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RESEARCH ARTICLE

ENHANCEMENT OF WOUND HEALING BY TOPICAL APPLICATION OF EPIDERMAL GROWTH FACTOR IN ANIMAL MODEL

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ABSTRACT

Objective: Wound healing is a complex process of biological events involving re-epithelialization and granulation that are mainly mediated by several endogenously released growth factors such as epidermal growth factor. This work was undertaken to study the effects of various doses of locally applied recombinant human epidermal growth factor (rhEGF) on wound healing in rats.

Methods: Recombinant human EGF consists of 53 amino acids. *In vitro*, rhEGF promoted its obvious cell growth and proliferation when added to cultured 3T3 cells using MTT assay. In the test groups, *in vivo*, wound sites were given daily with a solution containing 2, 5, 10, 50ug of EGF spray and 40ug of EGF ointment, respectively.

Results: Current study presented evidence that a significant decreased healing time in wound was observed in all rhEGF groups when compared with the control, and reach to its maximal efficacy at 10ug/ml of rhEGF spray. The rate of wound closure was over 50% at initial 3 days of treatment. Treatment with rhEGF significantly decreased the length of time to over 50% healing by approximately 4-5 days, and that to 70% and 90% healing by approximately 3-4 days and 3 days, respectively. A stimulatory, dose-dependent effect of EGF on wound healing was observed with increased rhEGF concentration. In toxicological group, higher doses of 100ug/ml of rhEGF spray was applied by local dorsal incision in rats. Moreover, a dose of single 200ug, single 300ug or 300ug within 24 hrs of subcutaneous and intramuscular rhEGF injection was given respectively. There were no significant adverse side effects.

Conclusion: Current study recommended a proposal of clinical drug doses in wound at 2µg, 5 µg and 10 µg /ml of rhEGF spray, and 10 µg and even higher 40 µg rhEGF/g of ointment. The results indicated that prepared rhEGF by genetic engineering in current study is safe, and is emerging in clinical effective use in assisting wound healing time.

Keywords: Animal wound model, gene engineering, Recombinant human epidermal growth factor (rhEGF), wound healing.

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INTRODUCTION

Epidermal growth factor (EGF) is a broad variety of cellular regulator. EGF was firstly isolated by Cohen S¹ from a mouse submaxillary gland, and further purified mouse EGF. Its amino acid sequences was found to the relationship to urogastrone^{2,3}. EGF is a single polypeptide chain of 53 amino acid residues and contain these intramolecular disulfide bonds that are required for biological activity. EGF is a heat stable protein and were destroyed during the isolation procedure by boiling. EGF could be still shown its biological activity when storage at room temperature for 2 years⁴ (Chen LQ's data in chinese, 2008). It has been reported that EGF promotes the proliferation and differentiation of cultured cells originated from ectoderm and mesodermal layers, such as epidermal

cells, fibroblasts, myofibroblasts, keratocytes, corneal epithelial cells and angiogenesis, and a key importance on maintenance and impact organ development and a cascade of cellular events. In recent, It has been suggested that EGF could be beneficial in burn^{5,6,7} wound healing⁸ diabetic foot ulcers^{9,10,11} and digestive ulcer¹², and provide an attractive perspective. In addition, cosmetic containing EGF was found to be effective to improve the plasticity, to remove wrinkle, to show whitening and anti-aging, and control of erythem amount and sebum amount on the human skin care. In local district, Zhu G in this field has successfully prepared a series of 53 bottles of Shampoo liquid (New Wash) into market, and responder rate with satisfied or perfect satisfied over 95 per cent. Actually, EGF is the secreted protein by epithelial cells

in epidermis. Based on these data, the present work was undertaken to study the effects of various doses of locally applied EGF on rat wounds.

MATERIALS AND METHODS

Wound model

10 female Kunming (KM) strain rats (age 8 weeks,

body weight 30-35g) were purchased from Hunan SJA Laboratory Animal Co., Ltd, Changsha, Hunan. The dorsal skin of the rats was sterilized with iodophor and dorsal hair was shaved with clippers. An incision, 2cm lengthx1.5cm width in diameter was made in the dorsal midline at the causal portion of the back using medical scissor and forceps.

Table 1A: The proliferative activity of 3T3 culture cells following a various of rhEGF concentration. MTT assays were completed in Lalchuang Biotechnology Co. LTD, Shanghai.

