Wound Medicine 23 (2018) 6–10

Contents lists available at ScienceDirect

Wound Medicine

journal homepage: www.elsevier.com/locate/wndm

Original research article

Regenerative effect of epiregulin-loaded hydrogel

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ARTICLE INFO

Keywords:
Hydrogel
Epiregulin
Wound healing
Gelatin
Crosslinking
Polyglutamic acid

ABSTRACT

Rapid and efficient wound healing response is essential to maintain whole organism integrity and homeostasis. Such process could be influenced by a plethora of factors that when interfering with the different healing phases finally result in impaired tissue repair. Furthermore, defective healing process is often associated with chronic and difficult to heal wounds, both conditions deeply affecting patients' quality of life.

Advanced wound management solutions are the new standard in difficult to heal treatment and their key feature is represented by their ability to protect the wound bed but also to direct cellular behavior.

The aim of the present work was to evaluate the effectiveness of a bioactive hydrogel in modulating wound healing in a rat excisional wound model.

Surgical wounds were treated with hydrogel matrix enriched with a keratinocyte specific growth factor (epiregulin), saline (control condition) or the different hydrogel components alone.

Results showed from both a macroscopic and microscopic point of view a faster wound closure in hydrogel treated wounds associated with a better quality of the regenerated tissue.

Such results thus support the use of the proposed hydrogel matrix as a springboard to develop innovative advanced wound dressings to treat difficult to heal wounds.

1. Introduction

By definition a wound represents a break in the normal anatomical structure, determining the loss of tissue function. In particular, skin integrity damage results in the loss of water, electrolytes and proteins, moreover it allows body invasion by pathogens. For these reasons, any wound must heal in a quick and efficient manner [1–5].

A rapid and efficient repair of the wounded skin is one of the basic tasks of the organism in order to reconstitute tissue integrity and homeostasis. Wound healing process is a dynamic and interactive series of events that must occur in a temporally and spatially overlapping manner and continue for a specific time at an optimal intensity. Cellular and biochemical events involved in wound healing process can be schematically divided in four overlapping stages, namely: homeostasis, inflammation, proliferation and remodeling. At the end, wound healing process results in the substitution of the damaged tissue with a newly formed functional tissue. Such tissue regeneration lays on the deposition of new collagen and on the proliferation and differentiation of preexisting cells in the tissue and/or stem cells [2,4,6–8].

Wound healing process could be affected by many factors interfering with one or more phases of the process, finally resulting in impaired tissue repair. Such factors could be broadly divided into local (directly influencing the wound itself, such as oxygenation, infections) and systemic (affecting the overall health state of the individual, such as age, stress, hormones, diabetes, smoking, obesity, alcohol consumption, nutrition status). Defective healing is generally associated with chronic wounds. Chronic wounds are, by definition, hard to heal wounds, taking substantial time to heal and being very often associated to major symptoms, deeply affecting patient's quality of life [2,7,9–11].

In normal conditions, skin wound healing involves soluble mediators as well as blood cells, extracellular matrix (ECM) components and parenchymal cells, resulting in specific interactions among them which are mediated by growth factors [2,4,7,8].

Growth factors are mitogenic stimuli involved in wound resolution as they are able to stimulate both cell proliferation and directed migration as well as to regulate their differentiation [2,8]. Wound closure is a dynamic process involving not only epidermal cells proliferation and migration, but also deep dermal rearrangements. Newly formed tissue, in fact, needs both structural support, assured by ECM deposition and remodeling and blood supply, assured by neoangiogenesis. Among the growth factors actively orchestrating these cellular responses, the Epidermal Growth Factor (EGF) family has long been considered one of the key players in regulation keratinocytes' functions at the wound edge [5,8,12,13].

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https://doi.org/10.1016/j.wndm.2018.09.002
Received 31 January 2018; Accepted 13 September 2018
Available online 14 September 2018

Epiregulin (Epi) is one of the latest identified members of the EGF growth factor family. It has been originally described as a tumor growth inhibitor but has rapidly been identified as an autocrine growth factors in keratinocytes. Epi is a 5.4 kDa single chain polypeptide composed of 46 aminoacids and showing a 24–50% sequence homology to the other EGF receptors ligands [12–17]. The main characteristic of such growth factor is represented by its ability to mediate a stronger biological response than EGF [17,18].

In individuals suffering from extensive or deep skin wounds, the primary therapeutic goal of wound management is represented by the restoration of the maximum functionality along with an aesthetically satisfactory result, allowing patient reintegration into the society [3,4].

In vivo studies showed that topical application of recombinant growth factors on wounds are able to stimulate the injury repair, significantly accelerating skin wound healing through an increase in re-epithelialization, granulation tissue formation and ECM deposition [12]. In the present study epiregulin has been used to dope a hydrogel matrix in order to obtain a drug delivery system to be used for the localized delivery of such growth factor directly at the wound site with the aim to develop a possible innovative wound management device.

