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Emodin, an anthraquinone derivative from *Rheum officinale* Baill, enhances cutaneous wound healing in rats

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Abstract

Emodin (1, 3, 8-trihydroxy-6-methyl-anthraquinone) is an anthraquinone derivative from the roots of *Rheum officinale* Baill, a Chinese herb widely and traditionally used for wound healing. Our objective was to determine whether topically applied emodin enhanced repair of rats' excisional wounds and its possible mechanism. Wounds were treated with either topical emodin (100, 200 and 400 μg/ml), recombinant human epidermal growth factor (rhEGF, 10 μg/ml), or vehicle for 7 or 14 days consecutively. At day 5 postinjury, wounds receiving emodin (400 μg/ml) were significantly smaller than those treated with vehicle. Emodin treatments had markedly more hydroxyproline content in day 7 wounds and tensile strength in day14 wounds than that of vehicle control. The level of transforming growth factor- β_1 (TGF-β_1) in wound tissues assessed by immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR), showed a dose-dependent increase in emodin-treated wounds compared with vehicle. Western immunoblotting analysis of wound tissues for Smad 2, 3, 4, 7 protein expression showed increase in Smad 2, 3 in the emodin-treated wounds compared with vehicle. In contrast, a reduction of Smad 7 was observed in emodin-treated wounds compared with vehicle and no change of Smad 4. In summary, our results showed that emodin promoted repair of rats' excisional wounds via a complex mechanism involving stimulation of tissue regeneration and regulating Smads-mediated TGF-β_1 signaling pathway.

Keywords: Emodin; Wound healing; Transforming growth factor-β_1; Smads

1. Introduction

Wound care can be traced back to early civilizations, and many of these treatments were based on the use of herbal remedies. Approximately one-third of all traditional medicines used for the treatment of wounds and skin disorders, such as Physic nut (*Jatropha curcas*, Villegas et al., 1997), St. John's Wort (*Hypericum perforatum*, Upton, 1997), Aloes (*Aloe barbadensis*, Davis et al., 1994), Red sandal wood (*Pterocarpus santalinus*, Biswas et al., 2004) and Cinnamon (*Cinnamomum zeylanicum*, Kamath et al., 2003), compared to only 1–3% of modern drugs, have been found effective in treating dermal wounds on different wound models.

Rheum officinale Baill (Chinese name Dahuang), is one of the most popular traditional medicinal herbs and is officially listed in the Chinese Pharmacopoeia (China Pharmacopoeia Committee, 1999). Its species are widely distributed in China, Kirgheeze desert and Europe. Chinese rhubarb is the only kind recognized by the United States Pharmacopeia, and pharmacological test revealed that *Rheum officinale* Baill had pharmacological activities such as cathartic, anti-psychotic, anti-inflammatory, antimicrobial, hemostasis and so on, so it has been used for the treatment of dysentery, cholera, uraemia, leukaemia, diabetes, lung cancer and widely used in combination with other crude drugs for the treatment of wound healing from ancient times in China (Wang et al., 1996; Li et al., 2000). The major active components of the herb are hydroxyanthraquinones. Emodin, 1, 3, 8-trihydroxy-6-methyl-anthraquinone (Fig. 1), is an anthraquinone derivative from the roots of *Rheum officinale* Baill (Kuo et al., 2001b). It has been reported that emodin possesses a number of the same biological activities as *Rheum officinale* Baill, such as inhibitory activity of monoamine oxidase (Kong et al., 2004), anti-inflammatory, anti-

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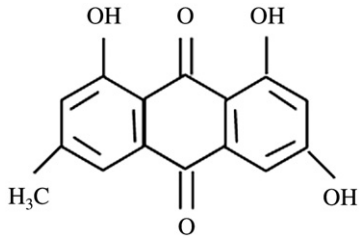

Fig. 1. Chemical structure of emodin (1,3, 8-trihydroxy-6-methyl-anthraquinone).

virus, vasorelaxative (Huang et al., 1991), immunosuppressive (Huang et al., 1992; Kuo et al., 2001a), hepatoprotective (Lin et al., 1996) and anti-tumor (Shi et al., 2001; Srinivas et al., 2003). Recently, emodin has been shown that the mechanism of it in treating acute pancreatitis might be by way of enhancing TGF-β_1 gene expression, regulating cell growth and differentiation, stimulating the formation of extracellular matrix components, increasing DNA synthesis and protein content, and taking part in pancreatic repairing and remodeling (Gong et al., 2002). There were many pharmacological researches about emodin but few were about promoting wound healing activity.