EGF ng/ml	12.8	6.4	3.2	1.6	0.8	0.4	0.2	0.1	0 (control)
OD	1.5137	1.4633	1.3657	1.3265	1.2907	1.2936	1.2836	1.2639	1.2131
	1.4446	1.4545	1.3729	1.3686	1.3486	1.3079	1.3151	1.2246	1.2779
	1.4446	1.4388	1.3581	1.3382	1.3242	1.3464	1.2877	1.2611	1.2555
	1.4354	1.4324	1.345	1.3423	1.3278	1.2865	1.2919	1.2761	1.2278
Mean	1.4596	1.4472	1.3604	1.3439	1.3228	1.3086	1.2945	1.2564	1.2436
Increase rate%	117.37	116.38	109.40	108.07	106.37	105.23	104.10	101.03	100.00
	±3.63	±1.42	±1.19	±1.78	±2.40	±2.67	±1.41	±2.22	±2.89

Table 1B: The proliferative activity of 3T3 culture cells following a various of rhEGF concentration. MTT assay was completed in Lalchuang Biotechnology Co.LTD, Shanghai.

EGF (ng/ml)	0	0.1	0.2	0.39	0.78	1.56	3.13	6.25	12.5	25	50	100
OD	1.1371	1.1662	1.2044	1.2470	1.2914	1.3405	1.4245	1.4218	1.4245	1.4030	1.4758	1.4822
	1.1813	1.1837	1.1893	1.1549	1.2402	1.2837	1.3614	1.4353	1.4427	1.4659	1.4442	1.4467
Mean	1.1573	1.1803	1.1729	1.2224	1.2543	1.3663	1.3711	1.3935	1.4142	1.4536	1.4575	1.4652
Increase rate	1.1586	1.1767	1.1889	1.2081	1.2620	1.3302	1.3856	1.4169	1.4271	1.4408	1.4592	1.4647
Mean	100.00	101.57	102.62	104.28	108.93	114.81	119.60	122.30	123.18	124.36	125.95	126.42
± SD	±2.21	±0.93	±1.58	±4.77	±2.65	±4.22	±3.40	±2.13	±1.44	±3.34	±1.59	±1.77

After full-thickness wound created on the back of the female back, wound sites were photographed daily. Wound area was measured with a ruler on day 0, 1, 2, 3, 4, 7, 8, 10, 13, 15 after initial treatment. The surface area of the wound was calculated using the formula for calculation of the regular mathematic geometric figure that best approximated to the wound shape (area=a.x.b), where a and b represent the perpendicular length and width dimension, respectively. During the experiments, the animals received a normal diet and water *ad libitum*, and were housed individually in cages in animal quarter.

Figure 1: Amino acid sequence of human epidermal growth factor.

All animal experiments were approved by the Animal Committee of Nanjing Origin Biosciences, Nanjing, China (OB1409).

Construction of pET-28a-hEGF plasmid and its high throughput expression in *Escherichia coli* BL21 (DE3)

rhEGF used in this study was obtained as a donation from Prof. Zhi QW¹³ in Institute of pharmacology and toxicology, academy of military medical sciences, Beijing. According to gene engineering technique¹³⁻¹⁷

in 2002-2004 period of previous prepared methods, synthetic hEGF gene was cloned in a T7/lac Z pET-28a expression vector. After identified by screening and sequencing, recombinant pET-28a-hEGF plasmid containing hEGF gene was transformed into *E. coli* strain BL21 (DE3) competent cells using CaCl₂ methods, plated on LB agar containing Kanamycin and incubated at 37°C overnight. An individual positive colony was selected from LB agar plate and incubated into LB liquid medium containing Kanamycin. The *E. coli* culture was incubated in shaking incubator 12-16 hrs at 37°C, then was continuously incubated to expanding at 1:100 ratio. After reaching to OD₆₀₀=0.4, protein expression was induced by the addition of IPTG with final concentration of 0.5 mmol/l. After 5 hrs of growth, cells were harvested partially in form of inclusion body, and resuspend in phosphate buffer solution (PBS). After smashing by ultrasonic disintegrator, the supernatant of cell lysate was collected by centrifugation and the residue was added with 8 mol/l urea lysis solution and recentrifuged. The supernatant fluids were collectively combined, and then purified by Ni²⁺-NTA affinity chromatography. The purified rhEGF was obtained (Figure 1), and its biological activity was detected by MTT methods (see appendix)

