implying a role for epilysin both in normal tissue homeostasis and in response to injury [35].

Human keratinocytes grown on Epi-enriched HG showed a reduced MMP-2 production and an increased MMP-9 expression, indicating that the presence of Epi induced a more proliferating epithelium while reducing the potential migratory ability of keratinocytes. So far there are no reports about a direct effect of Epi on MMP-9 or other MMPs expression in not-tumoral cells, even if it has been reported that EGF, the most important and studied member of the Epi family, can induce both cell proliferation and migration along with MMP-9 expression in HaCaT cells [36–38]. Moreover, the presence of Epi slightly increased the expression of MMP-28, normally expressed in basal proliferating keratinocytes.

5. Conclusions

In this paper we tested a novel strategy for skin tissue engineering. We showed that an anionic hydrogel loaded with epiregulin (Epi), a potent EGF family member allowed the growth of a stratified human epithelium. The ability of Epi in promoting keratinocytes proliferation along with the chemiophysical characteristics of hydrogels that promote cell adhesion could be an interesting new tool for skin tissue engineering.

Comments from One of the Reviewers

"As an overall comment, I do not think this new strategy would represent an option in the treatment of major skin losses as claim by the authors. I think that we are in front of two options: (1) produce *in vitro* an artificial skin that would then be grafted. In that case, growth factor as a supplement in the culture medium should

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be compared to the growth factor containing hydrogel; (2) produce a matrix that would be grafted and that would favour skin repair and/or skin regeneration. In that case, lessons from the clinical trials previously done with various growth factors should be taken into account. We learned that the use of growth factors in the treatment of wound healing is highly erratic because the window during which the wound requires the growth factor is extremely sharp, and not only the window but also its concentration. Higher or lower concentrations may result in a totally inverse effect. The use of an uncontrolled drug releasing hydrogel for this application appears highly hazardous. This also needs to be linked to one of the interpretations in the manuscript that suggests that Epi removes the cell-contact inhibition and, therefore, favours cell proliferation and, maybe, promotes cell transformation. A 'vertical growth' would not create a stratified epithelium as suggested in the discussion, at least in a physiological sense".

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