Wound healing is a multi-step complex process consisting of inflammation, granulation tissue formation, angiogenesis, re-epithelialization, and wound contraction. The cytokine transforming growth-beta factor 1 (TGF-β_1) has been widely recognized as a key mediator of wound healing (Massague, 2000). Its stimulatory effect plays a fundamental role in physiological tissue repair as well as pathological fibrosis (Sales et al., 2006). Recent investigation has revealed that all TGF-β isoforms signal through transmembrane serine/threonine kinases (Pham et al., 2004). The ligand is bound to TGF-β type II receptor, which then recruits and activates TGF-β type I receptor to form a heterotetramer of two TGF-β type I receptor and two TGF-β type II receptor molecules (Yamashita et al., 1994). Intracellular signaling mediators of TGF-β activity were identified by genetic screening, and the mammalian members of this highly conserved family have been named Smads (Sekelsky et al., 1995). Activated TGF-β receptors phosphorylate the cytosolic receptor-activated Smad 2 and Smad 3, which form heteromeric complexes with Smad 4, and enter the nucleus, bind to DNA, and regulate gene transcription in cooperation with DNA binding cofactors. Inhibitory Smads, Smad 7, block TGF-β signal transduction, in part, by preventing the interaction of Smad 2/3 with the activated TGF-β type I receptor (Attisano and Warana, 2000; Heidin et al., 1997; Massague and Wotton, 2000).

Keeping all pharmacological activities of this plant in view, as well as its utilization in the treatment of skin diseases, we investigated the effect of emodin isolated from the traditional Chinese medicine *Rheum officinale* Baill on cutaneous wound healing in rats. Our results showed that emodin had the exerted effect on cutaneous wound healing and its promoting effect may be due to the stimulation of tissue regeneration and regulating Smads-mediated TGF-β_1 signaling pathway.

2. Materials and methods

2.1. Plant materials

The dried roots of *Rheum officinale* Baill were purchased from a local drug shop and were identified by Dr. Mingxi Jiang of Wuhan Institute of Botany, Chinese Academy of Sciences, Wuhan, China. A reference sample (WIBC 40601) was retained at the Institute.

2.2. Extraction and purification of emodin

The roots of *Rheum officinale* Baill were air-dried and cut into small pieces before grinding. The roots (200 g) were ground to powder (about 30 mesh) and then extracted with methanol at 50 °C for 24 h. The solvent was removed under reduced pressure and the residue was mixed with distilled water (100 ml). The mixture solution was extracted with *n*-butanol (3 × 100 ml) and the solvent then evaporated to yield the crude extract (2.3 g). The sample, absorbed on silica gel (sample/adsorbent (v/v) = 1/8), was subjected to dry flash column chromatography. Sufficient hexane was passed through the column to expel all of the air. Extensive gradient elutions were then employed using hexane (250 ml), chloroform (300 ml) and 15% chloroform/methanol (v/v, 200 ml) and further separated to obtain emodin. The identification of emodin was definitively assessed by spectroscopic data as follows. 1H NMR data of emodin: ^{1}H NMR (400 MHz, C_2HCl_3): 12.80 (1H, s, C_3–OH), 12.30 (1H, s, C_1–OH), 12.12 (1H, s, C_8–OH), 7.63 (1H, s, C_5–H), 7.29 (1H, d, J=2.4 Hz, C_4–H), 7.10 (1H, s, C_7–H), 6.68 (1H, d, J=2.4 Hz, C_2–H), 2.46 (3H, s, CH_3). All the data coincided well with the previous reports (Chen, 2000; Liu et al., 2004). A hydrogel containing 2% carbopol was prepared incorporating 0, 100, 200 and 400 μg of emodin in 1 ml of the gel.

2.3. Animals

Male Sprague–Dawley rats weighing 250–280 g were purchased from the Experimental Animal Center of Wuhan University. The rats were maintained under standard laboratory condition at 25±2 °C relative humidity 50±15% and normal photo period (12-h dark/12-h light). The animals were fed normal diet and water ad libitum. All study protocols were approved by internationally accepted principles and the Guidelines for the Care and Use of Wuhan University, Wuhan, China.

2.4. Rat excisional wound model and drug treatment

For wound healing experiments, rats were anesthetized with an intraperitoneal dose of sodium pentobarbital (50 mg/kg). The dorsal skin was prepared by trimming the hair with electric clippers, depilating and treating with antiseptic. Full-thickness wounds were excised on each rat using an 8 mm biopsy punch to the depth of loose subcutaneous tissues. Hemostasis was achieved by even compression with sterile gauze.