Preparation of recombinant human epidermal growth factor (rhEGF)

When obtaining rhEGF crude, in the next further step, the drug formulation was prepared by manufacturer Dr. Zhu G in this study at dose of 2, 5, 10 µg of rhEGF

spray, and 10 and 40 µg rhEGF/g of ointment. The rhEGF dose(10µg/g) was selected according to the therapeutic guideline of Brown's in USA⁵ and Wong's in HongKong¹¹ and in Korea Kwon's¹⁸ previous reports. Accordingly, as Gregory RA² described urogastrone early, Dr. Zhu has also prepared rhEGF injection. Laboratory data: Anti-HIV 0.077. No bacteria growth was shown as to rhEGF spray and liquid Shampo in bacteriological examination. All animals with similar-sized wounds were randomly assigned to 7 groups of 10 rats. Group 1 was treated with saline solution twice daily as the control. Group 2-4 was treated with 2, 5, 10µg of rhEGF spray twice daily, respectively. High doses of testing group 5 and 6 received 50 and 100ug/ml of rhEGF spray, 5-10 times its clinical drug dosage, twice daily for 15 days, and then once a day up to one month. Group 7, the drug rhEGF ointment (40ug rhEGF/g) was also administered topically to the animal every 7-12 hrs for 15 days and then once daily for next 15 days beginning on the day of the incision. Macroscopic assessment was carried out by an independent observer and recorded. Animal body weights and adverse reaction were recorded after the end of experiments.

Data collection and statistical healing rate analysis

Initial study data were gathered in Table 2 and Table 3. Percentage wound closure was calculated using the formula:

$$\text{Wound healing rate} = 1 - \frac{\text{Area of present wound}}{\text{Area of initial wound}} \times 100$$

Wound healing time and crude healing rate of wound were used to evaluate the efficacy of the treatment. A t-

test was used to show the significant difference between the mean outcomes of rhEGF groups and the control at the level of significance less than 0.05(p). The overall period of experimental procedure including genetic engineering in crude product of rhEGF was beginning from November, 2018 to June 25, 2019, and pharmacological preparation of rhEGF spray and its animal wound test was from August 29, 2019 to February 5, 2020.

RESULTS

The biological activity of rhEGF

For investigation, to examine the biological activity of 3T3 culture cells to a various dose of rhEGF using MTT methods. It has been uncovered that purified rhEGF spray was a potent stimulation of cell growth and proliferation when added to the cultured 3T3 cells at 6.4ng/ml of rhEGF concentration (Table 1A). In homogenous preparation of the same batch drugs, when storage as a frozen solution (-20°C), rhEGF could be shown its potent proliferative activity even at 3.13ng/ml of EGF concentration (Table1B). As can be shown from table 1A and B, A dose-dependent cellular stimulation was observed with increased rhEGF concentration. In this study, when it was carried out the experiments at room temperature, rhEGF has still been found to maintain obviously its biological activity when stored at 4°C for over 5 months. Therefore, if condition permit, rhEGF drug was preferably selected to stored at 4°C refrigerator.

Table 2: Experimental data of the wound healing time following various doses of rhEGF [Drug doses(µg/ml)/healing area(cm)/days].

