**Original article**

**Effect of combination therapy with alginate dressing and mouse epidermal growth factor on epidermal stem cells in patients with refractory wounds**

BI Qing, ZHANG Qiong, MA Jun, XU Ming, ZHANG Shui-jun, QIU Bin-song, XIA Bing, GU Hai-feng, HONG Jian-fei, ZHAO Chen and ZHU Dan-jie

**Keywords**: alginates; epidermal growth factor; stem cells; wound healing

**Background**  The aim of this research was to determine the efficacy of combination therapy using an alginate dressing and mouse epidermal growth factor (mEGF) on proliferation and differentiation of epidermal stem cells (ESCs) in patients with refractory wounds.

**Methods**  Eighteen patients (12 males and 6 females, aged from 18 to 61 years (mean 36.4 years)) with various skin wounds, were treated by dressing changing for one month. The wounds were located in the foot (11), calf (3), thigh (2) and forearm (2). The patients were randomly divided into 3 groups: alginate dressing and mEGF (group A; n=6), mEGF (group B; n=6) and control (group C; n=6). Wound closure indexes were measured at 7, 14, 21 and 28 days. Samples were harvested for pathologic examination, at 7 and 14 days following treatment. Cytokeratin 10 (CK10) and cytokeratin 15 (CK15) positive cells were evaluated using the super-sensitivity (SP) immunohistochemical staining technique.

**Results**  Wound healing was promoted in groups A and B. In group A, the wound closure index was increased significantly ($P<0.05$), and in one case the maximum cure area reached 102 cm$^2$. Pathological examination identified a thicker epidermis, active angiogenesis and enhanced granulation in group A compared with groups B and C. Using the SP immunohistochemical staining technique, we showed that ESCs in group A were bigger in size and larger in number than in groups B and C. Overall, there was a significant difference in ESCs proliferation and differentiation between group A and group B (or C).

**Conclusions**  Combination therapy using an alginate dressing and mEGF shows increased proliferation and differentiation of ESCs in patients with refractory wounds compared with those treated with mEGF alone.

Skin wounds are common and can be caused by trauma, burn, infection, tumor, diabetes and peripheral vascular diseases. Clinically, healing the wound is difficult if the skin affected is large and with poor circulation. Further challenge occurs if the infection is in the exposed deep tissue such as bone, cartilage and tendon. At present, surgical debridement is still the preferred option to treat refractory wounds. Different approaches have been used, and to facilitate healing, the wound is commonly covered by autologous, allogeneic or heterologous skin grafts, or other biological materials. However, these methods cause psychological difficulties in many patients because of the invasiveness of the procedures. Furthermore, they can cause new trauma and increase the risk of rejection as well as other disadvantages. The promotion of self-healing for the treatment of refractory wounds has been a challenge to clinicians. In this study we jointly applied an alginate dressing and freeze-dried mouse epidermal growth factor (mEGF) to refractory skin wounds, and evaluated their combined effect using immunohistochemistry.

**METHODS**

**Drugs, reagents and equipment**

Alginate gel (Coloplast, Sinopharm, China; No. 2000-1694) was provided by the operating theatre. Freeze-dried mouse epidermal growth factor (Hangzhou Tianmu Beidou Bio-Pharmaceutical Co., Ltd, China; S19990001), was diluted ($5 \times 10^6$ IU/L) and stored at 4°C. SP immunohistochemical reagent boxes, diaminobenzidine (DAB), and monoclonal mouse anti-human cytokeratin 10 (CK10) and cytokeratin 15 (CK15) antibodies, were from Beijing Zhongshan Bio-Technology Co., Ltd. (China). Other reagents were provided by the Department of Pathology, Zhejiang Provincial People’s Hospital. For pathological analysis, an RM2135 microtome, ST5020 automatic stainer and ASP300 tissue processor (all Leica, Germany) were used. Images were taken using a micro-camera system (Olympus BX51, Japan), and analyzed using the MIAS pathological imaging analysis system.