To control for effects of wound location on the rate of healing, the wounds were randomized to receive once daily

topical treatment with either rhEGF (10 μg/ml), emodin 100, 200 and 400 μg/ml, or vehicle, for a period of 7 or 14 days. Each wound was treated with a 100 μl topical application of gel alone (vehicle control) or gel containing emodin or rhEGF. The rhEGF dose was derived from previously published animal and human wound models (Brown et al., 1989; Nanney, 1990). Following each treatment, the wounds were covered with a sterile, transparent, occlusive dressing to ensure that the formulations remained in contact with the wound bed. Each animal was individually housed to prevent external tampering with the wounds. All the wounds received daily standard wound cleansing prior to the next application of emodin, rhEGF or vehicle. Any rat showing a wound hematoma or wound infection was immediately killed with an overdose of pentobarbital to avoid any discomfort. Also, data from these animals were not used to assess wound healing. Half of the animals were euthanized at day 7 postinjury, this being the approximate timing of the peak of TGF-β_1 expression and half timing of complete wound closure, and their wounds harvested by complete excision and excluding any underlying muscle or extraneous tissue. Solitary wounds were either immediately placed in snap frozen in liquid nitrogen and stored at −80 °C until RNA isolation was performed, or fixed in 4% paraformaldehyde for 24 h and processed in a routine fashion for immunohistochemistry. Another fifty animals were euthanized at day 14 postinjury, the entire wound minus the small strips was used for tensile strength and histologic assessment.

2.5. Wound contraction determination

Wounds were serially photographed using a Kodak Digital Science DC260 Zoom camera (Eastman Kodak, Rochester, NY) on days 1, 3, 5, 7, 12 and 14. Wound area was measured using Zeiss ProPlus scientific image analysis software (Carl Zeiss Inc., Thornwood, NY). The wound contraction rate was measured as percentage reduction in wound area of the original surgical excision (Sadaf et al., 2006).

$$\%\ \text{wound contraction} = \frac{d1\ \text{wound area} - \text{unhealed wound area}}{d1\ \text{wound area}} \times 100\%$$

2.6. Histological examination

Following fixation, each wound was embedded in paraffin. Serial sections of 5 μm were cut and stained with hematoxylin and eosin for histologic evaluation of fibroblast proliferation, neovascularization, epithelial regeneration and collagen deposition. Two areas in each section were evaluated for neovascularization and fibroblast proliferation.

2.7. Hydroxyproline assay

The frozen excision sites were thawed, homogenized and hydroxyproline content determined per gram of tissue by the method of Edwards and O'Brien (1980). Collagen content was estimated by multiplying the hydroxyproline content by weight, since hydroxyproline is consistently 12–15% by weight of tissue collagen.

2.8. Tensile strength determination

8 mm strips of the wound from each rat were excised for measurements of tensile strength on day 14 wounds. The strips were removed from the lower half of the excision in all animals to assure consistency of the measurement. The strips were mounted on a tensiometer (Tensiometer 10, Monsanto, St. Louis, MO). Tensile strength in each strip was determined by measurement of maximum load in grams per mm^2. Tensile strength was performed immediately after wound excision.

2.9. RNA extraction and reverse transcription polymerase chain reaction

Total RNA was extracted from wound tissues using Tri Reagent (Sigma-Aldrich) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from total RNA using AMV reverse transcriptase (Promega, Madison, WI) and oligo $(dT)_{15}$ as primer. Polymerase chain reaction (PCR) was performed using cDNA product to detect gene expression of TGF-β_1. β-actin was included as an internal standard. Specific primers for TGF-β_1 and β-actin and amplification conditions were using as these: TGF-β_1: 5′-GCT AAT GGT GGA CCG CAA CAA CG-3′, 5′-CTT GCT GAT CTG TGT GTC CAG GC-3′ (Ishida et al., 2004); β-actin: 5′-CCA ACT GGG ACG ACA TGG AG-3′, 5′-GTA GAT GGG CAC AGT GTG GG-3′. These primers set yielded PCR products of 442 and 262 bp for TGF-β_1 and β-actin, respectively. A Perkine Elmer GeneAmp 9600 thermal cycler (Foster City, CA) was used with the following cycling program: 94 °C for 30 s, 59 °C for 1 min, 72 °C for 1 min for 30 cycles. The PCR products were electrophoresed through a 1% agarose gel and visualized by 0.2 μg/ml ethidium bromide staining and UV irradiation, and semiquantitated using image analysis by comparing TGF-β_1 with β-actin.

2.10. Immunohistochemistry

To evaluate the TGF-β_1 levels on wound tissues, immunohistochemistry was performed using human anti-mouse monoclonal antibody TGF-β_1 (1:100 dilution, R&D Systems, Minneapolis, MN, USA), we also used PBS as a negative control. Briefly, tissue sections were incubated with primary antibodies overnight at 4 °C, followed by incubation with biotinylated secondary antibody for 15 min and streptavidin–horseradish peroxidase (HRP) complex (DAKO LSAB+Kit, K090) for another 15 min. After three washes with PBS, slides were incubated with substrate-chromogen solution (3,3-diaminobenzidine DAB from DAKO LSAB) for 5 min and counterstained with Mayer hematoxylin for 3 min. Images including almost all the wounds were captured using a light microscope (Olympus BHS) and a digital camera (Olympus DP12), and the number