2. Materials and methods

2.1. Epiregulin loaded hydrogel synthesis

Epiregulin loaded hydrogel (HG-Epi) was synthesized as previously described in a 2012 paper [19] and in the patent EP2714113-B1. Briefly a 10% w/v gelatin type B (Lapi Gelatine, Empoli (FI), Italy) and 1% w/v polyglutamic acid (Xi'an Real in Biotechnology Co, Ltd, Xi'an, China) solution was prepared in sterile water and left to dissolve at 40 °C. Just before crosslinking with 0.5 µl/ml EDC (1-[3-dimethylamino)propyl]-3-ethylcarbodiimide, Sigma Aldrich, Saint Louis, MO, USA), 200 ng/ml epiregulin (Perprotech, London, UK) was added to the HG mixture. After crosslinking reaction, the resulting HG-Epi matrix was washed in sterile water before use.

2.2. Experimental animals

Ten male healthy adult Sprague Dawley rats were purchased from Envigo RMS srl (San Pietro al Natisone (UD), Italy) maintained in the faculty animal house. Each rat weighted between 250 g and 300 g (age 5–6 weeks) and was housed separately (one rat per cage). All the animals were maintained in controlled environmental conditions (22 ± 2 °C, relative humidity 45–55%, 12 h light/ 12 h dark cycle) allowing them to freely access both water and food until surgical procedure. Animal experimentation was approved by the Italian Ministry of Health (approval 1160/2015-PR). Animals received care according to the internationally approved guidelines for the use of laboratory animals. At the beginning of the experiment and at fixed time points (3, 7, 10, 14 days post-surgery) animals were weighted and wounded area were photographed in order to monitor wound healing process.

2.3. Experimentally induced excision wound in rats

Five excision wounds were created by surgical procedure on the back of each animal. The animals were anesthetized with intraperitoneal injection of a ketamine/xylazine solution (60–80 mg/kg ketamine (Ketavet 100, Intervet MSD) and 5–10 mg/kg xylazine (Rompum, Bayer DVM)). The skin on the back of the animals was shaved using an electrical razor and disinfected with 70% ethanol solution and betadine (Meda Pharma spa). Uniform wounds of ~28 mm^2 diameter were excised from the back of each animal using a 6 mm diameter biopsy punch (Kai Medical, Solingen, Germany). Surgical procedure was carried out paying attention to not injury the muscle layer. Wounded area was photographed immediately after surgery.

2.4. Experimental wound management

Wound were treated according to an internal protocol identifying each wound as control (Cnt, saline treated), HG-Epi (epiregulin loaded HG matrix), polyglutamic acid (PGA), gelatin (Gel) and epiregulin (Epi). The single HG components were tested at the same concentrations used to produce the HG scaffold (10% gelatin aqueous solution, 1% polyglutamic acid aqueous solution, 200 ng/ml epiregulin aqueous solution). HG matrix was cut to cover the corresponding wound while liquid components were added in drop-like manner. The back of the animals was then covered with a transparent surgical plaster in order to allow optical inspection of the wounded area. At the end of the surgical procedure the animals were housed separately with free access to food and water. Surgical wounds were optically inspected daily and 14 days after surgery all the animals were sacrificed by CO_2 euthanasia and the wounded area excised to allow further histological analysis.

3. Macroscopic and microscopic evaluation of wound healing process

On the day of surgical intervention (t = 0), wounded area was photographed and at fixed time points (3, 7, 10, 14 days post-surgery) digital images of the lesions were acquired. Macroscopic evaluation of wound closure was performed by measuring wounded area with ImageJ software (National Institutes of Health, Rockville, MD, USA). At the time of sacrifice (day 14 after surgery), skin specimen from wounded areas were surgically removed, washed in PPBS, fixed in 4% buffered formalin and processed to be embedded in paraffine. Wound area was then analyzed by staining 5 µm thick sections with hematoxylin and eosin. Stained sections were digitally acquired and epidermal thickness was measured using Pannoramic Viewer software (3DHistech, Budapest, Hungary). Dermal-epidermal interface length was measured using ImageJ software.

4. Statistical analysis

The statistical significance of the obtained results was assessed using ANOVA test followed by Bonferroni's post-hoc test. Probability of $p < 0.05$ was considered statistically significant.

5. Results

Animal weight was monitored during the whole experimental period, in order to verify the appearance of any physiological stress sign. All the animals used in the study did not show any sign of stress, as testified by their constant weight increase after surgery. The mean weight of the animals at the beginning of the experiment was 261.8 ± 2.21 g and reached 317.9 ± 5.2 g at the time of suppression ($p < 0.001$).

At day 3 post-surgery, animal weight showed a small decrease, even if not statistically significant, that could be explained by the surgical stress. The increase in animal weight start to become statistically significant from 7 days post-surgery (280.6 ± 4.65 g compared to 261.8 ± 2.1 g at the beginning of the experimental procedure, $p < 0.05$) and reach the maximum value at the time of the animal sacrifice (Fig. 1).

HG-Epi effectiveness in promoting skin wound healing was monitored daily by optical inspection of the animals to obtain a macroscopic information (Fig. 2). Macroscopic observations showed no statistically significant differences in wound healing process on the short time (3 and 7 days after surgery) while started to show a different wound closure speed for lesions treated with HG-Epi or single components starting from 10 days after surgery. At the end of the experiment, HG-Epi treated injuries showed the higher degree of wound closure ($p < 0.05$) compared to both control and single compound conditions, suggesting a synergistic effect of the HG-Epi matrix components