DOI: 10.3760/cma.j.issn.0366-6999.2012.02.018

Department of Orthopedics (Bi Q, Zhang SJ, Qiu BS, Xia B, Gu HF, Hong JF, Zhao C and Zhu DJ), Department of Operating Room (Zhang Q, Ma J and Xu M), Zhejiang Provincial People’s Hospital, Hangzhou, Zhejiang 310014, China

Correspondence to: Dr. ZHU Dan-jie, Department of Orthopedics, Zhejiang Provincial People’s Hospital, Hangzhou, Zhejiang 310014, China (Email: ZhuDJ@126.com)

BI Qing and ZHANG Qiong contributed equally to this work.
Clinical data
Informed consent was obtained from all patients. For study inclusion, we identified patients with skin wounds and defects due to acute trauma, whose wounds had not healed after 1 month of conventional daily sterile dressing change and anti-inflammatory treatment. Additionally, patients had wounds greater than 3 cm × 4 cm without infection. Patients with other co-morbidities such as diabetes, chronic heart failure, chronic kidney disease with renal failure, severe malnutrition and other chronic wasting diseases were excluded from the study. In total, 18 cases were identified and ordered according to their age, from young to old. Using the random number table method, patients were divided into three groups of six in each group: alginate gel and mEGF combined treatment group (Group A; 4 males and 2 females; 3 feet, 2 legs and 1 hand wound), mEGF treatment group (Group B; 2 males and 4 females; 5 feet and 1 leg wound) and conventional therapy group (Group C; 6 males; 3 feet and 2 hands wounds). There was no significant age difference among the three groups (P >0.05).

Treatment
Wound debridement finished in the operating room and the surrounding area was cleaned. Group A was treated with mEGF (100 IU/cm² wound surface area), by spraying the mEGF on alginate dressings of appropriate size, and then covering the wound with the dressing. Group B was treated with the same amount of mEGF (100 IU/cm² wound surface area), but using ordinary gauze instead of alginate dressings. Group C was treated with Vaseline gauze dressings. All groups were covered with ordinary gauze after treatment. Aseptic technique was strictly followed during the treatment. During the first week of treatment, abundant discharge was observed on the wounds; therefore dressings were changed every day. This was reduced to every second day after a reduction in discharge was observed.

Evaluation indices and methods

Sample preparation
Following 7 and 14 days of treatment, a small amount of tissue on the wound edge was excised. Tissue was fixed in 10% neutral formaldehyde, then dehydrated through an ethanol gradient, cleared with xylene and finally embedded in paraffin. Four-micrometer sections were cut and mounted for immunostaining.

Evaluation of wound healing index
The wound area was recorded with transparent tracing paper at the time of starting treatment and 7, 14, 21 and 28 days following treatment. The wound healing index was calculated using the following weighing method:

Healing index = (weight before treatment – weight after treatment) / weight before treatment

Histopathological examination
Paraffin was removed from sections using xylene. Slides were then rehydrated in gradient ethanol baths and stained with hematoxylin-eosin (HE) before light microscopy observation.

Immunohistochemical study
Paraffin was removed from sections and the slides rehydrated. Endogenous peroxidase activity was blocked using a 3% H₂O₂ methanol solution, 10 minutes, followed by washing in distilled water for 5 minutes, twice. Heat mediated antigen retrieval was performed using a citrate solution (0.01 mmol/L, pH 6.0), at 92–98°C for 10 minutes. After cooling, sections were incubated in phosphate buffered saline (PBS, 0.1 mmol/L), then in 5% fetal bovine serum at room temperature for 10 minutes, to block non-specific antigens. CK10 and CK15 mouse anti-human monoclonal antibodies were applied and sections stored overnight at 4°C. Next, sections were washed using PBS before incubating at 37°C, 30 minutes, in biotin-labeled secondary antibody. Sections were washed again using PBS, and then incubated in SP solution at 37°C, 30 minutes. Final PBS washes were performed, before DAB chromogenic detection under microscopic control. Sections were stained with hematoxylin, taken through gradient ethanol dehydration and xylene clearance, sealed with neutral balata and observed under the light microscope. Positive cells are brown in color. Positive cell numbers were counted in 5 randomly selected fields (400× magnification). Sections with no mouse anti-human monoclonal antibodies were used as negative controls.

Statistical analysis
Statistical data processing was performed using SPSS 12.0 statistical software (SPSS, IL, USA). Measurement data were expressed as mean±standard deviation (SD) and analyzed by one way analysis of variance (ANOVA, single-factor analysis of variance q test), and quantitative data by multiple independent samples rank sum test.