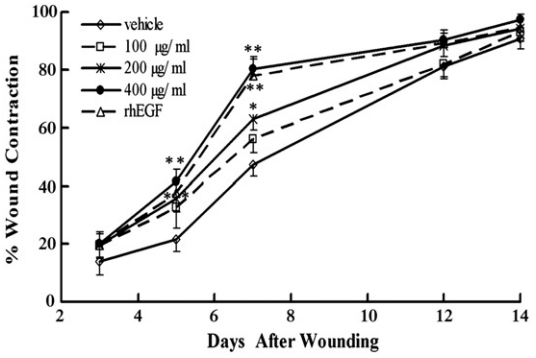

Fig. 2. Effect of emodin on wound healing by excisional wound model in rats. Full-thickness excisional wounds were treated once daily with either topical vehicle, emodin (100, 200 and 400 μg/ml), or rhEGF (10 μg/ml). Analysis of the wound healing was performed on days 3, 5, 7, 12 and 14 post-wounding. The wound contraction of each time point was measured as percentage reduction of original wound size. Values are expressed as means ± S.E.M. ($n=20$ for days 3, 5, and 7 and $n=10$ for days 12 and 14). Significance was determined by ANOVA followed by Dunnett's test. $^{*}P<0.05$, $^{**}P<0.01$ vs. vehicle-treated group.

$$\%\ \text{wound contraction} = \frac{d1\ \text{wound area} - \text{unhealed wound area}}{d1\ \text{wound area}} \times 100\%$$

of TGF-β_1-positive cells in the wound sites quantified per unit area in either section were measured for positive area using an image analyzing system (C-Imagining System. Compix Inc.), the positive area was then calculated from the data. The average value of every five fields was taken as the measure value of every section, and the rate of positive area was calculated as followed:

$$\text{Rate of positive area} = \frac{\text{Total area of positive reaction in unit area}}{\text{Total area of cells in unit area}} \times 100\%.$$

2.11. Western immunoblotting

Equal amounts of protein in wound tissues were homogenized for 10 min in Laemmli sample buffer (50 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 0.1% bromophenol blue) and centrifuged at 13,000 ×g for 5 min before electrophoresis through a 10% SDS-polyacrylamide resolving gel layered with a 4% stacking gel. Proteins were transferred to nitrocellulose membranes and nonspecific binding sites blocked with 5% skim milk powder for 2 h at room temperature. Membranes were incubated with Smad 2,3,4,7 polyclonal antibodies (1:500 dilution, Santa Cruz, CA) for 1 h at 4 °C. Membranes were washed in Tris-buffered saline and incubated in HRP-conjugated secondary antibodies immunoglobulins diluted in PBS containing 10% FCS and 0.05% Tween-20 for 1 h at room temperature. Membranes were washed in Tris-buffered saline and bound antibody detected using ECL™ Western blotting detection reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The resulting blots were digitized and subjected to densitometric scanning using a standard NIH image program.

