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Epiregulin-loaded PLGA nanoparticles increase human keratinocytes proliferation: preliminary data

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Abstract. – OBJECTIVE: Epiregulin is a member of the epidermal growth factor (EGF) family produced by keratinocytes: the aim of this study was to investigate the ability of biocompatible nanoparticles loaded with such growth factor to increase human keratinocytes proliferation.

MATERIALS AND METHODS: Different PLGA (Poly-d,l-lactide-co-glycolide)-nanoparticles (NPs) formulations have been characterized in size and zeta potential by dynamic light scattering (DLS) analysis. The ability of the different PLGA-NPs formulations to adhere onto dental surfaces has been tested, and epiregulin-enriched PLGA-NPs has been produced. Epiregulin release from NPs has been tested by enzyme-linked immunosorbent (ELISA) assay and the proliferative effects of epiregulin-NPs on human keratinocytes have been evaluated.

RESULTS: DLS analysis revealed a different size distribution depending on the PLA/PGA (poly lactic acid/poly glycolic acid) ratio used. 50:50 PLGA-NPs exhibited the smaller size and the best dental adhesive ability. Moreover, such epiregulin-loaded NPs was able to increase cell proliferation.

CONCLUSIONS: Direct dental pocket drug delivery implies the NPs solution loading onto the dental surface at the cement-enamel junction level: 50:50 PLGA-NPs, with their small size and excellent adhesive ability, represent an interesting tool to deliver epiregulin directly where there is the need for epithelial proliferation. These results describe a possible strategy for periodontal pocket delivery of Epiregulin-loaded PLGA-NPs and might provide a new approach for the treatment of gingival recession, where gingival epithelium proliferation is needed.

Key Words:
Epiregulin, Gingival recession, PLGA nanoparticles.

Introduction

Gum recession is defined as the shift of the marginal tissue apical to the cement-enamel junction (CEJ), with exposure of the root surface, leading to pain, unfavorable aesthetic appearance and root caries. Surgical root coverage is the only therapeutic choice if cause-specific measures are insufficient to correct the deformity of gingival mucose[1].

Epiregulin (Epi) is a broad specificity EGF (epidermal growth factor) family member with the unique characteristic to transmit a more potent mitogenic signal than EGF itself by binding all possible ErbB receptor complexes[2,3], thus, stimulating cell proliferation in various cellular linages, such as keratinocytes and fibroblasts[4,5].

Gum wound healing is important for both periodontal pathologies and surgery: for this reason, it is conceivable a beneficial effect of Epi as treatment of gingival recession, considering the mounting evidence suggesting also a crucial role of Epi in mediating proliferation and migration of gingival epithelial cells and fibroblasts[6].

The aim of the present study was to design an innovative nanotechnological approach to deliver Epi directly to the gingival pocket, where its positive effect on cell proliferation and migration is needed.

Nanotechnology has gained significant clinical interest in recent years, and nanoparticles (NPs) have become very attractive to the pharmaceutical and biomedical fields as drug delivery vehicles[7].

NPs can deliver a plethora of drugs, vaccines or biological macromolecules. By acting as drug delivery systems, NPs allow both a targeted administration of the active component to specific organ or cells and a controlled release of the drug[7-9].

Moreover, biodegradable polymeric NPs represent a useful delivery device as they can protect drug moieties from enzymatic disruption and provide sustained drug release over a certain time in a controlled manner, reducing side effects and dosage of therapeutic agents[8,10-12].

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Poly-d,l-lactide-co-glycolide (PLGA) is one of the most successfully used polymer to develop drug delivery systems, thanks to its attractive properties, such as favorable mechanical characteristics, biodegradability and biocompatibility, and FDA (US Food and Drug Administration) approval for human usage.

PLGA-NPs are biodegradable in the body: they undergo hydrolysis, leading to the production of the original monomers lactic acid and glycolic acid, that are metabolized via the Krebs cycle[8,9,12,13].