Area(cm ²)	Mice No.	Days									
		0	1	2	3	4	7	8	10	13	15
Control	2	2x1.5	1.5x1.5	1.5x1.4	1.5x1.3	1.5x1.2	1.4x1.1	1.4x1.0	1.1x0.8	0.7x0.4	0.5x0.3
			25.0%	30.0%	35.0%	40.0%	48.7%	53.3%	70.7%	90.7%	95.0%
		2.0x1.5	1.6x1.5	1.5x1.45	1.4x1.35	1.4x1.3	1.2x1.1	1.1x0.95	1.1x0.85		
			20.0%	27.3%	37.0%	39.3%	56.0%	65.2%	68.8%		
			1.7x1.4	1.5x1.2	1.5x1.15	1.5x1.1	1.15x1.0	1.1x0.9	0.9x0.8	0.65x0.5	0.5x0.4
			26.5%	44.4%	46.8%	49.1%	64.5%	69.4%	77.8%	90.0%	93.8%
2µg	1	2.5x1.5	1.5x1.4	1.3x1.3	1.3x1.1	1.3x1.0	1.2x1.0	1.0x0.8	0.7x0.5	0.5x0.3	0.3x0.2
			44.0%	55.0%	61.9%	65.3%	68.0%	78.7%	90.7%	96.0%	98.4%
5µg	2	2.5x2.0	1.8x1.5								
			46.0%								
		2.0x1.5	1.3x1.35	1.3x1.1	1.1x1.1	1.1x1.0	1.0x0.9	0.9x0.8	0.8x0.6	0.4x0.3	0.45x0.2
			41.5%	52.3%	59.7%	63.3%	70.0%	76.0%	84.0%	96.0%	97.0%
10µg	2	2.2x1.5	1.5x1.3	1.3x1.2	1.2x1.0	1.1x1.0	0.9x0.8	0.8x0.6	0.5x0.3	0.3x0.2	0.3x0.2
			40.9%	52.7%	63.6%	66.7%	78.2%	85.5%	95.5%	98.2%	98.2%
		2.0x1.5	1.3x1.45	1.1x1.05	1.1x1.0	0.9x0.9	1.0x0.9	0.7x0.65	0.7x0.6		
			37.2%	61.5%	63.3%	73.0%	70.0%	84.8%	86.0%		
		1.8x1.8	1.5x1.3	1.35x1.0	1.1x0.95	1.1x0.9	1.0x0.75	0.8x0.8	0.7x0.6	0.5x0.4	0.4x0.25
			39.8%	58.3%	67.7%	69.4%	76.9%	80.2%	87.0%	93.8%	96.9%
High Doses Test											
50µg	1	2.0x1.5	1.4x1.3	1.3x1.3	1.3x1.2	1.2x1.1	1.1x0.9	1.05x0.7	0.8x0.5	0.5x0.3	0.4x0.2
			39.3%	43.7%	48.0%	56.0%	67.0%	75.5%	86.7%	95.0%	97.3%
100µg	1	2.0x1.8	1.5x1.45	1.4x1.25	1.2x1.15	1.15x1.0	1.05x0.8	1.0x0.8	0.8x0.6		
			39.6%	51.4%	61.7%	68.1%	76.7%	86.7%	86.7%		
		1.5x1.5	1.1x1.0	1.0x0.9	0.95x0.9	0.9x0.9	0.8x0.7	0.7x0.6	0.6x0.55	0.4x0.3	0.3x0.2
			51.1%	60.0%	62.0%	64.0%	75.1%	81.3%	85.3%	94.7%	96.0%
40µg cream	1	2.0x1.5	1.3x1.1	1.1x1.0	0.8x0.7	0.3x0.3	0.7x0.6	0.8x0.5	0.7x0.4	0.4x0.3	0.3x0.2
			52.3%	63.3%	81.3%	97.0%	86.0%	86.7%	90.7%	96.0%	98.0%

Table 3: The Comparative data of wound healing rate at 2-10 µg doses of rhEGF spray (Days(healing per cent)).

Group	Exp. No.	Days												
		1	2	3	4	5	6	7	8	9	10	11	13	15
Control	3	23.8	33.9	39.6	42.8	52.1 (n=2)	53.2 (n=2)	56.4	62.6	67.2	72.4	84.9 (n=1)	90.4	94.4
2 µg	1	44.0	55.0	61.9	65.3			68.0	78.8		90.7		96.0	98.4
5 µg	1	43.8 (n=2)	52.3	59.7	63.3	63.3	66.7	70.0	76.0	77.8	84.0	92.0	96.0	97.0
10 µg	3	39.3	57.5*	64.9	69.7**	73.4 (n=2)	74.1 (n=2)	75.0***	83.5	84.5	89.5*	92.3 (n=1)	96 [△]	97.6
50 µg	1	39.3	43.7	48.0	56.0			67.0	75.5		86.7		95.0	97.3
100 µg	2	45.4	55.7	61.9	66.1	69.4	72.3	75.9	79.6	84.4	86.0	91.1	94.7	96.7