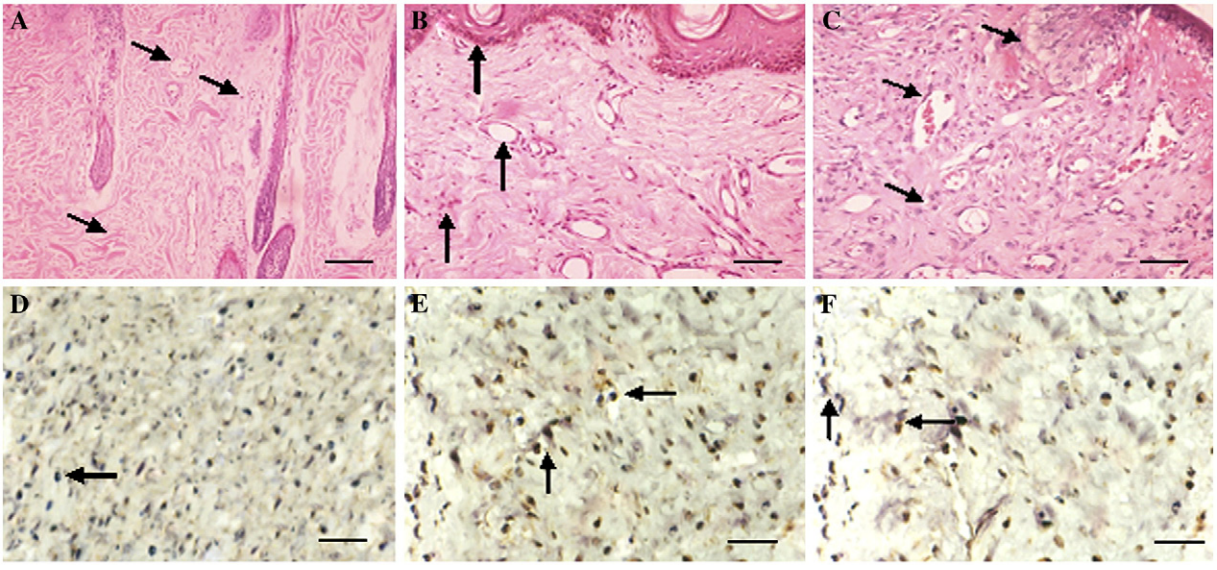

Fig. 3. Histopathology and immunohistochemical detection of TGF-β_1 in vehicle-, emodin-, or rhEGF-treated wounds. Representative images of an H and E-stained section of wounds treated once daily with either (A) vehicle, (B) emodin (400 μg/ml), or (C) rhEGF for 14 days. Resurfacing is complete after 14 days in all treatment groups. Vehicle control excision site (A) showed irregular arrangement of collagen bundles were loosely packed, and wounds were only moderately cellular with fibroblast cells. The site of both emodin-treated and rhEGF-treated wounds (B, C) showed more densely-packed and compactly arranged collagen bundles with more fibroblasts cells, increased blood vessel formation and on-going epithelialization, reflecting a more rapidly healing wound than that of vehicle-treated groups. TGF-β_1 immunoreactivity in day 7 (D) vehicle-, (E) emodin (400 μg/ml)- and (F) rhEGF-treated wounds. Vehicle-treated wounds exhibited TGF-β_1 immunoreactivity in approximately half of fibroblasts and rare macrophages in endochylema. Emodin-treated wounds were notable for TGF-β_1 immunoreactivity in all the two cells. The extent of immunoreactive TGF-β_1 in rhEGF-treated wounds is greater than that of vehicle control, but less than that observed in emodin (400 μg/ml)-treated wounds. Scale bar: (A, B, C) 200 μm; (D, E, F) 100 μm.

2.12. Statistical analysis

All results are expressed as mean±S.E.M. and statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's test. Data analysis was performed by using SPSS 11.5 (SPSS Inc., Chicago, IL, USA) statistical package programme. $P<0.05$ was considered as statistically significant.

3. Results

3.1. Effect of emodin on wound contraction

The progress of the wound healing induced by emodin- (100, 200 and 400 μg/ml), vehicle- and rhEGF- (standard drug) treated groups was shown in Fig. 2. There was no statistically significant difference in the rate of wound healing in rats treated with emodin 100, 200 and 400 μg/ml or vehicle by day 3. At day 5 after injury, the rate of wound contraction was 41.6% in animals treated with emodin 400 μg/ml as compared to 21.6% in vehicle-treated group. By day 7 post-wounding, rate of emodin (100, 200 and 400 μg/ml)-treated wounds contraction were 56.1%, 63.1% and 80.5% respectively, by comparison, those of rhEGF- and vehicle-treated wounds were 77.9% and 47.3% respectively. By day 12 or 14, both control and emodin-treated wounds were fully reepithelialized and there was no statistically significant difference in the rate of wound contraction.

3.2. Histopathological evaluation

The multiple sections studied in histopathological examination of the tissues of the wounds treated with the emodin (400 μg/ml), vehicle and rhEGF groups were shown in Fig. 3. The histological examination showed that the original tissue regeneration was much greater in the wounds treated with emodin (400 μg/ml) (Fig. 3B) and rhEGF treated group (Fig. 3C) with increasing in restoration of collagen bundles, fibroblasts and blood vessel formation. On-going epithelialization was observed in the rhEGF and emodin treated wounds with flattened rete ridges in the epidermis comparing with the wounds treated with vehicle (Fig. 3A), which showing irregular arrangement of collagen bundles were loosely packed, and wounds were only moderately cellular with fibroblasts cells and few irregularly shaped blood vessels was observed.

Table 1
Effect of emodin treatment on wound healing parameters

Treatment group	Concentration (μg/ml)	Hydroxyproline (mg/g/tissue)	Tensile strength (g/mm^2)
Vehicle control	–	71.1±3.0	110.2±1.2
Emodin	100	83.6±4.5[a]	114.7±1.5
Emodin	200	91.5±3.5[a]	120.6±2.6[a]
Emodin	400	110.2±5.4[b]	134.2±5.8[b]
rhEGF	–	109.7±6.3[b]	121.3±7.1[a]

Hydroxyproline content on day 7 wounds and tensile strength on day 14 wounds treated with topical vehicle, emodin 100, 200 and 400 μg/ml, rhEGF. Values are represent means±S.E.M. ($n=10$). Significance was determined by ANOVA followed by Dunnett's test. [a]$P<0.05$, [b]$P<0.01$ vs. vehicle-treated group.