In the present study, epiregulin-enriched PLGA-NPs was produced and characterized. Once identified the formulation assuring the best adhesion on the dental surface, epiregulin-loaded NPs was produced and tested to evaluate growth factor release and its ability to modulate cell proliferation in an *in vitro* model (human keratinocytes (HaCaT) cells).

Materials and Methods

Teeth Collection

Healthy adult teeth were obtained from "S.C.D.U. Odontoiatria e Stomatologia", of the "Maggiore della Carità" Hospital (Novara, Italy) after routine extraction with written informed consent obtained from patients. The study was approved by the Ethical Committee of "Maggiore della Carità" Hospital in Novara, ALS of Biella, Novara, Vercelli and Verbano-Cusio-Ossola (study n. CE 63/11).

Soon after extraction, teeth were immersed in a 0.2% chlorhexidine solution for 30 minutes and then stored at 4°C in phosphate-buffered saline (PBS) solution (pH = 7.4) during the study period.

PLGA-nanoparticles Production

PLGA-NPs were prepared by a modified double solvent evaporation method[14]. Briefly, 60 mg of PLGA (50:50, 65:35, 75:25) crystals (Sigma-Aldrich, Saint Luis, MO, USA) were dissolved in 1 ml of dichloromethane (DCM) (Sigma-Aldrich, St. Luis, MO, USA) at room temperature. To produce control NPs, 50 µl of 1% polyvinyl alcohol (PVA) (Sigma-Aldrich, St. Luis, MO, USA) aqueous solution were added to PLGA and the solution was sonicated for 1 min. After that, 5 volumes of 1% PVA aqueous solution were carefully added to the resulting emulsion in order to maintain phase separation. A further 2 min sonication was performed to obtain the final emulsion that was evaporated overnight under fume hood, to remove DCM. The resulting NPs were washed 5 times in distilled water by centrifugation at 13000 rpm for 5 min, resuspended in water and stored at 4°C.

NPs containing epiregulin were produced as above by adding 50 µl Epi (human recombinant epiregulin, *E. coli* derived, R&D Systems, Minneapolis, MN, USA, 50 ng/ml), instead of PVA, to the PLGA solution dissolved in DCM during the first step of the preparation.

Particle Size and Zeta Potential Measurement

Particle size and polydispersity index after water dispersion and the charge density exposed on the surface of PLGA-NPs were evaluated by using DLS (dynamic light scattering) technique. PLGA-NPs were dispersed in water (1 mg/ml) and measured through DLS analysis in order to determine the particle size distribution. DLS experiments were carried out using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd, Malvern, UK), operating in a particle size range from 0.6 nm to 6 µm and equipped by a laser He-Ne with $\lambda = 633$ nm. Zeta potential analysis (ζ-potential), carried out to determine the stability behavior of our NPs solutions, was performed using the same instrument.

NPs adhesion to Dental Surface

In order to evaluate the NPs adhesion onto the dental surface, teeth were photographed before and after NPs loading, testing three different PLA/PGA (poly lactic acid/poly glycolic acid) ratio formulations (50:50, 65:35 and 75:25) in order to determine the best PLGA grade in terms of dental surface adhesion. The NPs suspension was positioned at the CEJ level, let dry and photographed. NPs-loaded teeth were then incubated in simulated saliva[15] at 37°C for 3 hours in order to simulate the oral cavity real conditions. Then, digital pictures of each tooth were captured for comparison and NPs attachment areas were quantified using ImageJ software (U. S. National Institutes of Health, Bethesda, MD, USA). NPs covered area after incubation, was expressed as the percentage of the initial area at time zero ± standard deviation (SD).

Epiregulin Release Assay

As dental adhesion and particle size results indicate the optimal PLA/PGA polymer ratio to be 50:50, we produced 50:50 PLGA-NPs containing Epiregulin and analyzed its release profile by enzyme-linked immunosorbent assay (ELISA)

Table I. Size and zeta potential. Size and zeta potential of the different PLGA-NPs formulations analyzed by dynamic light scattering.