Note: *A t-test represents significant difference between means of 10 µg rhEGF group and the control. * $p < 0.02$, ** $p < 0.002$, *** $p < 0.05$, $\Delta p > 0.1$

rhEGF enhance wound healing

Data of the effect of rhEGF on wound healing parameters were shown in Table 2 and Table 3 and Figure 2. After daily application of various doses of rhEGF spray, a significant decreased healing time was observed when compared with the control. The rate of wound healing in all rhEGF groups was over 50% at initial 3 days of treatment. Treatment with rhEGF significantly decreased the length of time to 50% healing by approximately 5 days, and that to 70% and 90% healing by approximately 3-4 days and at least 3 days, respectively. In comparison of wound healing

curve analysis between these rhEGF groups (Figure 3), wound healing at 2, 5, 10 µg of rhEGF concentration was generally better effective than that in control, and reach to its maximum efficacy at 10 µg/ml of rhEGF spray [the healing rate: 57.5% at initial 3 days ($t=4.0305$, $p < 0.02$), 69.7% at days 5 ($t=7.3777$, $p < 0.002$), 75.0% at days 7 ($t=3.5643$, $p < 0.05$), and 89.5% at days 10 ($t=4.0728$, $p < 0.02$)], while no difference was found as to the effects of rhEGF spray at 13-15 days. More data, a higher dose of 100 µg/ml of rhEGF bid for 5 days appear to produce faster initial healing of the wound compared with control.

Figure 2: Wounds before experiments (a, b, c) in control and test pair groups.

After rhEGF treatment, an obvious wound closure in wound was shown at days 2(d, e) and days 5(f). From left to right in figure 1a control; figure 1b, 5 µg test rat; figure 1c, control (left) and 2 µg (right) test rat. In figure 2d and 2e, rhEGF spray accordingly at 10 µg, saline solution, or 5 µg concentration. In figure 2f, a solution containing 10 µg, 5 µg, saline and 2 µg/ml of rhEGF spray respectively. In figure 2g and 2h, wound area was measured with a ruler.

During the duration of wound closure, A stimulatory effect of local rhEGF daily on granulation tissue formation was observed, accompanied with occasional microvillus hair (data not shown). In contrast, two wounds were treated with only saline solution as control. There were 3 times of repeated bleeding in local surface of a wound, whereas superficial seeping

blood was found in the next day of incision in another mouse wound. This bloody wound was trying to iodophor and hemostasis with cotton ball pressure. The results suggested that rhEGF spray may play an antibiotic role and its possible hemostatic effect. Surprising finding, the wound site was treated topically with 1% silver-sulfadiazine cream containing rhEGF

(40 μ g/ml). It has been noted a significant higher efficacy of wound closure. Wound closure in wound was obviously accelerated at initial 3 days, and at 5 days the most parts of wound were scabbed, whereas scab abscission occurred at 7 days. Therefore, study concluded that the EGF has the ability to become an efficient therapeutic drug for superficial or deep partial-thickness wound in skin.

Figure 3: The wound healing rate curve.

It was recorded in various solution containing 2 μ g (black), 5 μ g (purple), 10 μ g (red) of rhEGF spray. Control received injection of saline solution (pencil color line). An obvious difference between rhEGF spray and control was distinctly shown

In the toxicological study, by using topical application of high doses of rhEGF spray at doses between 50 μ g and 100 μ g/ml in dorsal wounds, no significant adverse side effects has been observed in association with the use of EGF regarding body weight and other (hair) growth-inhibiting effects even the incidence of infection in rats. Otherwise, the complete wound healing including hair-growth was observed at 25-28 days in all experimental rats. To further test whether rhEGF can be used as EGF saline injection, current study carried out the additional experiments of subcutaneous and intramuscular injection of rhEGF solution in 2 rats, respectively. Initial dose of rhEGF was 20 μ g daily for 5 days, and escalated to 30 μ g daily for 5 days, then 50 μ g daily for 5 days, and the final amount 100 μ g daily for 5 days. Total dosage of rhEGF was 1mg within 20 days. In toxicological test, a dosage of single 200 μ g, single 300 μ g or 300 μ g/24hrs of subcutaneous and intramuscular rhEGF injection was given respectively in 5 rats, which 20–150 times its clinical drug at doses between 2-10 μ g.

As it was expected, there was no significant adverse side effects, except that a suspicious subcutaneous nodule was noted in one rat receiving continuous application of rhEGF subcutaneously (Figure 4). Animal behavior of rats after a single injection at dose of 200 μ g, 300 μ g or at 300 μ g/24hrs of rhEGF solution was blunt in movement. The rats recovered their activity within 24 hrs, whereas a single 400 μ g or an injection of rhEGF at doses of 400 μ g a day, 40-200 times higher than its use in clinical drug doses, is lethal within 30 minutes-24 hrs in another 3 rats. Other more, no any nodule was noted in one control rat receiving a subcutaneous injection of 2-4 ml per time of continuous saline solution for 36 days. The results

indicated that prepared rhEGF is safe and available in clinical wound healing.