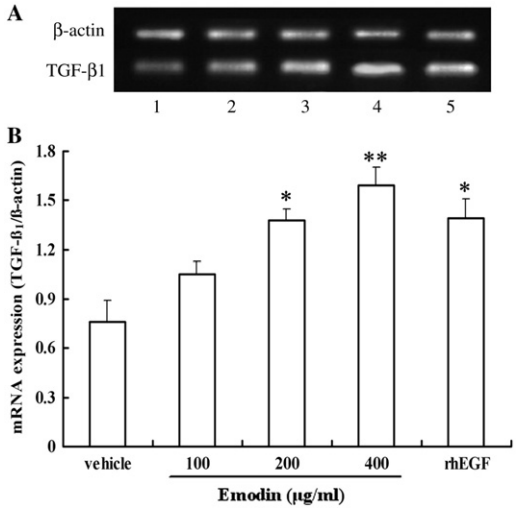

Fig. 4. RT-PCR detected TGF-β_1 mRNA expression in wound tissues treated with vehicle, emodin 100, 200 and 400 μg/ml, rhEGF for 7 days. (A) Representative gels are shown with the upper band displaying β-actin (262 bp) and the lower band displaying TGF-β_1 (442 bp). Lane 1: vehicle control; Lanes 2–4: emodin 100, 200 and 400 μg/ml; Lane 5: rhEGF. (B) The PCR products were semiquantified for relative levels of mRNA using image analysis by comparing TGF-β1 with β-actin. Bar graph shows the mean±S.E.M. value of relative TGF-β_1/β-actin mRNA expression ($n=10$). Significance was determined by ANOVA followed by Dunnett's test. *$P<0.05$, **$P<0.01$ vs. vehicle-treated group.

3.3. Biochemical and physical measures of wound healing

The hydroxyproline content on day 7 and the tensile strength measurements on day 14 wound tissues were presented in Table 1. A concentration-dependently increase in hydroxyproline content of wound tissue was observed in emodin-treated

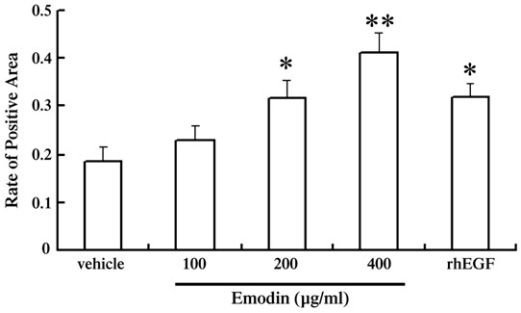

Fig. 5. Quantitative immunohistochemical analysis of TGF-β_1 in day 7 vehicle-, emodin (100, 200, 400 μg/ml)- and rhEGF-treated wounds. The number of TGF-β_1-positive cells quantified per unit area in either section was measured for positive area using an image analyzing system and the positive area was then calculated. Values are expressed as mean±S.E.M. ($n=10$). Significance was determined by ANOVA followed by Dunnett's test. *$P<0.05$, **$P<0.01$ vs. vehicle-treated group. The rate of positive area was calculated as follows:

$$\text{Rate of positive area} = \frac{\text{Total area of positive reaction in unit area}}{\text{Total area of cells in unit area}} \times 100\%$$

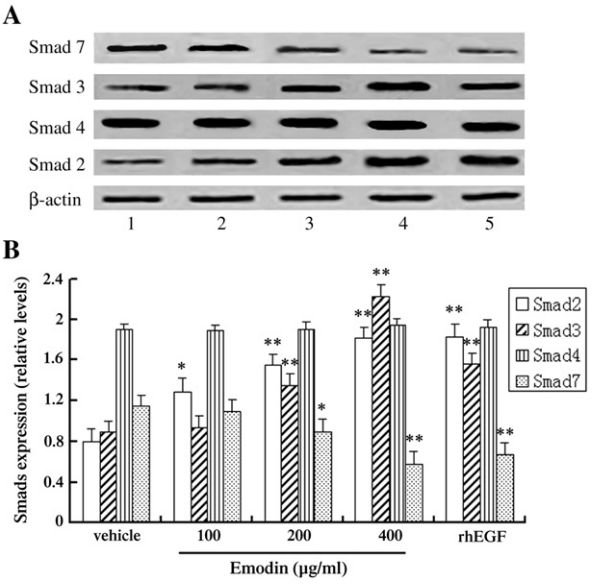

Fig. 6. Effect of emodin on Smads 2, 3, 4, 7 protein expression in wound tissues. Wounds treated with topical vehicle, emodin 100, 200 and 400 μg/ml, rhEGF for 7 days and protein expression of Smads 2, 3, 4, 7 were detected by Western blotting. (A)Representative images are shown of Smad 2, 3, 4, 7 expression at the wound tissue ($n=10$). Lane 1: vehicle control; Lanes 2–4: emodin 100, 200 and 400 μg/ml; Lane 5: rhEGF. (B) The bar chart shows the semiquantitative analysis of Smads 2, 3, 4, 7 protein expression. Data represent means ± S.E.M. ($n=10$). Significance was determined by ANOVA followed by Dunnett's test. *$P<0.05$, **$P<0.01$ vs. vehicle-treated group.

wound compared with vehicle group. Emodin at 400 μg/ml has more or less equivalent effect to that of rhEGF increasing the hydroxyproline content. Furthermore, there was a significant increase in wound tensile strength of wound tissue in emodin-treated group at concentration of 200 and 400 μg/ml, as compared with vehicle group. Emodin at 400μg/ml also showed slight increase of tensile strength than that of rhEGF group.