PLGA NPs	Size	Zeta potential
50:50	190 nm (PDI=0.093)	-5.91 mV (SD=3.95 mV)
65:35	220 nm (PDI=0.235)	-3.20 mV (SD=3.54 mV)
75:25	370 nm (PDI=0.617)	-4.49 mV (SD=11.10 mV)

(Uscn Life Sciences Inc., Wuhan, China). Briefly, Epi-enriched NPs have been incubated in simulated saliva at 37°C and at fixed time points (3h, 6h, 1, 2, 10 days) simulated saliva aliquots of 100 µl for each sample have been assayed following manufacturer's instructions; the optical density (O.D.) was read at 450 nm on a microplate reader. Results were expressed as mean values ± standard deviation (SD).

Cell Culture

Spontaneously immortalized keratinocytes (HaCaT, CLS Cell Lines Service GmbH, Eppelheim, Germany), isolated from human adult skin[16], were grown in culture flask (75 cm^2) in DMEM medium (Euroclone, Milan, Italy) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Euroclone, Milan, Italy), penicillin (100 U/ml), streptomycin (100 mg/ml) and L-glutamine (2 mM) (Euroclone, Milan, Italy) in a humidified atmosphere containing 5% CO_2 at 37°C.

Cell Proliferation Assay

To evaluate the proliferative effects of epiregulin released from PLGA-NPs, HaCaT cells were seeded in 24-well plates at a density of $2x10^4$ cells/well and incubated in the presence or absence of PLGA-NPs supernatant, obtained after Epi-loaded and unloaded NPs solution centrifugation. Such samples were diluted 1:4 in Dulbecco's Modified Eagle's Medium (DMEM) before cell treatment. As NPs supernatant main component is represented by water, cells were also treated with the same volume of water in order to exclude vehicle interference. After 72h of incubation, cells were fixed in 3.7% formaldehyde-3% sucrose solution and stained with 1% toluidine blue solution. Stained samples were photographed at 10X magnification, using an optical microscope (Leica ICC50HD, Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) and cell proliferation was evaluated by counting cells in 10 random fields in three samples for each experimental condition. Results were expressed as cells/mm^2 ± standard deviation (SD).

Statistical Analysis

Unpaired Student's t-tests were used for statistical analysis. Statistical evaluation was performed with the Prism 4.0 statistical software (GraphPad Software Inc., La Jolla, CA, USA). Probability values of $p<0.05$ were considered statistically significant.

Results

NPs Characterization

NPs size distribution analysis by DLS approach revealed a different size distribution depending on the formulation (i.e. PLA/PGA ratio). In particular, the 50:50 PLGA-NPs formulation displayed the smallest size among the three formulations analyzed (Table I). In general, particle aggregation is less likely to occur for charged particles (high zeta potential) due to electric repulsion. The zeta potential found here for the different NPs formulations suggest that there was no difference in the surface charge characteristics of these NPs, but might allow particles aggregation. Nevertheless, these data do not indicate that NPs suspension is unstable, as the hydrophilicity of PLGA is sufficient to maintain the particles suspended.

PLGA NPs Adhesion to Dental Surface

NPs ability to adhere to the dental surface is an essential feature allowing their direct placement at the CEJ level; for this reason, the different PLGA-NPs formulations ability to adhere on teeth was evaluated.

As shown in Figure 1, 50:50 PLGA-NPs exhibited the best dental adhesion ability. Indeed, after 3h of incubation in artificial saliva, 50:50 PLGA-NPs were still present onto the dental surface covering an area of 87.6 ± 1.2% compared to time zero (NPs freshly applied onto the teeth). On the contrary, the other PLGA formulations (65:35 and 75:25) have shown a statistically significant reduction ($p<0.001$) in the attachment area.

As 50:50 PLGA-NPs displayed the better adhesion ability on dental surface, growth factor loaded NPs were produced using such scaffold.