RESULTS

Wound healing index
After 7 days of treatment, the wound size was significantly reduced in Groups A and B, compared to Group C (P <0.05). After 14 days of treatment, significant differences among the 3 groups were observed. Namely, in Group A, 3 cases were predominantly healed, but only one case in Group B healed (P <0.05). After 21 days, only one wound in Group A was not closed; In group B, 3 cases were almost healed, but not a single wound in Group C was closed. After 28 days, all wounds in group A were closed, and in one case, the maximum cure area reaches 102 cm². In group B one wound was still open, and in group C, wound closure occurred in only 2 cases (Table 1, Figure 1).

Histopathological examination
After 7 days of treatment, Group A demonstrated a relatively well organized tissue structure, with basal cells...
Table 1. Wound closure indexes at different time points (% mean±SD; n=6)

<table>
<thead>
<tr>
<th>Groups</th>
<th>7 days</th>
<th>14 days</th>
<th>21 days</th>
<th>28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>37.8±4.2*</td>
<td>85.0±9.1†</td>
<td>94.3±6.3†</td>
<td>99.2±1.0*</td>
</tr>
<tr>
<td>B</td>
<td>32.2±4.5*</td>
<td>73.2±7.4*</td>
<td>85.2±9.1*</td>
<td>95.5±5.5*</td>
</tr>
<tr>
<td>C</td>
<td>23.8±5.3</td>
<td>54.8±8.5</td>
<td>64.8±6.1</td>
<td>85.0±7.8</td>
</tr>
</tbody>
</table>

*P <0.05 vs. group C, †P <0.05 vs. group B.

Immunohistochemical examination

After 7 days of treatment, an increase in CK15 positive cells was observed in both Groups A and B. This increase was greater in Group A than Group B, but did not reach statistical significance (P >0.05). Microscopic observation showed the CK15 positive cells were generally smaller and rounder in shape, with big nuclei and granules in the cytoplasm. In group C, CK15 positive cells were rarely found (P <0.05). After 14 days of treatment, numbers of CK15 positive cells continued to increase in Groups A and B, with the cells showing increased volume and a markedly stratified appearance. The statistical difference between Groups A and B is more significant than that observed at the 7 days time point. There were CK15 positive cells observed in Group C, but at reduced numbers compared to Groups A and B (P <0.05). Microscopic observation also revealed increased epithelial prominence and stratification in the cells of Group A, than Groups B and C (Table 2, Figure 2).

Table 2. CK10+ and CK15+ cell counts at different time points (% mean±SD; n=6)

<table>
<thead>
<tr>
<th>Groups</th>
<th>CK10 7 days</th>
<th>CK10 14 days</th>
<th>CK15 7 days</th>
<th>CK15 14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>69.8±3.7*</td>
<td>96.0±3.1†</td>
<td>70.3±4.3*</td>
<td>97.2±1.5†</td>
</tr>
<tr>
<td>B</td>
<td>63.7±2.5*</td>
<td>71.2±6.4*</td>
<td>62.2±8.1*</td>
<td>79.5±5.5*</td>
</tr>
<tr>
<td>C</td>
<td>20.8±6.3</td>
<td>31.8±7.5</td>
<td>26.8±5.1</td>
<td>33.0±7.6</td>
</tr>
</tbody>
</table>

*P <0.05 vs. group C. †P <0.05 vs. group B.

Figure 1. The skin defect was treated by dressing change for one month (A), and by alginate dressing and mEGF for 14 days (B and C). A: The skin defect of a male patient (41 years) treated by dressing changing for one month, was big and indolent. B and C: Following combination therapy using an alginate dressing and mEGF for 14 days, the wound was nearly closed.
and barely present in group C. In Group A, epidermal cells were increased and stratification of cells was apparent.

We observed an increase in CK10 positive cells in Groups A and B after 7 days of treatment, but similar to CK15, there was no statistical difference between the two groups ($P > 0.05$). In Group C, significantly less CK10 positive cells were observed ($P < 0.05$). After 14 days of treatment, all three groups expressed increased numbers of CK10 cells, with Group A showing the greatest increases. Furthermore, in Group A, CK10 expression showed increased epithelial prominence and stratification of cells than Groups B and C (Table 2, Figure 3).