3.4. Effect of emodin on TGF-β_1 mRNA expression in wound tissue

TGF-β is released at the site of injury and is activated from its latent complex (Grainger et al., 1995). In addition, TGF-β acting on wound fibroblasts, stimulates the transcription of a variety of matrix proteins including fibronectin and collagen (Howell and McAnulty, 2006). As detailed above, treatment of wounds with topical growth factors causes substantive enhancements in both the epidermis and the dermis. Thus, we hypothesized that topically applied emodin or rhEGF would up-regulate TGF-β_1, resulting in accelerated healing of treated wounds. To test this hypothesis, the effect of emodin treatment on TGF-β_1 mRNA expression was determined by RT-PCR as shown in Fig. 4. There was a dose-dependent increase in TGF-β_1 mRNA expression in emodin-treated wounds than vehicle-treated wounds. At 400 μg/ml, emodin treatment caused a marked up-regulation of TGF-β_1 mRNA expression in wound tissue, and emodin at 200 μg/ml behaved identically to that of rhEGF treatment. We also performed immunohistochemistry for TGF-β_1 on day 7 wound. Vehicle-treated wounds exhibited TGF-β_1 immunoreactivity in approximately half of fibroblasts and rare macrophages in endochylema. Emodin-treated wounds were notable for TGF-β_1 immunoreactivity in all the two cells. The extent of immunoreactive TGF-β_1 in rhEGF-treated wounds is greater than that of vehicle control, but less than that observed in emodin (400 μg/ml)-treated wounds (Fig. 3 and Fig. 5).

3.5. Effect of emodin on Smads protein expression

Members of the Smad family of intracellular proteins are phosphorylated by TGF-β receptors and convey signals to specific TGF-β-inducible genes (Mori et al., 2000). Thus, we evaluated the expression of Smads 2, 3, 4, 7 (Fig. 6) in wound tissue, and determined if emodin treatment selectively altered their expression. Western blotting detection revealed that emodin treatment increased the level of Smad 2 and Smad 3 protein expression with concurrent decreased in Smad 7 protein expression in wound tissue in a dose-dependent manner. The level of Smads expression reached statistical significance for Smads 2, 3 and Smad 7 in emodin-treated wounds compared with vehicle-treated control. With regard to Smad 4 expression no significant difference was found between emodin- and vehicle-treated wounds.

4. Discussion and conclusion

A number of studies indicate that plant products are potential agents for wound healing and largely preferred because of their widespread availability, absence of unwanted side effects and their effectiveness (Jagetia and Rajanikant, 2004). In this report, we show for the first time that topical application of emodin, an anthraquinone derivative isolated from the roots of *Rheum officinale* Baill accelerates cutaneous wound healing in rat excisional wound model.

Wound contraction can be defined as the centripetal movement of the edges of a full-thickness wound to facilitate closure of the defect (Suguna et al., 2002). The progression of wound healing can be judged by the periodic assessment of the contraction of excision wounds. Topical emodin markedly improved wound contraction and closure, and the effects were distinctly visible from day 5 post-wounding. Histopathological evaluation of the wound site provides evidence of a more desirable histological organization of the tissue in response to emodin treatment. Treatment of rats with emodin resulted in an enhancement of wound healing, as evidenced by increased wound contraction and the formation of new blood vessels. In contrast, very recent studies reveal that emodin inhibits tumor-associated angiogenesis (Kaneshiro et al., 2006; Srinivas et al., in press). This discrepancy could be due to differences of physiological and pathological conditions (wound healing versus cancer and metastasis) but more probably reflects the dual regulation of emodin in the different diseases. Thus further work will be necessary to define emodin's angiogenic effect and the critical mechanism of action in wound healing. In addition, our results show that emodin (400 μg/ml) stimulates wound healing more potent and more effective than rhEGF. There are numerous animals and clinical studies of cutaneous wounds,

may be reported several years ago, on the positive wound healing effects of rhEGF (Sun et al., 2000), which has been shown to accelerate wound repair by a modest 20% (Huang and Wei, 2003), and this well-characterized ligand was included in our studies as a basis for comparison. The mechanism of the rhEGF that displayed more rapid wound closure was considered to result from increasing collagen synthesis in the earlier studies.

Hydroxyproline content is an index of collagen and measure the synthesis of collagen. The healing process depends to a large extent, on the regulated biosynthesis and deposition of new collagens and their subsequent maturation (Gao et al., 2006). Collagen is produced by fibroblasts and helps the wound gain tensile strength during repair (McFarlin et al., 2006). In addition, several studies reported an increase in wound tensile strength, which depends on factors in addition to collagen deposition, namely matrix deposition and cell migration (Demling, 2000). Recent studies found that emodin significantly inhibited tumor necrosis factor-á (TNF-á)-induced expression of matrix metalloproteinase-1(MMP-1) which is one of the key enzymes involved in the degradation of the extracellular matrix, a process closely related to tissue remodeling (Lee et al., 2006). Our results also showed emodin treatment caused a significant increase in wound collagen content measured as hydroxyproline and wound tensile strength, suggesting that effect of emodin influencing wound healing is responsible for enhancing collagen synthesis and deposition.