In vitro Epiregulin Release

Quantification of epiregulin release from PLGA-NPs performed by ELISA assay indicates that Epi-enriched NPs incubated in simulated saliva at 37°C were able to produce a peak in Epi release after 3 hours of incubation (Figure 2). At this time point, 88 ± 25 pg/ml of Epi have been detected,

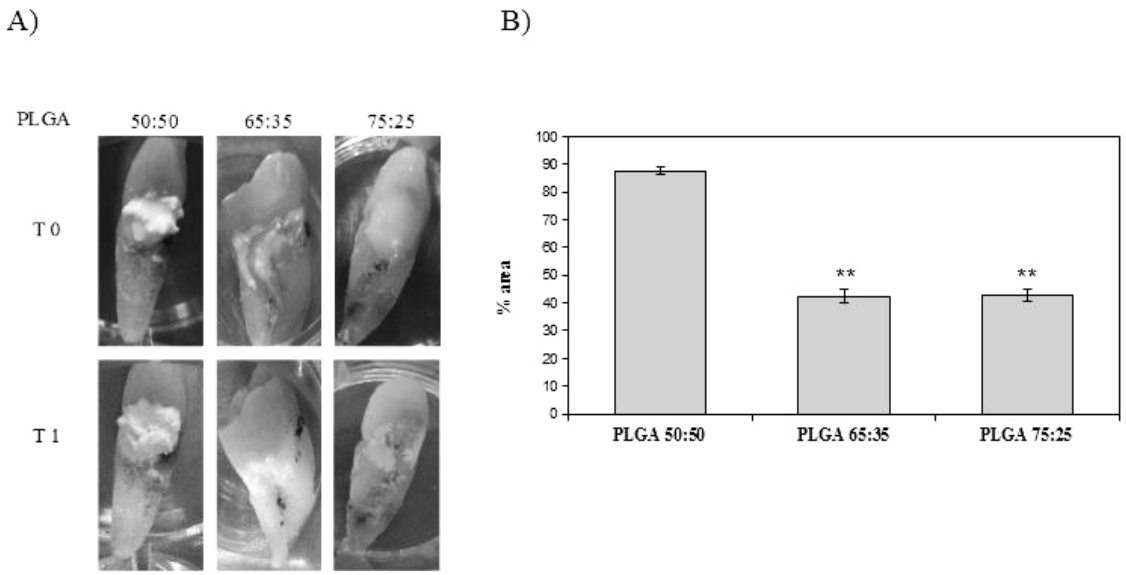

Figure 1. NPs adhesion to dental surface. ***A)*** Digital pictures of teeth immediately after NPs loading (T0), and after 3 hours (T1) of incubation in simulated saliva. *B)* Quantification of NPs attachment areas on the different PLGA formulations. Data are expressed as percentage ± SD of the initial filled area ($^{**}p<0.001$).

while a significant reduction ($p<0.05$) and a stabilization of the Epi release levels occurred at the following time points.

Epiregulin Released from PLGA NPs Stimulates Cell Proliferation

As shown in Figure 3, a statistically significant ($p<0.01$) increase in cell proliferation occurs only in the samples treated with Epi-enriched PLGA-NPs supernatant while unloaded NPs supernatant did not affect cell proliferation. Therefore, even if epiregulin released by NPs is very low, it was sufficient to induce an increase in cell proliferation compared to control conditions.