**DISCUSSION**

EGF is a potent cell proliferation factor. Following trauma, aggregated platelets release large quantities of inactive EGF. The released EGF promotes wound healing through a series of downstream signal transduction processes, leading to cell repair and extracellular matrix proliferation, and also more importantly, regulates proliferation and differentiation of epidermal stem cells to repair skin wounds. Thus, a threshold increase in EGF levels in local wounds is necessary to accelerate wound healing. However, although there are post-traumatic increases in EGF receptors on local wounds, as well as a rapid local build-up of endogenous EGFs, EGF levels are still not sufficient for self repair process because of low EGF levels in tissue and the inhibition of EGF release due to wound inflammation and tissue necrosis. Therefore, it has been suggested that supplementation of exogenous EGF to the wound may be required to increase endogenous EGF levels. Moreover, exogenous EGF may also promote expression of endogenous EGF, thereby stimulating cells to secrete additional EGF, and maximizing the biological effect of EGF on accelerated wound healing.

Tanaka et al. reported that for wound treatment, EGF has to be present on the wound continuously at an adequate concentration in order to achieve optimal results. In general, using an EGF solution to treat wounds is not ideal, as liquid solutions are easily drained, especially in wounds with excessive secretions, resulting in rapid dilution and elimination of EGF with the wound discharge. The use of EGF gels is therefore more suitable for the treatment of wounds. The gel forms a thin layer on top of the wound and acts as a barrier to prevent bacterial invasion. In addition, the gel is present at the wound for longer than a liquid solution, enabling continuous EGF and accelerated wound healing. Furthermore, gels are softer, reducing direct irritation of the wounds by gauze dressings, and they also keep the wound moist and lubricated in order to facilitate and benefit the healing process. However, despite these advantages, EGF gels are not at present widely used in clinical practice for a number of reasons.

Compared to ordinary gauze, the alginate dressings we have used in this study are very soft. The main component is an algae protein acid, a type of water insoluble polysaccharide similar to cellulose, which is extracted from seaweed and converted into a calcium salt during dressing production. Alginate is highly absorbent, and can absorb up to 20 times its own weight in liquid, allowing it to effectively absorb the wound discharge and increase the dressing longevity. Upon contact with the wound, the calcium ions in alginate are exchanged for sodium ions in the wound discharge, forming a stable mesh-like gel layer on the surface of the wound. This keeps the wound moist and prevents the dressing from sticking to the wound surface. Moreover, the wound is isolated and put in a closed oxygen-free environment, thus accelerating the proliferation of new capillaries, enhancing epithelial cell regeneration, speeding up epidermal cell migration, protecting exposed nerve endings and promoting wound healing. These characteristics of alginate dressings are similar to those of EGF gels.

There are high levels of CK15 expression in epithelial stem cells, allowing CK15 to be used as an ESC
molecular marker. CK10 on the other hand, mainly exists in terminally differentiated cells.18-20 Our study found higher levels of CK15 and CK10 expression and a significantly increased cell count in the group given alginate dressings in combination with mEGF treatment, compared to treatment with either mEGF alone or conventional dressings. Furthermore, HE staining showed that fibroblasts and collagen fibers had also increased significantly in the combination therapy group, with better integrity in tissue structure and organization. The results indicate that alginate dressings can be combined with mEGF to significantly improve epidermal stem cell proliferation and differentiation, accelerate wound tissue granulation and angiogenesis, and facilitate epithelialization of the wound, thereby promoting wound healing, as seen by the significantly improved wound healing index. Overall, it appears that the combination therapy has a synergistic effect on wound healing. The alginate dressing not only directly promotes epidermis recovery, but by forming a thin layer of gel, provides a matrix to prolong the retention time of mEGF on the wound surface. This maximizes the treatment effect of mEGF, promoting epithelialization and wound healing. Thus, combination therapy using alginate dressings and mEGF, has significant advantages compared with single application treatment using either agent individually, and is worthy of more widespread use in clinical practice.

REFERENCES


(Received September 25, 2011)  
Edited by HAO Xiu-yuan