DISCUSSION

Current study demonstrated the potential efficacy of rhEGF as an adjunct to conventional wound repair, which have previously been confirmed by a number of *in vitro* and animal experiments^{18,19}, even though strain-specific differences in wound healing rates may influence the true effect of EGF in the mouse models. The results are encouraging.

From the dose effects curve, It has been shown that wound healing rates were generally better effects at 2-10 μ g of rhEGF, and reach to a peak value of 10 μ g/ml of rhEGF concentration. A comparison of dose-response rates also appears to the length of the best healing time at 10 μ g/ml of rhEGF. Certainly, daily application of 2 μ g and 5 μ g of EGF were also available. The results were consistent with others Brown's⁵ and in HongKong Wong's¹¹ observation.

Brown and colleagues⁵ carried out a trial on 12 chronic wounds, they treated their patients initially with silvadene alone, which was ineffective in spite of its antibacterial action, for a period of 3 weeks to 6 months. This was followed by treatment with topical silver sulfadiazine cream containing EGF(10 μ g/g). EGF significantly decreased the average length of time to 50% healing by approximately one day and that to 75% and 100% healing by approximately 1.5 days ($p < 0.02$). The addition of EGF was producing a highly significant response. The same group²⁰ in Finland also proved the stimulation of wound healing by EGF in dose-dependent effects. In the test group, wound were injected daily with a solution containing 0.2, 1 or 5 μ g of EGF in 0.1% albumin. EGF significant increases the accumulation of DNA and RNA, and accumulation of granulation tissue cells, collagen and glycosaminoglycans in experimental rat wounds. Dr. Zhu once experienced a 2.5x 2.5cm brush burn, the wound healed without scar at 5 μ g of rhEGF spray for 1 week. Indeed recent, my colleague Dr. Tang HL caught a knife wound (2x2.5cm) by a table knife accident. The entire epidermis was stripped off his forefinger. Blood flowed profusely from his right index finger. The wound healed without scar by the continued combination of 5 μ g of rhEGF spray every other day and Yunan Baiyao spread within 20 days. Moreover, in Zhu's personal communication, a bone fracture attack by accident, caused severe swollen on the right leg of an animal chicken. The obvious disappearance of serious swollen was found at 3 days after local use of 5 μ g/ml of rhEGF spray, and 1 week late, the chicken could go on foot with a broken leg. The wound was gradually healing through the continued combination of local rhEGF spray and topical EGF-silvadene ointment (10 μ g/g) for 6 days. The results suggested that rhEGF may play its antibiotic role and its some benefits in a bone fracture.

A 73-year-old female with her right lumbar burn, the superficial burn area reached 12 x 8cm², the burn wound was recently healed following cefuroxime sodium 2g/day for 8 days, and local 5 μ g and 10 μ g/ml of rhEGF spray over 20 days. Silver sulfadiazine (Ag-SD)

is an adjunct for the prevention and treatment of wound sepsis in patients with second- and third-degree burns. It is bactericidal for many gram-negative and gram-positive bacteria in wound treatment. However, recent findings²¹ indicate that Ag-SD delays the wound-healing process, while the addition of rhEGF could reverse the impairment. Therefore, a drug delivery system containing both EGF and Ag-SD, such as 1% silver sulfadiazine containing EGF (10 μ g/g), may be clinically relevant. In this study, it was shown that EGF-Silvadene in the dual collaboration of both rhEGF on wound granulation tissue formation and antibacterial of silvadene. EGF-Silvadene ointment

significantly decreased the length of healing time within initial 3 days, and that to over 90% scabbed healing by 5 days. But at 1 week scab abscission occurred. Therefore, it is need to cover the wound with sterile gauze after topical rhEGF-silvadene cream, then fixed it with medical tape in order to its better drug efficacy. Many topics on the mechanism of hEGF action^{5,12,22,23}. Exogenous EGF could selectively bind to its receptor (EGFR) on cell membrane of keratocytes and fibroblasts in skin, and EGF induce initiation of DNA synthesis, activation of RNA and protein synthesis, and activation of the synthesis of extracellular macromolecules.

Figure 4: Observations in different rats.