Discussion

Gingival recession or marginal tissue recession is a pathological condition defined as the location

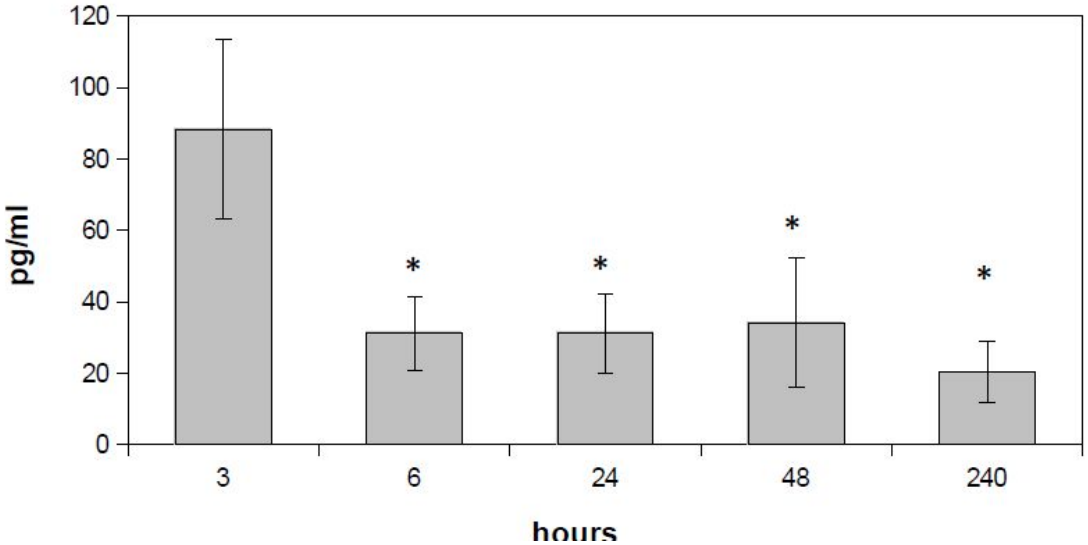

Figure 2. Epiregulin release assay. Quantification of epiregulin release from 50:50 PLGA-NPs performed by ELISA assay ($^{*}p<0.05$).

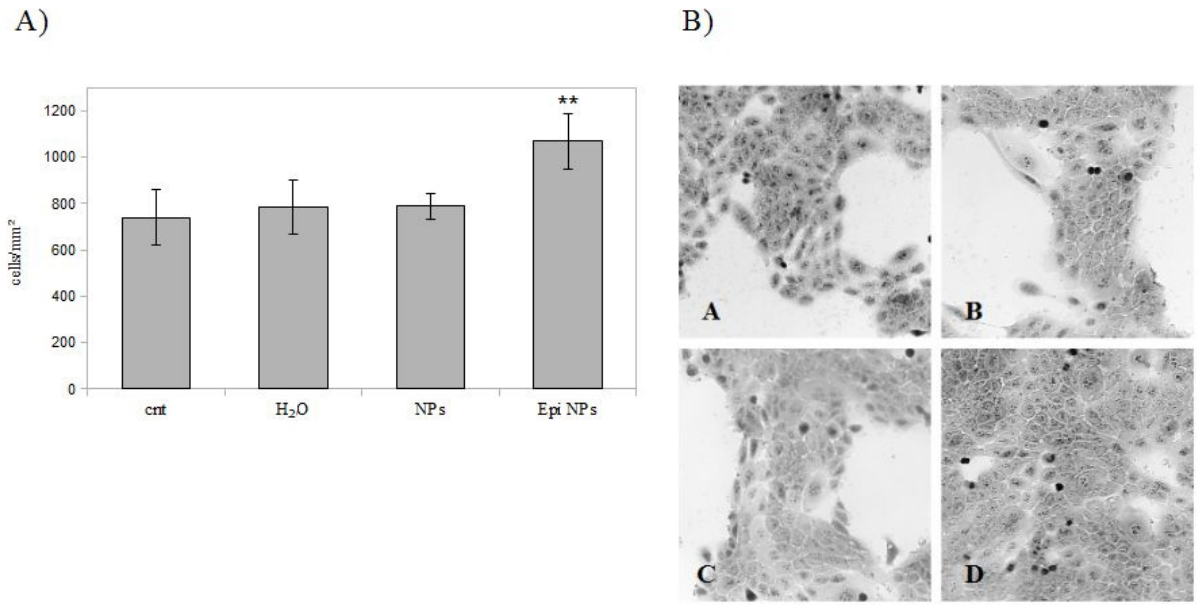

Figure 3. Cell proliferation. ***A)*** Cellular proliferation of treated cells assessed by manual count. Results are expressed as means ± SD ($^{**}p<0.01$). ***B)*** Optical microscopy images of HaCaT cells grown in presence or absence of NPs supernatant. A= cnt, untreated cells; B= H_2O-treated cells; C= NPs supernatant treated cells; D= Epi-NPs supernatant treated cells.