Wound healing processes are controlled by a multitude of cell–cell and cell–matrix interactions, but also by various growth and differentiation factors. Although expression of many of these factors has been described in the healing wound, their precise roles and mechanisms of action have been poorly defined (Werner and Munz, 2000). One of the most important players in the wound repair process is transforming growth factor-β (TGF-β) of which three isoforms exist in mammals: TGF-β_1, β_2, and β_3. These factors released from degranulating platelets at the site of injury, provide the early signals for the activation and infiltration of fibroblasts, macrophages and neutrophils, spearheads of the wound tissue repair phase. In turn, cells migrate into the wound site and release cytokines/chemokines that trigger granulation tissue formation (Martin, 1997). Furthermore, these factors are highly expressed by macrophages, fibroblasts, and keratinocytes in the healing wound, whereby each isoform has a characteristic pattern of expression. TGF-β_1 was chosen because it is the most abundant isoform in the healing wound (Frank et al., 1996). Its stimulatory effect on collagen synthesis plays a fundamental role in physiological tissue repair as well as pathological fibrosis (Sales et al., 2006). Numerous evidence suggested exogenous application of TGF-β_1 to cutaneous wounds, either directly or indirectly, significantly enhanced the wound healing process, particularly in animals with wound healing defects by stimulating recruitment of inflammatory cells, the production of extracellular matrix, the formation of new blood vessels, and wound contraction (Sales et al., 2006; Lee et al., 2004). Furthermore, increased granulation tissue formation enhancement of reepithelialization was also observed in these animals (Ishida et al., 2004).

The mechanism of emodin in treating acute pancreatitis might be by way of enhancing cytokine TGF-β_1 gene expression, regulating cell growth and differentiation, stimulating the formation of extracellular matrix components, increasing DNA synthesis and protein content, and taking part in pancreatic repairing and remodeling (Huang et al., 1991). Chan et al. (2003) also demonstrated that emodin ameliorated concentrated glucose-induced matrix synthesis via suppression of TGF-β_1 secretion in human peritoneal mesothelial cells. Our finding was that emodin increased TGF-β_1 expression of mRNA in wound tissue compared with vehicle. In accordance with gene expression, emodin enhanced TGF-β_1 protein expression in a dose-dependent manner when used at concentrations of 100, 200, and 400 μg/ml. This may explain the promoted effects of emodin on wound healing are by modulating the activity of TGF-β_1 within the wound environment.

The mechanism of TGF-β-mediated signal transduction through the Smad proteins has been well described previously (Sekelsky et al., 1995; Attisano and Warana, 2000; Heidin et al., 1997). The present study demonstrated that Smad 2, Smad 3 protein expressions were both up-regulated by emodin with concentration-dependently compared with vehicle control, while Smad 4 protein expression was not altered. This confirms and extends previous reports that TGF-β receptor-induced activation of Smad 3 and interaction with Smad 4 accompanied by their translocation into the nucleus to direct specific transcriptional responses to TGF-β actions (Chegini et al., 2003). This suggests that alteration of Smads expression and Smad 2, Smad 3 activation due to emodin therapy could result in changes in transcriptional activation of specific TGF-β-inducible genes, which are known to modulate of cell growth and proliferation, angiogenesis, apoptosis and extracellular matrix turnover (Brunner and Blakytny, 2004). Smad 7, an inhibitory Smad, which like Smad 3 interacts with activated TGF-β type I receptor, however, Smad 7 forms a stable association with the receptor complex and prevents receptor-mediated phosphorylation of pathways — restricted Smad 3, resulting in disruption of TGF-β mediated signaling (Zimmerman and Padgett, 2000; Massague and Wotton, 2000). The level of Smad 7 we saw in wound tissues, furthermore, was down-regulated by emodin. This result may imply emodin can act directly by activating TGF-β_1 and indirectly through modulating Smads, the only downstream substrates of TGF-β type I receptor known so far (Verrecchia et al., 2001). These signaling pathways amplify the effect of emodin and may explain its actions in wound healing, although the exact signaling mechanisms involved remain to be elucidated.

In summary, our current study provides firm evidence to support that topical application of emodin represents a feasible and productive approach to support cutaneous wound healing. Taken together, this report of in vivo findings indicate that emodin, an anthraquinone derivative isolated from the roots of *Rheum officinale* Baill has ability to accelerate healing of cutaneous wounds which is related to TGF-β_1/Smad signaling pathway and improves reorganization of the regenerating tissue.

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