A subcutaneous nodule was clearly shown in the application of continuous rhEGF injection, whereas no sign of nodule formation was observed following intramuscular rhEGF injection in another rat within 20 days (Figure 4a-Figure 4d). Each rat was injected with a total amount of 1 mg of rhEGF solution. The subcutaneous nodule was progressive regression after stopping rhEGF injection for one week (Figure 4e-Figure 4f)

It was reported that interaction between rhEGF and its receptor has to be maintained for 10-12 hrs in order to achieve an effective cellular response in terms of better-organized granulation tissue, a greater DNA and protein content and a higher rate of cell replication²². Using acetic acid-induced gastric ulcer in rats, Liu and Xu¹² have reported that the numbers of EGF and its receptors obviously increased around ulcer margin at 2 days and reach its increased expression to a peak value at 4 days, and consequentially the peak was decline to a normal pattern. Gu *et al.*, (Gu YM's data in Chinese) also observed that in health skin EGF and EGFR were weak positive, with its distribution of epithelial basal cells, and afterward the expression in wound became positive at 4 days, and at 10 days the EGF and EGFR were strong positive, with mainly distribution of epithelial basal cells around wound edge, dermal endothelial cells and fibroblasts. In addition to EGF-EGFR complexes, other increased PDGF and hydroxyproline contents were also locally acting factors. To evaluate the further stimulation of rhEGF in malignant ulcer, *in vitro* transfection of NIH3T3 with a

functional EGF receptor resulted in no significant alteration (0.4% colonies in soft agar) in growth properties^{24,25}. The same observation was shown that the 32D/EGFR cells which exhibited high levels of functional EGFR remained non tumorigenic during a 2-months period²⁶. However, EGF addition led to the formation of densely growth transfected foci (~2x10² ffu/pM of DNA) in liquid culture and colonies (19.7%) in semisolid medium²⁵. Moreover, isolation of two distinct epithelial cell line K248C and K248P from a single feline mammary carcinoma with different tumorigenic potential in athymic mice, the K248C cells with amplification of the EGFR gene and elevated levels of RNA and protein were highly tumorigenic. The K248P were poorly tumorigenic²⁷. An oncogenic receptor EGFR (for examples, oncogenic receptor EGFRvIII, oncogenic HMGA2-EGFR fusion)²⁸⁻³³ which was first identified in primary human glioblastoma was revealed to be capable of abrogating IL-3-dependent pathway with tumorigenic activity²⁶. Thus, these results were consistent with the autocrine loop model postulated by Professor DF Stern and RA

Weinberg³⁴ at Massachusetts Institute of Technology in which constitutive production of a mitogenic growth stimulatory signals by the EGFR in response to its normal ligand (EGF) or oncogenic mutations of EGF receptor constitutively activating EGF receptor can lead to uncontrolled proliferation and cell transformation. EGF receptor over expression appears to amplify EGF signal transduction.

In Zhu's further communication, in March 28, 2019, he consulted a patient with ulcerative laryngocarcinoma. The patient experienced the chief complaint of cough, dyspnea and severe hoarse voice for over one month duration. A harden 5x7.5cm mass was palpable in his right neck region. After being applied with topical herbal paste by a doctor, the tumor was ulcerative and hemoptysis in sputum. There was a lot of exudate emitted from ulcerative wound, and the clothing neckline was wet almost every day. Meanwhile, the patient felt that exudate of the wound penetrated the lower part of his throat. The ulcer troubling caused him to undergo ulcer healing to improve his symptoms. It is not useful to spread Yunan Baiyao on the wound for more than two months, and other erythromycin ointment was also ineffective. Moreover, the patient has used silver sulfadiazine ointment in another drug store for over one week, and the tumor tissue was bright red. When the patient was admitted to Zhu's clinic in June 8, 2019, the protocol was consisted of the prescription of traditional medicine (TCM). After obtaining his consent, in August 29, 2019, the patient was trying to topical EGF-Silvadene cream (rhEGF at initial 4μg, then escalated to 7μg/g, twice daily) for 15 days, and then intermittent rhEGF cream use. At the third consultant, the hoarse voice of the patient was obviously improved, and the exudate of the wound was also significantly decreased. A close scrutiny uncovered granulation tissue hyperplasia and three neovascular nodules formation in ulcer wound. These data suggested that in addition to autocrine loop by tumor itself, exogenous rhEGF might accelerate the proliferative activity of malignant cells. Whether EGF can promote the growth of normal cells, accelerating the wound healing or to induce the incidence of tumor, it may depend on cell types of EGF action, internal and external environment and EGF concentration.