of the marginal tissue apical to the cement-enamel junction with exposure of the root surface.

Gum recession is a quite common condition affecting a large number of persons worldwide, including both dentally aware populations and those with limited access to dental care. Its etiology is multifactorial and its management is generally based on a thorough assessment of the causative factors and the degree of involvement of tissues.

From a clinical point of view, different surgical procedures exist to manage such condition.

When gum recession is treated by a surgical approach, techniques used for root coverage mainly focus on tissue displacement to increase the width of keratinized tissue, in order to solve poor aesthetics or root hypersensitivity[1].

One of the major drawbacks of surgery is represented by the presence of wounds, along with creeping attachment of free gingival grafts and possible complications.

Literature data show that localized drug delivery systems might represent a very interesting tool for the treatment of periodontal diseases as they can lead to lower incidence of side effects and enhanced patient compliance[17,19].

Epiregulin is a member of the EGF growth factor family known to stimulate keratinocytes proliferation[19]. As it can also regulate epithelial cells and fibroblast proliferation, it might also play a crucial role in gingival tissue wound healing, finally leading to the reconstruction of the damaged extracellular matrix and to fill damaged connective tissue forming the granulation tissue[6].

An innovative nanotechnological approach for the treatment of gum recession could be represented by the direct delivery and release of Epi directly in the periodontal pocket.

To obtain such effect, polymeric NPs could represent an interesting vehicle, as their size represent an important determinant for drug release and NPs degradation, finally affecting the efficacy of the therapeutic agent[14,20-22].

In this study, different PLA/PGA ratio formulations were tested. DLS analysis demonstrated that 50:50 PLGA-NPs exhibit the lowest size compared to 65:35 and 75:25 formulations, suggesting a more efficiently cellular uptake than larger size formulations.

The degradation rate of NPs depends on the hydrophilicity of the polymer. As lactic acid is more hydrophobic than glycolic acid, it slows down the degradation process of lactide-rich PLGA copolymers[21]. Therefore, 50:50 PLGA-NPs also display the fastest degradation rate *in vivo*, compared to the other PLGA formulations, which is a desirable result for patients[13,21].

As direct dental pocket drug delivery implies the NPs solution loading onto the dental surface

at the CEJ level, their ability to adhere onto dental surface is a characteristic of a major significance.

In the present study, different PLGA-NPs formulations ability to adhere onto the dental surface in experimental conditions simulating the oral cavity condition were tested, in order to identify the best NPs to be loaded with epiregulin.

50:50 PLGA-NPs showed the lowest particle size, associated to the better adhesion ability; for these reasons they were chosen as the vehicle for epiregulin.

The *in vitro* Epi release from PLGA-NPs revealed a peak in growth factor release after 3 hours of incubation in simulated saliva. Epi released from NPs was tested for its ability to modulate keratinocytes proliferation, demonstrating that, although released levels were low, a significant cell proliferation stimulation occurs.

Results described herein agree with a previous study [4] indicating that an epiregulin dose as low as 0.05 ng/ml is sufficient to induce a significant increase in cell proliferation in human keratinocytes.

Conclusions

Even if more *in vitro* and *in vivo* investigations are needed, preliminary data described herein suggest that 50:50 Epi-enriched PLGA-NPs could provide a new tool for the treatment of gingival recession.

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Conflicts of interest

The authors declare no conflicts of interest.

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