Thus current study was not yet able to draw a clear boundary^{23,24,35} between normal and abnormal cell growth. At present, because it is not necessary to target the normal receptor of health human cells, commercial antibodies for anti-oncogenic receptors within tumors (also anti-oncogenic receptor Abs) (Fu YX said in his Yang-xin Fu lab in UT South western Medical Center, Dallas, Texas; Icotinib- Drug Bank)³⁶⁻⁴⁷ have been developed available into market. Therefore, like G-CSF, GM-CSF, IGF-1, growth hormone and sex hormones (eg. estrogen and androgen), at least, use of G-CSF and GM-CSF are not without risk (Estey EH, 1990). And more in experimental group, in one rat, a suspicious subcutaneous nodule was noted in the continuous application of rhEGF injection subcutaneously. Long term application of EGF should therefore be paid

attention to this action, especially in the most destructive trauma, such as a ulcerative cancer³⁶⁻⁴¹.

In the present study, EGF administered topically by dorsal incision at a dose of 50 and 100μg/ml, which is 5 to 10 times higher than its use in clinical doses, was evaluated for its toxic effects for 30 days in skin. Additional toxicological tests, an injection of single 200μg or 300μg/24hrs of subcutaneous and intramuscular rhEGF solution was given respectively. No adverse side effects related to rhEGF was observed. Others, a stable body weight or behavioral signs in all groups indicated no obvious toxicity. In literature reports, another toxicological test was applied 6g (200μg EGF/g) rhEGF gelatin to the skin wound for 36 days on the back of a rabbit, 20 times its clinical dose, no adverse side effects regarding the local skin and organ toxicity was observed (data not shown). Moreover, *in vivo* over expression of epidermal growth factor in transgenic mice could lead to growth retardation, but no tumor was observed in transgenic animals⁴²⁻⁴⁸.

In current study realized that in preparation of rhEGF medicine based on the earlier experiments of an oncogenic pml/retinoic acid receptor alpha fusion (retinoid pharmacology) in APL and an aberrant androgen receptor with its methyltestosterone drug in the induction of breast tumors^{29,30} and subsequently the earliest discovery of normal or proto-oncogenic receptor kinase EGF receptor in cell proliferative signaling in wound healing, and in June,1991 patent application for rhEGF powder and its specific Shampo liquid, and its newly Band-Aids. Here, from the principle of drug action, the EGF receptor to its EGF ligand seemingly compared to 'the key in the lock'. In conclusion, in spite of a number of confounding factors, current study results and preliminary clinical trials support the intention that prepared hEGF by current study's group is more effective therapeutic agent in improving objective parameters of wound healing, and that it may assist in wound healing time.

APPENDIX

MTT assay

MTT assay is a rapid, convenient and safe method of widely used to measure cell proliferation by detecting viable cell number. The assay is performed by the addition of dye solution of culture cells of a 96 well plate usually containing various concentrations of growth factors. Living cells can convert a tetrazolium component of dye solution into a formazan product that is easily detected using a 96-well plate reader. Experimental procedure in brief is according to protocol in (Lalchuang) NEST Biotechnology Co. LTD, Shanghai:

1. Add 100 ul 3T3 cell suspensions to each well with a 96-well plate (5×10^3 cells/well).
2. Incubate 37°C for overnight in 5% CO₂ incubator.
3. Add a various concentration of 100μl rhEGF to the culture wells, and repeat four times in each rhEGF concentration.
4. Incubate 37°C for 48hrs in 5% CO₂ incubator.

5. Add 100ul MTT dye solution (1mg/ml) to each well and mix well, put back to incubate 4 hrs continuously.
 6. Add 150ul DMSO solution to each culture cells to solubilise the formazan product.
 7. Record the absorbance at 570nm wavelength using a multi-well spectrophotometer (ELISA reader).
 8. Calculate proliferative rate in all groups:
- $$\text{Cell proliferative rate \%} = \frac{\text{Experimental OD value}}{\text{Control OD value}} \times 100$$

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AUTHOR'S CONTRIBUTION

All authors have worked equally for this work.

CONFLICTS OF INTEREST

The authors declares that there are no conflicts of interest regarding the publication of this paper

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